Forest Fungi and their Interactions

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Presented in Partial Fulfillment of the Requirements for the

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with a

Major in Natural Resources

In the

College of Graduate Studies

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by

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Authorization to Submit Thesis

This thesis of Maria Marlin, submitted for the degree of Master of Science with a Major in Natural Resources and titled "Forest Fungi and their Interactions" has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Fungi are ubiquitous in a forest setting; they can function as endophytes in plant tissues, as plant pathogens, and as wood decomposers. Plant endophytes can play a tremendous role in plant ecology, fitness, and evolution. Plant pathogens are microbes with a sinister ability to weaken or completely destroy precious and prized forest tree species. Fungi that decompose wood are crucial in nutrient cycling. This thesis explores different guilds of forest fungi and how their interactions provide insight into overall forest dynamics and health.

The first chapter contains results from a study conducted to determine where the most antagonistic endophyte interactors are found within different plant tissues. An important forest tree, *Pinus monticola* or Western White Pine, was used as a model system. The seed contains specialized endophytes that were stronger interactors than their vegetative counterparts. This information is applicable to the current quest of locating effective antagonistic microbes to fight damaging plant pathogens.

Secondly, we report that copy number variation in hybrid poplar affects response to infection by *Melampsora* rust. Rust inoculations on mutated *P. deltoides* × *P. nigra* lines demonstrated that there are dosage-dependent genes for both rust resistance and rust susceptibility. Additionally, a novel *Melampsora* hybrid rust is discussed and characterized. Combined, these first two chapters offer insights into microbial interactions with both each other and their hosts, and how we can harness this information to reduce disease susceptibility.

The third chapter details a study that investigates host ranges of both nematophagous fungi and fungivorous nematodes. Nematophagous *Pleurotus pulmonarius* consumes some nematodes; other nematodes, however, are resistant to the *P. pulmonarius* toxin. Resistant nematodes begin to consume *Pleurotus pulmonarius*, even though they are currently only viewed as ‘bacterial feeders’.

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Dedication

I would first like to dedicate this thesis to my grandfather and Aunt Yummy, two important people I lost during the course of my Master's program. Both these people demonstrated unconditional love and their beautiful memory inspires me daily to serve others and be my best self.

Additionally, this work is dedicated to my family and close friends, who have been my constant cheerleaders and who were my shining lights during dark days. To my parents, John and Stephanie, thank you for the countless hours and immeasurable effort you devoted to ensuring I had an incredible education and foundation on which I could build. To Ed, the greatest and most knowledgeable botanist I have ever met: your contagious enthusiasm and exceptional teaching and mentoring inspired my path. Thank you so much for your priceless friendship, for brightening my days, and for always supporting and believing in me. To Alys, Kathleen, and Dominee, thank you for so many fond memories that I can always look back on and smile. I am forever grateful for the constant laughs, your steadfast camaraderie, and advice when all seemed lost.

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Statement of Contribution

Dr. Alexey Shipunov (Minot State University, North Dakota) contributed to Chapter 1 by providing Figure 1.4 as well as advice on other statistical analyses. Diana Cervantes (UI), Brenda Schroeder (UI), Sarina Heitmann (OSU), and Posy Busy (OSU) contributed by extracting DNA and sending samples for sequencing. Maria Marlin wrote the thesis chapter, conducted all experiments, collected all the data, and performed all other data analyses.

Dr. Heloise Bastiaanse (USFS, California) contributed to Chapter 2 by performing the data analysis and producing Figure 2.1 and the graphs. Dr. Pascal Frey (INRA, France) contributed to Chapter 2 by providing the details regarding the molecular and phenotypic characterization of the putative hybrid *Melampsora* rust. Maria Marlin wrote the thesis chapter, propagated all plants at the University of Idaho greenhouse, conducted all inoculations, and collected all data.

Avery Wolf (University of Idaho) contributed to Chapter 3 by collecting the data. Lynn Carta (USDA, Maryland) contributed the nematodes, Figures 3.1 and 3.2, and edits on the chapter. Mr. Shiguang Li (USDA, Maryland) provided excellent technical assistance with cultures and many sequences of the nematodes used in this study. Maria Marlin wrote the thesis chapter, maintained the nematodes, devised, and oversaw the study.

Dr. George Newcombe contributed advice and edits to all three chapters.

Chapter 1: Interactions among members of endophyte communities in needles and seeds of *Pinus monticola*

Abstract

Endophytes can antagonize pathogens and could thus be used as disease-reducing inoculants in agriculture and forestry. But where in the highly diverse microbiome of a plant can we find the strongest endophytic antagonists? The seed and foliar (needle) endophytes of *Pinus monticola* were used to test the hypothesis that the seed community should comprise stronger antagonists than the foliar, because exclusionary interactions have been reported for former but not for the latter. Eight needle fungal endophytes, eight seed bacterial endophytes, and eight seed fungal endophytes were selected as representative of the three communities; needle bacteria were not isolated into culture and thus not represented. All 576 possible pair-wise combinations, both intracommunity and intercommunity, were tested *in agaro* for interaction, either growth inhibition (i.e., antagonism) or stimulation; three replicates of each combination brought the total number of plates to 1,728. Seed bacteria (*Bacillus* spp.) were the strongest antagonists and interactors; the overall interaction average of the strongest individual interactor, *Bacillus pumilus*, was ten times higher than that of the weakest interactor, and three times higher than the strongest needle fungus, a species of *Coniothyrium*. Overall, needle fungi, including its dominant member, *Lophodermium nitens*, were the least antagonistic community, and were themselves antagonized the most. Some stimulatory activity (about 2/3 of all possible pair-wise combinations) is reported, with seed bacteria again proving to be the strongest interactors, in this case as stimulators, and seed fungi the most stimulated. Hierarchal clustering revealed a tree that clearly separated most microbes into their communities of origin, just based on their *in agaro* interactions. *Lophodermium nitens* is known to be in high relative abundance in needles and thus in the overall crown of mature trees, yet it was both a weak interactor (22nd out of 24 in average interaction strength), and it was commonly and substantially antagonized by seed endophytes. In contrast, seed endophytes must be rare at the crown level, but they are strong interactors. We discuss the implications of these findings for the search for the best inoculants.

Introduction

Plant endophytes, or microbes that live within plant tissue, have been found in tissues ranging from leaves and stems to seeds and roots. Foliar endophytes are particularly well-researched. They have wide-ranging effects on their host plants; some are beneficial to the plant while others are harmful. For example, some foliar endophytes are capable of reducing disease severity by

antagonizing pathogens (Ganley et al., 2008, Busby et al., 2016, Ridout & Newcombe, 2016). This may be due to inhibitory metabolites (Sturz et al., 1998, Malinowski et al., 1999, Vazquez-de-Aldana, 2011, Weber, 1981, Miller, 1985), although other direct and indirect mechanisms are possible. However, foliar endophytes can also have negative consequences on plant fitness. They can stunt growth or inhibit seed germination (Newcombe et al., 2009) or repel natural enemies of herbivores (Preszler et al., 1996).

Seed endophytes have been conserved during the 9,000-year domestication history of the wild ancestors of maize (*Zea mays*), demonstrating the adaptive roles these microbes play in contributing to overall plant fitness (Johnston-Monje & Raizada, 2011). However, seed endophytes have been less studied than foliar and root communities. Some fungal seed endophytes, like their foliar counterparts, have displayed antagonism towards plant pathogens. For example, Herrera et al. (2016) demonstrated that wheat seed endophytes inhibited growth of the pathogen *Fusarium graminearum*. Similar findings have been reported from rice (Mukhopadhyay et al., 1996) and eggplant (Ramesh et al., 2009). Bacterial seed endophytes, in particular, have been shown to be powerful symbionts. Some produce plant growth-promoting hormones such as IAA (Truyens et al., 2014, Herrera et al., 2016). Others help plants in environments contaminated with heavy metals (Truyens et al., 2014).

Optimal defense theory builds on this idea of strongly antagonistic endophytes in seeds. In plants, it is expected that costly seeds will be more strongly defended than more expendable leaves from invading microbes. Individual seeds do most often host single, culturable endophytes, whereas individual leaves host diverse endophytes (Newcombe et al, 2018; Ganley & Newcombe, 2006); seed endophyte diversity for bulked seeds is also lower overall than its vegetative counterpart (Compant et al., 2011). However, strong host defense of seeds by itself does not imply that seed endophytes will be especially strong antagonists but, the exclusion of one seed-infecting microbe by another does (Raghavendra et al., 2013).

As would be expected, seeds and needles of *Pinus monticola* host little and great diversity of endophytes, respectively (Ganley & Newcombe, 2006). Isolation frequency is low overall among seeds so no dominant seed endophyte is known, whereas needles host the dominant *Lophodermium* complex, of which *L. nitens* is the most common member (Ganley and Newcombe, 2006). Here, we extend our prior, descriptive research on endophytes of *Pinus monticola* by hypothesizing that seed endophytes of *Pinus monticola* will be stronger antagonists, and interactors generally, than their needle counterparts including the dominant *L. nitens*. This difference in interaction strength should further allow individual microbes to be correctly assigned to their community of origin.

Materials and Methods

Endophyte isolation and identification

Three hundred *Pinus monticola* needles were collected from the R.T. Bingham Seed Orchard in Moscow, Idaho. A total of 1,100 *P. monticola* seeds were provided by the Intermountain Forestry Cooperative, also from the Bingham Seed Orchard. Both the seeds and needles were separately surface-sterilized in 96% ethanol for 1 minute, followed by 6% sodium hypochlorite (NaOCL) for 5 minutes, and finally, 96% ethanol for 30 seconds (Ganley & Newcombe, 2006). The needles and seeds were then plated onto 4% potato dextrose agar (PDA) plates. A random selection of seeds and needles were imprinted on PDA plates in order to ensure successful surface-sterilization (the lack of fungal and/or bacterial growth on these plates was confirmed). Plates were incubated at 25°C while the endophytes grew out of their respective tissues. After 14-21 days, pure cultures were obtained.

Since there have been reports of bacteria in conifer needles based on 16S rRNA sequencing (Carrell & Frank, 2014, Carrell et al., 2016), we attempted to isolate them. *Gluconacetobacter*, directly sequenced in needles, has also successfully been cultured on selective media, such as LGI medium (Cavalcante & Dobereiner, 1988). Based on this, twenty-five needles of *Pinus monticola* were surface-sterilized using the above method and plated on LGI medium to obtain bacterial isolates. Twenty-five, surface-sterilized needles were also plated on both nutrient agar plates and LB plates (5 grams tryptone, 2.5 grams yeast extract, 5 grams NaCl, 7.5 grams agar, 500 mL water).

Overall design

Three communities were considered: seed bacteria, seed fungi and needle fungi. We also attempted to include needle bacteria but were unsuccessful in culturing such endophytes on PDA. With eight representatives of each of three communities to be confronted in *in agaro* interactions, and with three replicates of each interaction, a total of 1,728 plates were used to test the hypotheses. Examples of the different assays performed can be seen in Figure 1.1. Percent inhibition (i.e., antagonism) of growth was calculated for each organism in each interaction. Stimulation of fungal growth was seen in several endophyte-endophyte interactions; this was reflected in a negative value for inhibited growth. Because our primary focus in this study was growth inhibition and since bacteria were treated in a different manner due to their growth patterns, we did not record and measure growth stimulation of bacteria.

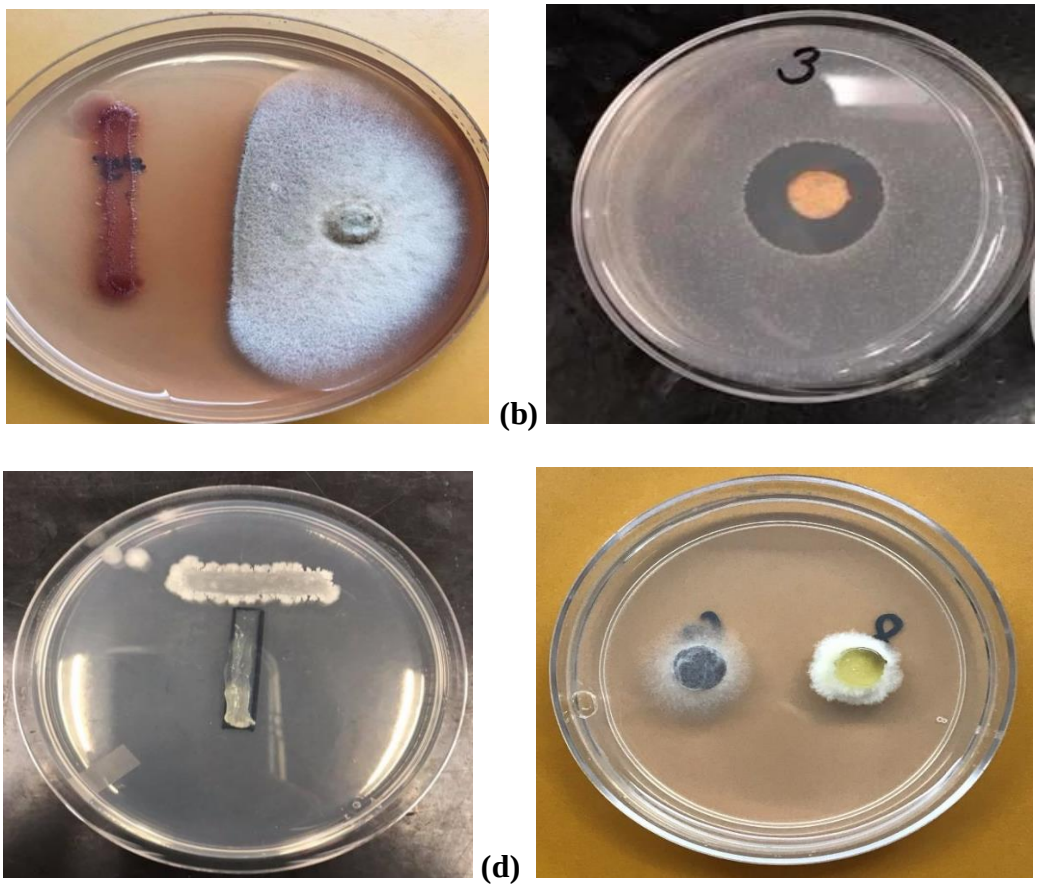

Figure 1.1. Different dual culture assays that were carried out to measure percent inhibition of growth. (a) Bacterium vs. fungus. (b) Fungus vs. bacterium. (c) Bacterium vs. bacterium. (d) Fungus vs. fungus.

Antagonism (growth inhibition) assays *in agaro*

Antagonism of seed bacteria towards seed/needle fungi

Bacteria were grown in LB broth for 48 hours. A 40 mm streak was made at one end of a 60 x 15 mm PDA plate. A 7 mm agar plug of actively growing fungus was then placed 2 cm away from the streak. All plates were repeated in triplicate. Plates were incubated at 25°C for 6 days.

Antagonism of seed/needle fungi towards seed bacteria

The agar overlay method (Schmalz, 1988) was used with the following modifications: Bacteria were grown with shaking (200 rpm) at 25°C in LB broth for 24 hours. Pre-solidified 60 x 15 mm plates of PDA were utilized for the bottom layer in this procedure. The top agar layer was composed of 1.5% PDA that had been autoclaved and slightly cooled, but not solidified. 20 µL of liquid bacteria culture were added per 1 mL of top agar. 3 mL of each top agar/bacteria combination was added to each solidified plate of PDA. After the top agar solidified, a 7 mm agar plug of actively

growing fungus was placed in the center of the plate. Control treatments received a plain PDA 7 mm plug. All plates were repeated in triplicate. The plates were incubated at 25°C for 48 hours.

Antagonism of seed bacteria towards each other

The cross-streak method was used according to Velho-Pereira & Kamat (2011). Two perpendicular rectangles, each measuring 3 cm x 0.5 cm were drawn on the bottom of a PDA plate. The distance between the rectangles was 1 cm. Separate bacteria were then inoculated in the respective rectangles. All plates were repeated in triplicate. The plates were kept at 25°C for 48 hours. The bacterium streaked across the horizontal rectangle was considered the antagonist while the bacterium streaked across the vertical rectangle was considered the bacterium being antagonized. Inhibition was calculated as the percent area of the vertical rectangle that was lacking bacterial growth.

Antagonism of seed/needle fungi towards each other

7 mm agar plugs of actively growing fungi were plated in dual culture. The plugs were plated 2 cm away from each other. All plates were repeated in triplicate. The plates were incubated at 25°C for 6 days.

Control plates

Fungi:

Since inhibition of growth may occur solely as a result of nutrient competition with other fungi *in agaro* and not due to the presence of antagonistic chemicals, two different plates were used to obtain the overall control value for fungal vs. fungal interactions. The first plate consisted of a 7 mm plug of a fungus co-cultured 2 cm away from a 7 mm plug of the same fungus taken from the same original culture. Any growth inhibition of self was viewed as nutrient competition, as the fungus won't chemically antagonize itself. The second control plate consisted of only one 7 mm fungal plug to determine growth in the absence of intraspecific competition. The overall control value was taken by using the following equation for the eight seed fungi and the eight needle fungi when plated against other fungi:

$$\frac{\text{Avg. diameter of growth in solo assay} - \text{Avg. diameter of growth in co-cultured assay}}{\text{Avg. diameter of growth in solo assay}} \times 100 = \text{Average control value}$$

When antagonism of seed bacteria towards fungi was being measured, similar control plates were used; however, only one plate was used, and the fungus was co-cultured with a streak of LB broth. The average fungal growth was used as a control value.

Bacteria:

A similar method was used in bacterial control plates, except the cross-streak method was used (see above). The first control plate consisted of the bacterium co-cultured with itself. Again, any growth inhibition was viewed as nutrient competition. The second control plate consisted of only one rectangle with the bacterium. The overall control value was taken by using the following equation for the eight seed bacteria:

$$\frac{\text{Avg. area of growth in solo assay - Avg. area of growth in co-cultured assays}}{\text{Avg. area of growth in solo assay}} \times 100 = \text{Average control value}$$

Data Collection

In all interactions between different endophytes, the following formula was used to determine percent inhibition:

$$\frac{\text{Control} - \text{Treatment}}{\text{Control}} \times 100$$

Statistical Analysis

All descriptive data analysis was conducted in Microsoft Excel. Statistical analyses (Kruskal-Wallis and Wilcoxon rank sum test, and clustering) was conducted in RStudio (Version 1.1453; https://www.r-project.org). Hierarchal clustering was completed using the 'plotbest' command to determine the best distance and clustering method. The 'hclust' and 'Jclust' commands were used to build the actual tree (Shipunov, 2019).

Fungal DNA Extraction and Sequencing

Fungal genomic DNA was extracted using the REDExtract-N-Amp Plant DNA Kit. The manufacturers protocol was followed with a few exceptions: less than <0.5 cm^2 fungal tissue was added to 20 μL of the Extraction Solution and 60 μL of the Dilution Solution was used. The ITS1-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) forward and LR3 (5′-CCGTGTTTCAAGACGGG-3′) reverse primers were used to amplify the internal transcribed spacer (ITS) and a portion of the nuclear large subunit (LSU) region for each fungal isolate (Raja et al. 2017). The PCR reactions were 25 μL in volume, including 4 μL of genomic DNA. Gel electrophoresis was used to visualize PCR products. If bands were present, products were sent to MCLAB (San Francisco, California) for sequencing and ExoSAP PCR clean-up. SeqTrace was used to trim and pair forward and reverse reads. The final reads were used in BLAST queries to determine taxonomic identity.

Bacterial DNA Extraction and Sequencing

Bacterial strains used in this study were grown for 24 hours in 5 mL of nutrient broth yeast extract (NBY) broth (Vidaver, 1967) at 28°C with agitation (200 rpm) and used for DNA isolation. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Fitchburg, WI) following the protocol for Gram-positive bacteria. Amplification of the 16S rRNA gene was completed using the universal primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') (Lane, 1991) and 1492r(l) (5'-CCTTGTTACGACTTC-3') (Fessehaie et al., 2002) and an Eppendorf Mastercycler ep gradient S thermocycler (Eppendorf AG, Hamburg) programmed with a 2 min denaturation at 95°C followed by 31 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 2 min, a final extension at 72°C for 5 min followed by a 15°C hold. The reaction mixture contained 5 µl 5X Promega GoTaq Buffer, 0.3 mM dNTPs, 1.25 µl DMSO, 2 µM of both 27f and 1492r(l) primers, 1.25 U Promega GoTaq, 1 µl of genomic DNA (40 ng/µl), and 12.5 µl sterile water for a total volume of 25 µl. PCR products were electrophoresed and visualized on 0.8% agarose gels, cleaned with ExoSAP-IT (USB Corp., Ohio, USA) and sequenced by Elim Biopharmaceutical (Hayward, CA) using the GC rich protocol. 16S rRNA sequences obtained from two separate amplicons were assembled using Geneious software (Biomatters Inc., Newark, NJ). BLAST analysis (Altschul et al., 1990) was used to identify 16S rRNA gene sequences with significant homology to the obtained sequences.

Results

As in previous studies (Ganley and Newcombe, 2006), endophytes were much more frequently isolated from needles than from seeds of *Pinus monticola*. A total of eight bacterial isolates (one per each of eight seeds) were obtained from an initial lot of 300 seeds, whereas almost all 300 needles yielded multiple fungal isolates from which we chose the most common. To obtain seed fungi for hypothesis-testing, an additional set of UI Bingham Orchard seeds were used, and those 800 seeds yielded eight fungal isolates in total (again, one per seed). Six of the eight seed fungi belonged to *Penicillium*; single isolates of *Fusarium* and *Aspergillus* were also obtained, and all were identified at the Westerdijk Fungal Biodiversity Institute (Table 1.1). *Lophodermium s.l.* was the most common foliar endophyte as it was present in over 75% of the needles; the isolate that we used as representative belonged to *L. nitens*, which is the most common taxon in the endophytic *Lophodermium* complex in *Pinus monticola* (Ganley and Newcombe, 2006). Of the other isolates representing six genera of needle fungus, two belonged to *Elytroderma* but they differed in ITS sequences (Table 1.2). Their culturability on PDA distinguished them from the unculturable *E. deformans* that is common to the region as a pathogen of species of *Pinus* subgenus *Pinus* (Ganley et

al, 2004; Ganley and Newcombe, 2006), so we refer here to each of the culturable, endophytic isolates of *Elytroderma* as *Elytroderma sp. nov*. The seed bacterial isolates all belonged to *Bacillus* (Table 1.3) with placement there based on 16S sequencing. The only pair of identical 16S sequences belonged to SB5 and SB6, isolates of *B. amyloliquefaciens*. SB5 seed isolate was identical to SB6 in 16S sequence but the two differed in colony morphology; subsequently, they also proved to differ in interaction (Table 1.4). Thus, each of the 24 total isolates used in the interaction studies was a unique entity. Finally, bacteria were not isolated into PDA culture from needles.

Table 1.1. Morphology- and sequence-based identification and GenBank accession numbers for *Pinus monticola* seed fungal endophytes.

Isolate	**Species identification**	**GenBank accession number**	**Most similar GenBank accession**	**Source of most similar GenBank accession**
SF1	*Penicillium sajarovii*	MK226542	KP152491	*Pinus ponderosa* foliar tissue
SF2	*Penicillium* sp. 5 (*yarmokense-arizonense* species complex)	MK226541	MF974901	*Pseudotsuga menziesii* root tissue
SF3	*Penicillium hordei*	MK226540	KC175293	*Lupinus albus* seed
SF4	*Fusarium pseudocircinatum*	MK211243	MG838067	*Swietenia macrophylla,* Mexico
SF5	*Penicillium sp. nov.*	MK226539	KP152491	*Pinus ponderosa* foliar tissue
SF6	*Penicillium palitans*	MK410955	AY674363	Mouldy liver paste
SF7	*Penicillium sajarovii*	MK226537	KP152491	*Pinus ponderosa* foliar tissue
SF8	*Aspergillus proliferans*	MK211244	MK267410	Unpublished

Table 1.2. Morphology- and sequence-based identification and GenBank accession numbers for *Pinus monticola* needle fungal endophytes. Note that NF2 and NF4 differed in ITS sequence, and interaction (Table 1.4) and that neither could be assigned to the common but PDA-unculturable *Elytroderma deformans*.

Isolate	**Species identification**	**GenBank accession number**	**Most similar GenBank accession**	**Source of most similar GenBank accession**
NF1	*Aureobasidium pullans*	MK211236	JX188096	*Vitis vinifera*, Washington State
NF2	*Elytroderma sp. nov.*	MK211237	AF203469	unpublished
NF3	*Coniothyrium sp.*	MK211238	MH871969	*Sarothamnus* dead twig/pod, Netherlands
NF4	*Elytroderma sp. nov.*	MK211239	KP152488	*Pinus ponderosa* foliar tissues, Idaho
NF5	*Anthostomella conorum*	MK188934	EU552099	*Protea neriifolia*, South Africa
NF6	*Cladosporium herbarum*	MK211240	JF311953	*Cicer arietinum* rooting soil, Saskatchewan
NF7	*Alternaria sp.*	MK211241	KR094465	*Echinacea purpurea* seed
NF8	*Lophodermium nitens*	MK211242	MG877446	*Pinus monticola*, California

Table 1.3. 16S-based identification and GenBank accession numbers for *Pinus monticola* seed bacterial endophytes.
Note that SB5 was identical to SB6 in sequence but differed in morphology and in interaction (Table 1.4).

Isolate	Species identification	GenBank accession number	Most similar GenBank accession	Source of most similar GenBank accession
SB1	*Bacillus velezensis*	MK214998	MG547922	Activated sludge, China
SB2	*Bacillus pumilus*	MK214999	MK342521	Unpublished
SB3	*Bacillus velezensis*	MK215000	MK097357	Bioaerosol, India
SB4	*Bacillus subtilis*	MK215001	LR535809	Marine water, India
SB5	*Bacillus amyloliquefaciens*	MK215002	MK337677	Unpublished, Car Nicobar Island
SB6	*Bacillus amyloliquefaciens*	MK215003	MK337677	Unpublished, Car Nicobar Island
SB7	*Bacillus sp.*	MK215004	MK229036.1	Wheat grain, Israel
SB8	*Bacillus sp.*	MK215005	MK229036.1	Wheat grain, Israel

Antagonism by seed bacteria

As hypothesized, seed bacteria (*Bacillus* spp.) were the strongest antagonists of the three communities (Figure 1.2). Seed bacteria inhibited seed fungi more than seed fungi inhibited themselves (22.03% ± 2.09% vs. 7.03% ± 1.05%; p= 0.002). Seed bacteria inhibited the needle fungi the most, with an average growth inhibition of 42.7% ± 2.66% (Figure 1.2). This inhibitory activity towards needle fungi was almost threefold the antagonistic activity of needle fungi among themselves [15.60% ± 1.89%] ($p=1.16 \times 10^{-14}$). Seed bacteria were also the least antagonized community, and significantly more antagonized by themselves, the seed bacteria, than they were by the other two communities of seed and needle fungi. The strongest individual antagonist overall, among all three communities, was SB-2, an isolate of *Bacillus pumilus*.

Antagonism by seed fungi

Seed fungi (six isolates of *Penicillium* taxa, *Fusarium pseudocircinatum* and *Aspergillus proliferans*) were not as antagonistic as seed bacteria but more strongly antagonistic than needle fungi (Figure 1.2). Seed fungi inhibited or antagonized needle fungi the most [17.12% ± 2.10%], although there was no significant difference between this antagonism and the antagonism of needle fungi towards

each other (15.60% ± 1.89%; p= 0.37). Seed fungi displayed very little antagonism towards seed bacteria (1.27% ± 0.17%); however, it was a significantly higher level of antagonism ($p = 3.53 \times 10^{-6}$) than the needle fungi displayed towards seed bacteria (0.35% ± 0.09%).

Antagonism by needle fungi

Needle fungi (*Lophodermium nitens*, two isolates of *Elytroderma sp. nov*. and five other common foliar endophyte taxa: *Aureobasidium*, *Coniothyrium*, *Anthostomella*, *Cladosporium*, and *Alternaria*) were the least antagonistic of the three communities. Average percent inhibition was highest against members of their own community (15.06% ± 1.89%) (Figure 1.2).

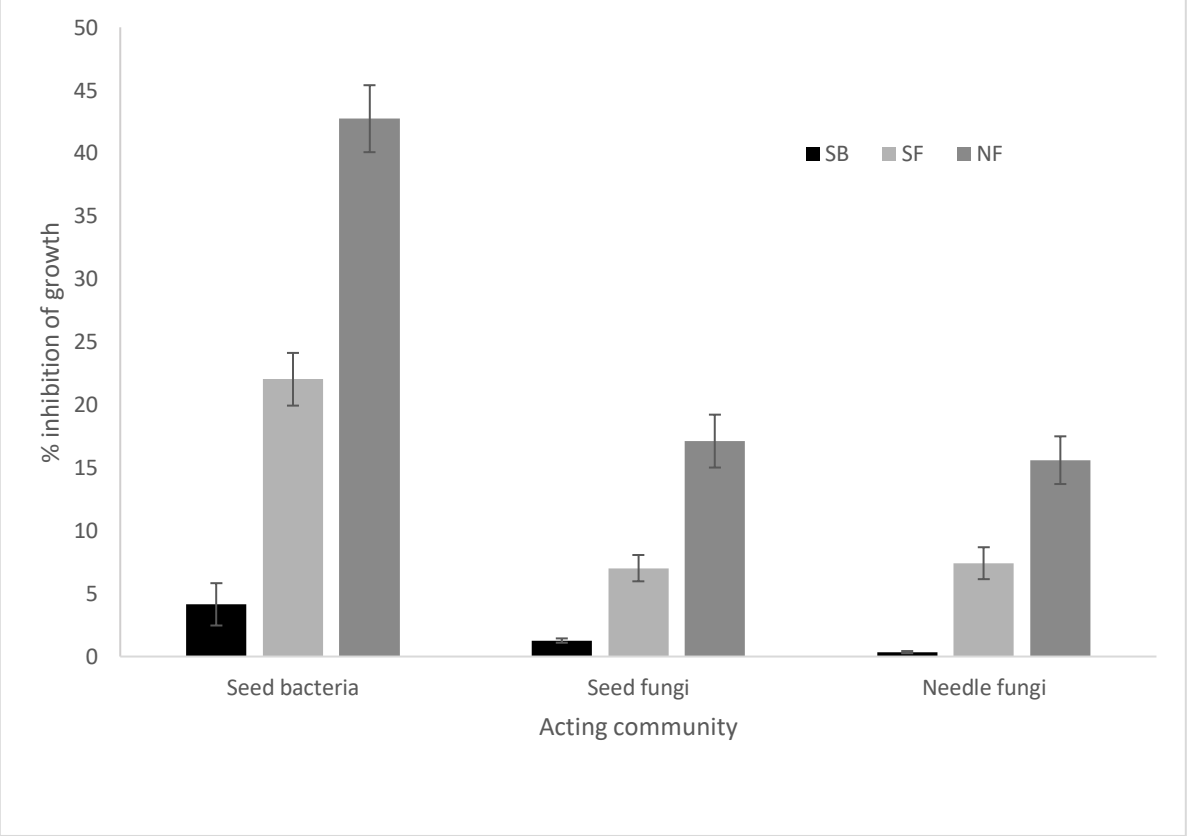

Figure 1.2. Antagonism (inhibition of growth) on the y-axis by each acting community on the x-axis. Thus, each cluster of three shaded bars represents the communities that are being antagonized by a particular acting community. Error bars represent ± SEM. Seed bacteria were the strongest antagonists and the least susceptible community to antagonism.

Stimulation

Stimulation of growth (negative percent inhibition) was also observed (Figure 1.3), although it was somewhat tangential to the study of antagonism, and stimulation of seed bacteria was not observable with our current assays. Only stimulation of the two fungal communities was observed. Seed bacteria stimulated seed fungi more than they stimulated needle fungi [22.42% ± 3.13% vs. 8.94% ± 4.05%] (p= 0.00024). Seed fungi stimulated seed and needle fungi equally (p= 0.46). Needle fungi significantly stimulated members of their own community more than they stimulated seed fungi (12.11% ± 2.39% vs. 5.29% ± 0.82%; p= 0.02), the opposite of the effect of seed bacteria.

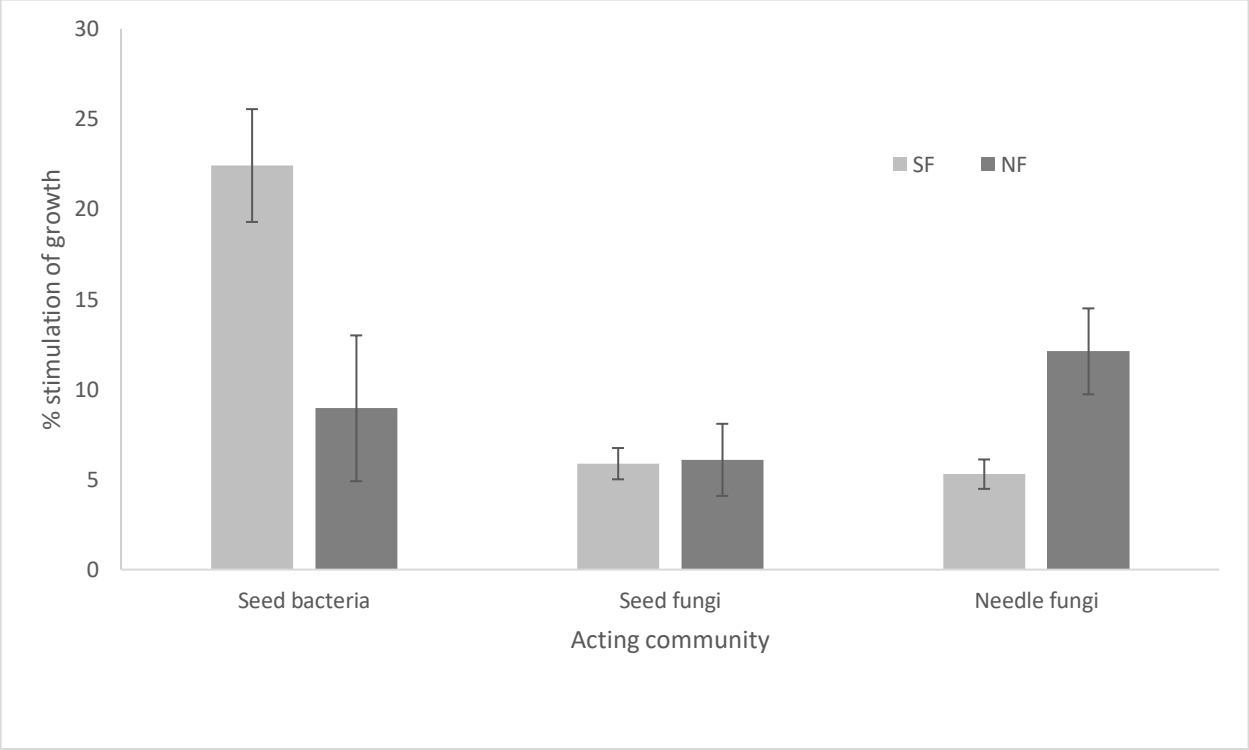

Figure 1.3. Average percent stimulation of growth by each acting community. Respective communities along the x-axis represent the community that is doing the stimulating. The different shaded bars represent the community that is being stimulated. Error bars represent ± SEM.

Of the two fungal communities, seed and needle, tested for stimulation by individual endophytes (Table 1.4) only the former was stimulated by seven isolates. The greatest individual stimulator of seed fungi was the 'SB-6' seed bacterium, an isolate of *Bacillus amyloliquefaciens*.

Table 1.4. Ranking and interactions of endophytes. The absolute value of interactions was used to calculate interaction strength for each endophyte, which is represented in the second column. The average interaction strength was used to rank the endophytes accordingly. The third through fifth columns represent average inter- and intracommunity interactions for each individual endophyte. Both inhibitory and stimulatory interactions were used in these columns. Negative values represent overall negative inhibition (or stimulation of growth).

Endophyte ID (from strongest down)	**Average effect on seed bacteria**	**Average effect on seed bacteria**	**Average effect on seed fungi**	**Average effect on needle fungi**
SB2	30.74	13.89	19.51	58.84
SB1	29.30	6.81	26.67	54.45
SB8	26.08	8.89	18.04	43.39
SB4	24.78	0.00	4.36	48.67
SB5	24.14	3.61	20.27	45.11
SB7	17.04	0.00	3.09	29.25
SB3	13.82	0.00	-1.06	26.33
SB6	12.94	0.00	-9.07	10.01
SF8	10.87	0.76	1.31	25.16
SF2	10.14	2.76	-2.58	19.83
SF4	9.61	0.00	2.73	22.25
SF5	9.24	2.04	-0.38	18.75
NF3	9.22	0.20	1.31	21.84
SF7	9.15	2.05	1.72	16.67
NF6	9.12	0.16	3.74	0.49
NF2	8.00	1.06	0.92	12.08
NF5	7.35	0.84	-3.23	2.23

NF1	6.75	0.00	0.33	10.86
NF7	6.49	0.00	5.72	5.54
SF3	5.45	1.45	-0.47	6.74
NF4	5.32	0.44	0.90	4.72
NF8	4.49	0.00	0.40	1.49
SF6	4.45	0.32	-1.49	3.60
SF1	3.70	0.79	5.34	0.79

Overall interaction strength of individual endophytes

Microbial growth varied little between the three replicate plates; the average coefficient of variation between replicates for all 576 interactions was 5.37%. Average interaction strength of each endophyte reveals that the strongest interactors overall were seed bacteria. The strongest interactor, SB2 (*Bacillus pumilus*) had an average interaction strength (30.74) that was ten times higher than that of the weakest interactor (3.70), SF1 (*Penicillium sajarovii*) (Table 1.4). The gap was greater when only antagonism was considered. 90.3% of the effects of individuals on communities were antagonistic overall. 9.7% were stimulatory and are represented by negative values (i.e., stimulation of growth) (Table 1.4).

A Kruskal-Wallis test showed that the means of the overall interactions (whether inhibitory or stimulatory) of each community also differed (χ^2= 36.47, p= 1.204 x 10^{-8}). Pairwise comparisons using Wilcoxon rank sum test (Bonferroni p-value adjustment) indicated that seed bacteria interactions significantly differ from both seed and needle fungi (p= 8.9 x10^{-5} and p= 2.9x 10^{-8}, respectively.) Seed fungi and needle fungi interactions also differ from each other, but on a smaller scale (p= 0.014).

Table 1.5. Summary of inhibitory and stimulatory activity of each community towards foliar endophyte *Lophodermium.*

	Seed bacteria	**Seed fungi**	**Needle fungi**
Number of inhibitory interactions (out of 8)	7	7	5
Identity of inhibiting microbes	*B. velezensis* (SB1), *B. pumilus* (SB2), *B. velezensis* (SB3), *B. subtilis* (SB4), *B. amyloliquefaciens* (SB5), *Bacillus sp.* (SB7), *Bacillus sp.* (SB8)	*Penicillium.* sp. 5 (*yarmokense-arizonense* species complex) (SF2), *P. hordei* (SF3), *F. pseudocircinatum* (SF4), *P. sp. nov* (SF5)., *P. palitans* (SF6), *P. sajarovii* (SF7), *A. proliferans* (SF8)	*A. pullans* (NF1), *Elytroderma. sp. nov* (NF2), *Coniothyrium. sp.* (NF3), *A. conorum* (NF5), *Alternaria sp.* (NF7)
Average percent inhibition	44.66% ± 9.76%	18.95% ± 5.53%	32.37% ± 5.38%
Number of stimulatory interactions (out of 8)	1	0 (the 8th interaction was neither inhibitory or stimulatory)	3
Identity of stimulating microbes	*B. amyloliquefaciens*	---	*Elytroderma sp. nov* (NF4), *C. herbarum* (NF6), *L. nitens* (NF8)
Average percent stimulation	3.44%	---	28.57% ± 12.89%

Lophodermium nitens

We concentrated on *L. nitens* because it is be the most abundant endophyte overall at the crown level in trees of *Pinus monticola* (Ganley et al, 2004; Ganley and Newcombe, 2006). In spite of their overall abundance in tree crowns, neither L. *nitens* nor any other *Lophodermium* has ever been isolated from seed of *Pinus monticola*. Table 1.5 displays the number of inhibitory and stimulatory interactions for each of the three communities towards *L. nitens*. Overall, 14/16 individual seed bacteria and seed fungi were inhibitory toward *L. nitens.* Seed bacteria strongly inhibited *L. nitens* growth [44.66% ± 9.76%].

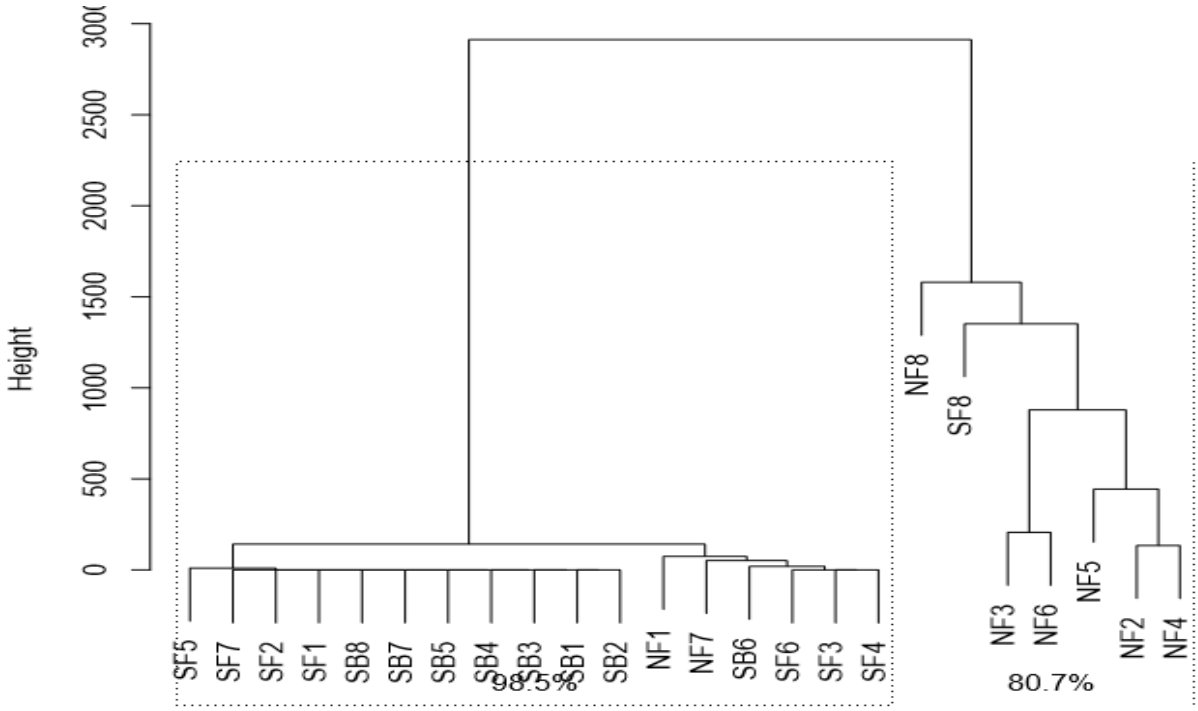

Figure 1.4. Bootstrapped consensus tree (1000 replicates) produced by hierarchal clustering of pair-wise endophyte interactions.

Clustering

Hierarchal clustering of all individual endophytes based solely on their interactions, and constrained to two clusters, resulted in a first cluster of 15 of the 16 seed microbes and a second comprised of 6 of the 8 needle fungi (Figure 1.4).

Discussion

A broad definition of community typically describes a unit or group of living organisms with common behaviors and/or identities that share a territory. Most definitions emphasize interactions (Fauth et al., 1996). However, with respect to endophyte communities, the interaction component is most often absent, and in its place are lists of species, genera and families, or just operational taxonomic units derived from high-throughput sequencing. Some of these taxa might then be assigned to guilds, but these assignments are impossible for undescribed and even for many described fungi (Nguyen et al, 2016), and again, interaction is not directly determined even for assignable taxa.

In this study, we defined communities as either bacteria or fungi (identity at the level of kingdom) and as either needle- or seed-based (i.e., territory). The justification for the first criterion is as codified as the traditional separation of bacteriology and mycology; bacteria and fungi have different growth patterns and utilize resources differently (Frey-Klett et al, 2011). Seeds and needles of *Pinus monticola* are distinct territories in terms of known endophyte community membership (Ganley and Newcombe, 2006); they also differ in a more general sense in that seeds are strongly defended and the sites of exclusionary, endophyte-endophyte interactions in *Centaurea* and possibly many other plants (Raghavendra et al, 2013; Newcombe et al, 2018). Seeds and needles also differ as territories at the crown level of a white pine tree in that seeds must be much less common and more variable across years than needles, even though quantified, seed:needle ratios are not known.

Our communities, defined as above, were distinct in terms of interaction *in agaro*. As hypothesized, the eight strongest antagonists, and interactors overall, were the eight individual seed bacteria, all of which belonged to *Bacillus* (Table 1.2). Seven of the eight had stronger antagonistic effects on needle fungi than any of the 16 individual fungal isolates did. Seed fungi were moderate interactors as a group, although two of them were the weakest overall (Table 1.4). Needle fungi were the weakest overall, although all eight were stronger interactors than the very weakest seed fungi (i.e., SF1, *Penicillium sajarovii*, and SF6, *Penicillium palitans*). NF8, *L. nitens*, the most abundant endophyte in crowns of *Pinus monticola* (Ganley and Newcombe, 2006), was nearly as weak an interactor as SF1 and SF6.

Microbes are known to engage in chemical warfare with one another (Schulz et al., 2002, Cho et al, 2007). These interactions are commonly observed *in agaro* by all microbiologists, and they have been the basis not only for Fleming's famous discovery of penicillin (1929), but for many other useful microbes and their products (e.g., Kunova et al, 2016). Interactions can also be strong *in planta*, and the strongest example of which we are aware is the *in planta* exclusion of specific microbes by others that are seen at the level of individual seeds (Raghavendra et al., 2013). *In agaro* results often predict *in planta* outcomes with endophytes (Ramesh et al., 2008, Passari et al., 2015, Senthilkumar, 2008), but not always (Bevivino et al., 1998, Long et al., 2004).

Strong interactors, especially strong antagonists, would seem to be ideal candidates to control plant pathogens in agriculture and forestry. However, our results suggest that strong interactors are relatively rare and weak interactors relatively abundant in the foliage of trees of *Pinus monticola*. For any endophyte to be relatively abundant at a canopy level it would have be to present in needles, and we did not obtain even a single isolate of *Bacillus*, *Penicillium*, *Aspergillus* or *Fusarium* from needles. From seeds of *Pinus monticola* the only endophytic bacteria and fungi that we isolated were these species. Yet not a single one of them was found at high isolation frequency,

so their relative abundances must be very low at the crown level compared to *L. nitens* and other members of the needle fungus community. Species of *Bacillus* are not only known from other studies to be strong antagonists of pathogens (e.g., Walker et al., 1988; Dong et al., 2004; Chaurasia et al., 2005) but our isolates of this taxon were far and away the strongest interactors in our study. One obvious inference to be drawn is that relative abundance in foliage is not a function of interaction with other microbes. Instead, ability to infect young needles may be paramount. This would mean that relative abundance in needles would be more a function of traits typically studied by plant pathologists: the timing of sporulation in relation to emergence of needles and weather conducive to infection of tissues that are still susceptible.

Seed bacteria such as the species of *Bacillus* of this study might have to be used inundatively as antagonists to overcome their natural rarity. We have experimented with an isolate of *Streptomyces* from white pine seed that when applied inundatively to poplar leaves prevented the development of any *Melampsora* rust (Marlin et al, unpublished). We are thus contemplating the use of both that *Streptomyces* as well as the *Bacillus* endophytes of this study to antagonize the most serious pathogen of white pine: the white pine blister rust fungus. Needle endophytes and pathogens might be especially susceptible to strong antagonists to which they have been minimally exposed.

The absence in seed of needle endophytes (Deckert & Peterson, 2000, Sumarah et al., 2015) might be explained by the combination of the optimal defense of seeds and the antagonistic abilities of *Bacillus* species and other members of the seed community. *Lophodermium nitens* was not isolated once from 1,100 *P. monticola* seed in this study, nor in a prior study (Ganley & Newcombe, 2006), but it was present in almost every single one of the needles of both studies. It is interesting that seven of the eight seed bacteria inhibited the growth of *L. nitens* (Table 1.3). Seed fungi were also antagonistic towards *Lophodermium*, whereas needle fungi were less so. Three needle fungi even stimulated the growth of *L. nitens.*

The seed niche is less dependable for endophytes than foliage. Seeds only appear with tree maturity; *Pinus monticola* does not produce cones until seven years of age at the youngest (Graham, 1990). Cone crops also vary considerably across years, and cone insects and weather events can result in the loss or destruction of a cone crop (Owens & Molder, 1977). One might therefore think that seed microbes ought to be found in foliage occasionally. Yet, none of the seed taxa from this study were isolated from needles. This was true even of *Penicillium*. Ganley & Newcombe (2006) also did not isolate any *Penicillium* from 750 *P. monticola* needles. Ridout et al. (2017) isolated, albeit rarely, *Penicillium* from both *Pinus ponderosa* and *Pseudotsuga menziesii* needles, but not from *P. monticola* needles. Additionally, Larkin (2012) found only negligible amounts of

Penicillium in his study of *P. monticola* needles. Interestingly, *Penicillium* appears to be seed-specialized in *Pinus monticola*.

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Chapter 2: The Effect of Gene Dosage on Hybrid Poplar Resistance to Rust

Abstract

Poplar rusts belonging to the genus *Melampsora* can have devastating effects on commercial poplar yields. Qualitative, major-gene resistance is often not durable, as the pathogen continues to evolve in ways that allow it to go unrecognized by the plant. Quantitative resistance can be more durable, but little is known about such resistance in poplar. Copy number variation (CNV) especially of genes critical in resistance can alter gene dosage and the resulting proteins that are transcribed. This study investigated the effects of CNV on genetic resistance to *Melampsora* rust. Irradiation was used to create mutant *P. deltoides* × *P. nigra* lines that had insertions or deletions (indels) collectively spanning >99% of the genome. 509 individual genotypes were propagated in the University of Idaho greenhouse and then challenged with a novel hybrid *Melampsora* rust, *M.* ×*sp. nov.,* from coastal California. Rust severity and leaf incidence differed significantly between the genotypes with indels and those without. Significant quantitative trait loci (QTL) for both rust resistance and susceptibility were located on six chromosomes. Rust severity was also found to differ based on leaf position, with LPI 5 often being the most susceptible. This information will be beneficial in making informed decisions regarding breeding poplar for resistance. In this chapter, we also report morphological and genetic characteristics of *M.* × *sp. nov.*, a hybrid between *M. larici-populina* and *M. occidentalis*, that has never before been reported.

Introduction

There are two types of resistance recognized by plant pathologists, qualitative (vertical, or gene-for-gene) resistance and quantitative (horizontal) resistance (van der Plank, 1968). Qualitative resistance is based on molecular recognition between host and pathogen. It occurs when a single plant gene codes for a protein that matches the single-gene protein product of an avirulence gene in the pathogen. When the pathogen tries to infect, the plant recognizes the pathogen effector and mobilizes the plant defense system. Qualitative resistance is not always complete, and it typically involves hypersensitive responses that manifest as necrotic spots (Warren et al., 1998 & Andaya et al., 2003). Regardless, qualitative resistance is usually not durable; pathogen genotypes without the avirulence alleles in question will go unrecognized by the plant and thus be selected and amplified in

the pathogen population (Jones & Dangl, 1992). Pathogenic variation and rapid emergence of new pathogen races can have disastrous consequences, especially in agriculture (Stokstad, 2007).

However, plants can also possess quantitative resistance to help in reducing potential damage from pathogen attack. Quantitative resistance is usually considered the result of multiple plant genes interacting (reviewed in Poland et al., 2008). This type of resistance has historically been known as incomplete, broad-spectrum or non-race specific resistance and can provide partial resistance to different pathogenic species or many races of one pathogen (Wisser et al., 2005, Century et al., 1997). For example, Moore et al. (2015) demonstrated that an allele of wheat gene *Lr67* codes for a protein (Lr67res) that is deficient in glucose transport; thus, there is less sugar available to invading pathogens. Wheat varieties that produce Lr67res are resistant to multiple wheat pathogens, including rusts and powdery mildews (Moore et al., 2015).

Quantitative resistance has been hailed as more durable than qualitative resistance. Scientists are particularly keen on combining genes for resistance into what is known as gene pyramids (Mundt, 2015). However, the seemingly clear-cut difference between quantitative and qualitative resistance may be blurred, as some quantitative resistance is race-specific (Parlevliet, 1978, Cho et al., 2004, Perchepied, et al., 2005). In other words, there is emerging evidence that even quantitative resistance, although in theory more robust, can be overcome by certain races of pathogens. For example, Dowkiw et al. (2010) demonstrated that R_{US}, a supposed quantitative resistance gene in poplar had no effect on the size of uredinia and urediniospore production of six isolates of *Melampsora larici-populina.* The authors hypothesized that R_{US} is actually a "defeated qualitative resistance gene" (Dowkiw et al, 2010). Other pathogens, such as powdery mildews and oomycetes have been shown to defeat quantitative resistance (James & Fry, 1983, Newton & McGurk, 1991). Li et al. (2006) even went as far to propose that race-specific quantitative resistance genes are merely "weaker R-genes." Even though pathogens are adapting to quantitative resistance and not just qualitative resistance factors, quantitative resistance is still considered a valuable option in breeding programs (Jorge et al., 2005).

Qualitative resistance and to a lesser extent, quantitative resistance has received significant attention in the poplar (*Populus*) system. Poplar rust, a polycyclic plant pathogen, causes widespread destruction, especially in cultivated poplar stands (Pinon & Frey, 2005). In fact, it is recognized as the most devastating disease to poplar and their close relative, willows (Vialle et al., 2011). *Melampsora larici-populina* is especially destructive, especially in Europe, its native range (Pinon & Frey, 2005). Some poplar species, such as *Populus deltoides* display qualitative, gene-for-gene resistance to *Melampsora* rust (Dowkiw & Bastien, 2004). However, due to the resulting selection

pressure on the pathogen, as well as the rust's ability to easily adapt even within a growing season, these complete resistances are easily broken down as new races emerge that overcome the host's single-gene recognition factor (Pinon & Frey, 2005). Aiding in the rust's astounding success are two sources of variability in *M. larici-populina*: virulence and aggressiveness (Pinon & Frey, 2005). There are over 200 pathotypes that have emerged, and currently ten virulences of *Melampsora larici-populina* exist that correspond to single R-genes in poplar. (Pinon & Frey, 2005, Frey, personal communication). Compounding the problem for plant pathologists is that single *M. larici-populina* pathotypes can accumulate multiple virulences. In this way, the rust is able to attack more host trees. Miot (1999) found that there is no apparent fitness cost to fungal pathotypes that have accumulated multiple virulences. In addition, aggressiveness can vary between isolates of the same pathotype. (Pinon & Frey, 2005)

Quantitative resistance has been studied in poplar, and hybrid poplar's (*P. deltoides* x *P. trichocarpa*) quantitative resistance to the poplar rust *M. larici-populina* conflicts with the historic definition: only two (not many) genetic factors control this type of resistance (Dowkiw & Bastien, 2004). Quantitative trait loci (QTL) mapping did find that there are minor QTLs in the vicinity of the two major genomic regions, but these did not have broad-spectrum effects (Jorge et al., 2005). The two major quantitative factors, R_{US} and a second region co-localized with qualitative resistance gene R_1, do confer resistance to some races of rust, but the additive effects are strain-specific (Jorge et al., 2005).

Populus nigra, the European black poplar has evolved with *Melampsora larici-populina.* However, scientists have failed to locate either exact qualitative or quantitative factors from this poplar species (Pinon & Frey, 2005). Some studies though have found trends of variation in *M. larici-populina* infection on *P. nigra* clones (Pinon & Frey, 2005, Benetka et al., 2005, & Stochlova et al., 2015). This suggests that *P. nigra* may possess quantitative resistance that has yet to be characterized.

Given the constant flux of pathogen virulence and host resistance, it is now even more critical to explore unfamiliar territory with regards to quantitative resistance. Manipulating copy number variation (CNV) can be a handy tool in this process. CNV refers to chromosomal deletions, insertions or duplications that are at least 1 kb long (Scherer et al., 2007). In plants, these have been known to occur in areas of the genome that are important in defense, such as nucleotide-binding leucine-rich repeat genes and receptor-like kinase genes (Saxena et al., 2014). Leucine-rich repeat areas play a critical role in recognition of a pathogen (Marone et al., 2013). Altered gene dosage because of CNV can also have cascading effects on transcription, posttranscriptional biochemical

pathways and protein interactions and degradation (Veitia et al., 2013). In addition, CNV of candidate genes can change gene expression, increasing or decreasing the proteins transcribed, and possibly resulting in complex phenotypic differences (Stranger et al., 2007). For example, gene expression that results from CNV can affect phenology in wheat (Diaz et al., 2012), stress and disease resistance in barley (Muñoz-Amatriaín, 2013) and nematode resistance in soybean (Cook et al., 2012).

Poplar trees are in high demand for their use as lumber, pulp, and more recently, biofuel (Balatinecz & Kretschmann, 2002). Poplar trees grow rapidly, but regardless, native species are susceptible to rust which can cause widespread tree mortality. The advent of hybrid poplar plantings solved this problem by producing trees that demonstrated rust resistance and were hardy and fast growing (Balatinecz & Kretschmann, 2002). Pure poplar species have massive water requirements; hybrid poplar can also potentially be used to reduce this requirement without sacrificing productivity (Monclus et al., 2005). Europe especially utilizes hybrid poplar; in 2009, 90% of poplar sold by nurseries were *P. deltoides* x *P. trichocarpa* hybrids or *P. deltoides* x *P. nigra* hybrids (Dowkiw et al., 2012). Because of the superior qualities of hybrid poplar, we used a *P. deltoides* x *P. nigra* mutant population of over 500 different genotypes in our study to determine the effect of gene dosage on phenotypic response to rust pressure. This population was provided to us by our collaborators at the University of California Davis and is extensively discussed in Henry et al., 2015. Briefly, gamma-irradiated *P. nigra* pollen was used to fertilize *P. deltoides;* about 55% of the resulting F1 generation possessed dosage lesions (indels) ranging from entire chromosomes to "small fragments". 99.5% of the genome was found to contain at least one indel or CNV. After propagating this population in the University of Idaho Greenhouse, we subjected the hybrid trees to *Melampsora* rust. We conducted three separate inoculations. The first inoculation yielded only slight infection pressure, so for the second inoculation, we did many successive inoculations. The third inoculation focused on leaf plastochron index (LPI), and whether there was differential rust infection based on leaf position. In order to define significant QTLs, we need clear resolution, and the third inoculation searched for which leaf position was most susceptible to rust. Plants such as poplar that can be maintained vegetatively are advantageous to use in studies where genetic mutations are formed. Because the genotypes can be easily cloned, preferred mutations that confer phenotypic benefits can be sustained.

Materials and Methods

Plant Material

Poplar cuttings from the *Populus deltoides* x *Populus nigra* population derived by Henry et al. (2015) were propagated in 1-gallon pots (1 cutting per pot). They were grown in sphagnum peat moss (Premier Horticulture Inc.), supplemented with 4 tablespoons Micromax® micronutrient fertilizer, 2 tablespoons of gypsum, and 1 cup of dolomite per 20 gallons of peat moss. Soil was slightly moistened before cuttings were placed.

Rust Collections

Poplar leaves with rust presumed at the time to be *Melampsora larici-populina* were collected from Andrew Molera State Park (Big Sur, California) in October 2017. Upon further microscopic and genetic analysis (conducted by Pascal Frey's group at INRA), it was determined that spores of this rust did not consistently display pure *M. larici-populina* morphology; instead some morphology consistent with *M. occidentalis* was also observed. Because of this inconsistency, the spore mixture was then sprayed onto leaves of a large variety of poplar clones, including mostly *P. deltoides* x *P. nigra* (D×N) and *P. trichocarpa* x *P. deltoides* (TxD); a low spore density (5000 spores/ml) was used to obtain well-separated individual uredinia. This process was then repeated, but with a higher density of spores (100,000 spores/ml).

A second rust field collection was made at Andrew Molera State Park in October 2018. Five hundred spores from each of eight trees were studied under the microscope. The ratio of spores with *M. larici-populina* morphology to those with *M. occidentalis* morphology was recorded. One hundred spores from five separate uredinia for each of the eight trees were sampled from each of eight trees.

A third rust field collection was made at the same location in December 2018. Samples were observed for the presence of telia.

Genetic Analysis

To investigate this further, qPCR was performed with several primer pairs of different specificities:

Mel F/R/P (Boutigny et al., 2013a) is located in the 28S and amplifies all species of *Melampsora.*

ITS-Mlp is specific to *M. larici-populina.*

Mmd (Boutigny et al., 2013a) is located in the 28S and amplifies *M. medusae (f. sp. deltoidae)* but not *M. medusae (f. sp. tremuloidae)*, nor *M. occidentalis.*

ITS-Mm (Boutigny et al., 2013b) is located in the 28S and amplifies both *M. medusae* races

as well as *M. occidentalis*.

Thirteen monouredinial isolates of this putative hybrid were maintained for further characterization. All were tested by qPCR using the species-specific primer combinations specific to *M. larici-populina* or *M. occidentalis* as outlined above. After two inoculations on our mutant D×N population, rust spores were harvested for a new collection. The morphology of this rust isolate was almost 100% *M. larici-populina*. This collection, hereafter referred to as 17US19, was also sent to Pascal Frey at INRA in France. The 13 monouredinial isolates from the original California sample, as well as 17US19, were then genotyped using microsatellite markers.

Rust Inoculations

Inoculation 1

Due to greenhouse spatial constraints, only one replicate of each genotype was planted. The first rust inoculation took place in late April 2018 when the cuttings were approximately 2 months old. To mimic a natural infection, we used a mister hose attachment to dislodge spores of heavily-rusted leaves over the cuttings. The inoculated cuttings were then covered with painter's plastic overnight.

Inoculation 2

After Inoculation 1, the poplar trees were cut back and allowed to regrow for 2 months. After this time, we inoculated the trees with the hybrid rust in the manner described above. However, instead of doing just one inoculation, we did multiple successive inoculations in an attempt to increase rust severity. Fifteen inoculations were completed over the course of 3 weeks. After data collection, all plants were discarded.

Inoculation 3

A subset of genotypes was constructed based on Inoculation 2's severity results. To make this list, the full set of genotypes was ranked from most resistant to least resistant. Then, every third genotype was selected for testing in Inoculation 3. A total of 166 genotypes were selected, and two replicates of these genotypes were planted. Cuttings were grown for 2 months. To determine whether there was differential rust infection based on LPI, we inoculated LPIs 3, 4, and 5 with a spray suspension of rust spores. The suspension was made by agitating 40 heavily-rusted leaves in a container with 500 mL deionized water. Spray bottles were then utilized to deliver the inoculum to the plants.

Data Collection

Inoculation 1

Uredinia density (uredinia/cm^2) on the most heavily rusted leaf for each plant was counted (severity). Uredinia were counted manually and leaf area was captured using Leafscan Mobile Application (www.leafscanapp.com). In addition, the percentage of leaves on each genotype with rust was also calculated (leaf incidence).

Inoculation 2

While we still only had one replicate per genotype, we considered three stems on each plant as replicates in this experiment. The percentage of the leaf covered with disease (severity) was recorded for the most heavily rusted leaf on each of three stems per genotype. Disease severity was captured by the Leaf Doctor Mobile Application, Version 1.1 (Pethybridge & Nelson, 2015). In addition, the number of leaves with rust on each of three stems was also counted and recorded (leaf incidence).

Inoculation 3

Uredinia density (uredinia/cm^2) of each inoculated LPI (3, 4, 5) was recorded. Uredinia were counted by hand, and leaf area was captured using Leafscan Mobile Application.

Statistical Analysis

Statistical analysis was completed by Heloise Bastiaanse from the United States Forest Service in Davis, CA. The following figure and caption (H. Bastiaanse, personal communication) explains the creation of bins and the calculation of relative gene dosage.

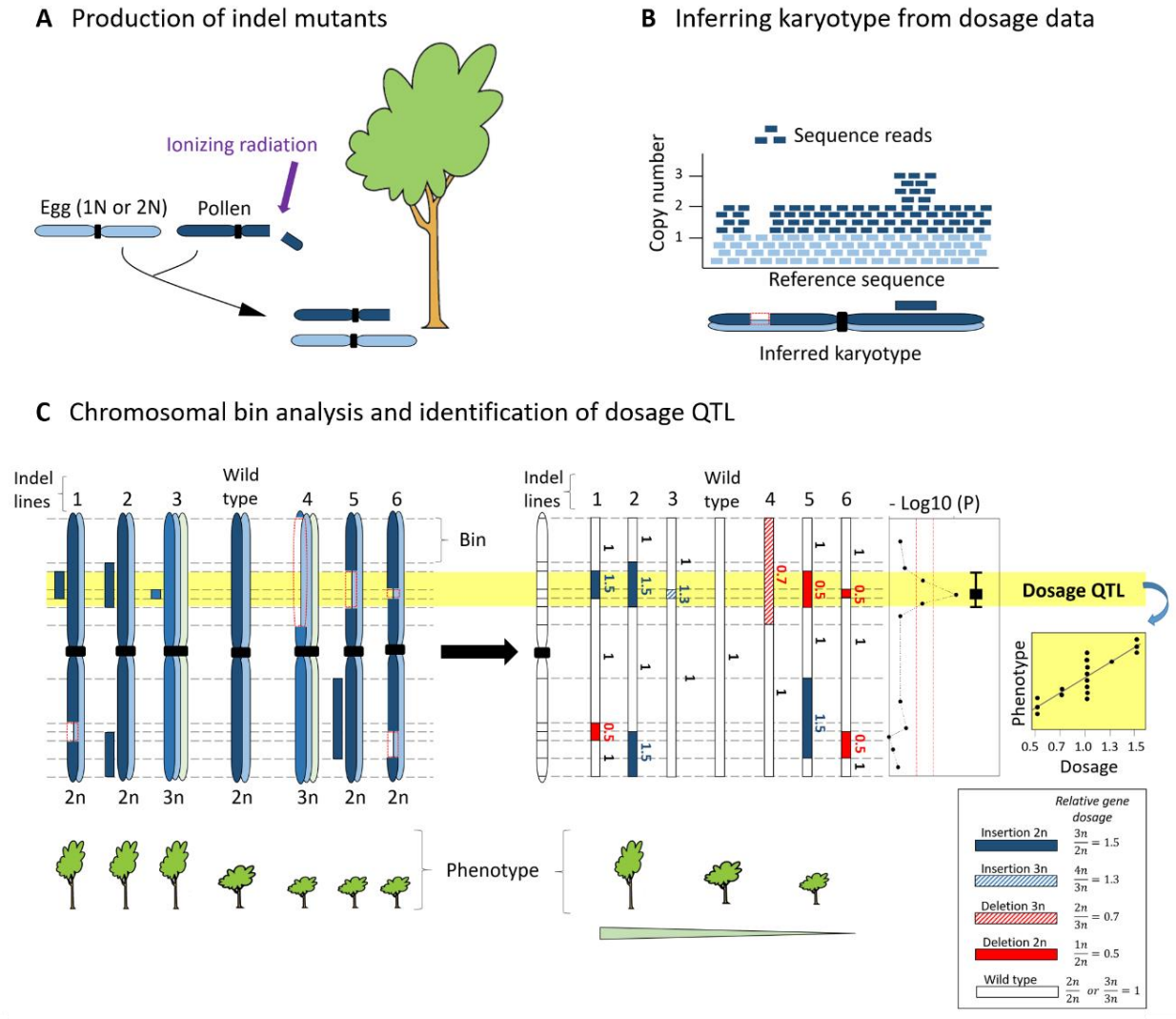


Figure 2.1. Production of irradiation hybrid lines and chromosomal bin analysis for the identification of dosage quantitative trait loci (dQTL). (*A*) poplar clones with high frequency of dosage variation were obtained by fertilizing a *Populus deltoides* female with gamma-irradiated *P. nigra* pollen. (*B*) F1 seedlings resulting from that cross were subjected to high-precision dosage analysis using Illumina sequencing (Henry et al, 2014). Insertions and deletions spanning the whole genome were detected in diploid and triploid lines. (*C*) Left: chromosomal bins are defined by the breakpoints of the indels tiled onto each chromosome. Right: Each line is assigned a relative dosage value for each bin, reflecting both the background ploidy level and indel type. dQTL are detected by calculating the correlation between phenotypic traits of each line (here tree height) and their relative gene dosage values along each chromosomal bin.

Data were transformed using Box-Cox transformation to achieve normality before conducting significance tests. For the first inoculation, p-values of the Kendall rank correlation test for each individual bin along the chromosomes were calculated. Then, in order to control for false discovery error, the p-values were adjusted using the Benjamini-Hochberg method. For the second and third inoculations, a different approach was utilized. The phenotypic responses by genotype were permutated 10,000 times to produce the Kendal rank correlation coefficient of each bin. The p-value was determined by dividing the number of simulated statistics greater than or equal to the observed value by the number of permutations.

For the second inoculation, after the QTL analysis was completed, a Kendall rank correlation test was run for each bin where significant genetic signal occurred. BLUP (Best Linear Unbiased Prediction) was used to normalize data. For optimal QTL analysis, only one data point per genotype should be utilized. However, we had multiple replicates per genotype, as well as two different populations of hybrids. Therefore, BLUP accounted for the variance across these two factors to provide a one-point estimate of the phenotypic response for each genotype.

Results

Rust Morphology

Some spores from the original California isolate had typical *Melampsora larici-populina* morphology (40-50 µm long x 15-20 µm wide) with a smooth apex, but most spores were wider (40-50 µm x 20-30 µm) and totally echinulate, without any smooth apex; this morphology is more typical of *Melampsora occidentalis* (Newcombe et al., 2000). It was estimated that the ratio of *M. larici-populina/M. occidentalis* morphology was about 10% or less. Because spores with apical bald spots (*M. larici-populina* morphology) sometimes occurred, the putative hybrid was named *Melampsora* ×*interdumglabra*.

The rust spores from the second field collection yielded similar results.

Rust Infection

When the isolate was sprayed onto different poplar trees to determine infection ability, only a few *M. occidentalis*-like uredinia formed on D×N cv. 'Brabantica' and on *P.* × *jackii* cv. 'Aurora'. No *M. larici-populina*-like uredinia appeared, even on the clone D×N 'Robusta' that is considered to be universally susceptible to *M. larcici-populina*. The second inoculation with a heavier spore density yielded similar results to the first inoculation: only scant uredinia formed on ‘Brabantica’ and

'Aurora'. In addition, there was rust infection on the *P. trichocarpa* clone 'Fritzi Pauley'. Again, all uredinia had *M. occidentalis* morphology, except for a *M. larici-populina* uredinium on 'Brabantica'.

qPCR and Genotyping Results

The original Californian rust isolate gave a positive result with the Mel F/R/P primers (positive control), the ITS-Mlp, and the ITS-Mm primers, but a negative result with the Mmd primers. The cycle threshold ratio was consistent with a Mlp/Moc ratio of about 1/10.

All monouredinial isolates but one, 17US07, were positive with both *M. larici-populina* and *M. occidentalis* primer pairs. The cycle threshold for both primer pairs were almost identical and ranged from 19 to 22. Therefore, for these isolates the hypothesis of F1 hybrids is highly probable.

The 17US07 isolate, which was considered to be pure *M. larici-populina* based on morphology was also positive with both primer pairs. However, the cycle thresholds were different: 18 for *M. larici-populina* and 36 for *M. occidentalis*. Thus, this isolate is either pure *M. larici-populina*, slightly contaminated with DNA from *M. occidentalis*, or a second-generation hybrid.

The *M. larici-populina*-like isolates (17US07 and 17US19) had almost the exact same multilocus genotypes (MLG) as a pure *M. larici-populina* isolate collected in Washington State in 1995 (Newcombe & Chastagner, 1993). One locus (Mlp_55) was the exception; 17US07 and 17US19 are heterozygous 152/155 at this locus whereas the pure *M. larici-populina* strain is homozygous 152. Based on these results, we can conclude that the *M. larici-populina* collected in California in 2017 is the same genotype (with one SSR mutation at one locus) as the one sampled in Washington State 24 years ago.

The twelve *M.* ×*sp. nov.* isolates 17US02-17US18 (except 17US07) retained only one *M. larici-populina* allele at each locus, again providing strong evidence of a hetero-dikaryotic hybrid.

Inoculation 1

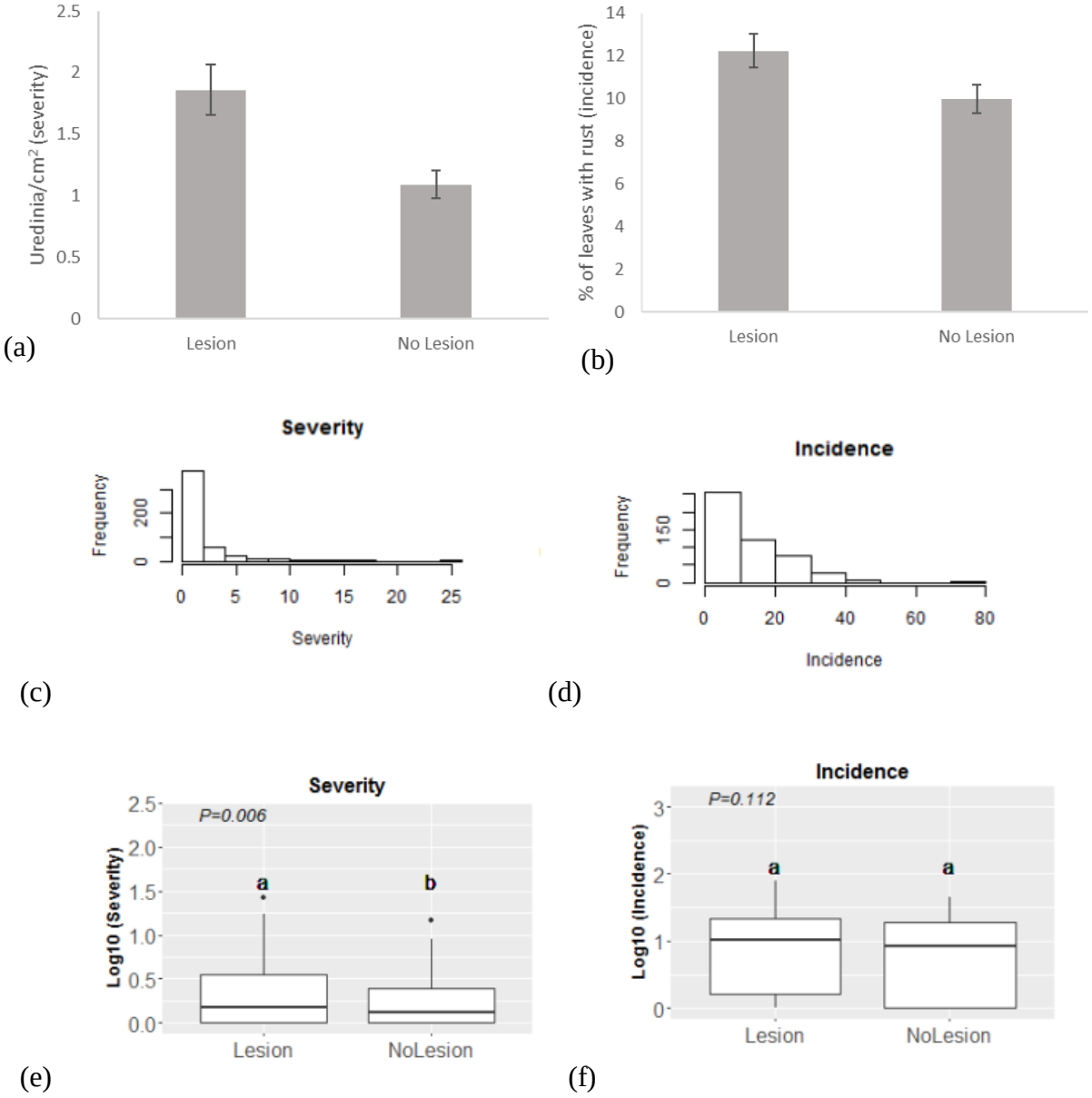


Figure 2.2. Severity and leaf incidence data from Inoculation 1. Panels (a) and (b) display average severity (uredinia/cm^2) and leaf incidence (percentage of leaves with rust) across all genotypes. Panels (c) and (d) display histograms showing severity and leaf incidence frequency. Data were transformed to achieve normality in panels (e) and (f), which demonstrate the differences in severity and leaf incidence between genotypes with lesions and those without lesions.

California's *M.* ×*sp. nov.* was not as aggressive as pure *M. larici-populina* would presumably be on the D×N clones; 27% of the inoculated genotypes did not display any rust signs. Overall, genotypes with lesions had higher rust severity and leaf incidence. Genotypes with lesions had an average of 1.86 ± 0.20 uredinia/cm^2 while genotypes without lesions had an average of 1.09 ± 0.11 uredinia/cm^2

(Figure 2.2a). An average of 12.25% ± 0.79% of leaves from genotypes containing lesions had rust, while only 10% ± 0.67% of the leaves from genotypes without lesions had rust (Figure 2.2b). Of the 63% genotypes that were infected, genotypes with lesions had significantly lower rust severity than those genotypes with no lesions (Figure 2.2e). On the other hand, there was no significant difference in leaf incidence between the genotypes with lesions and the genotypes without lesions. However, no peaks indicating significant QTLs were found (data not shown).

Inoculation 2

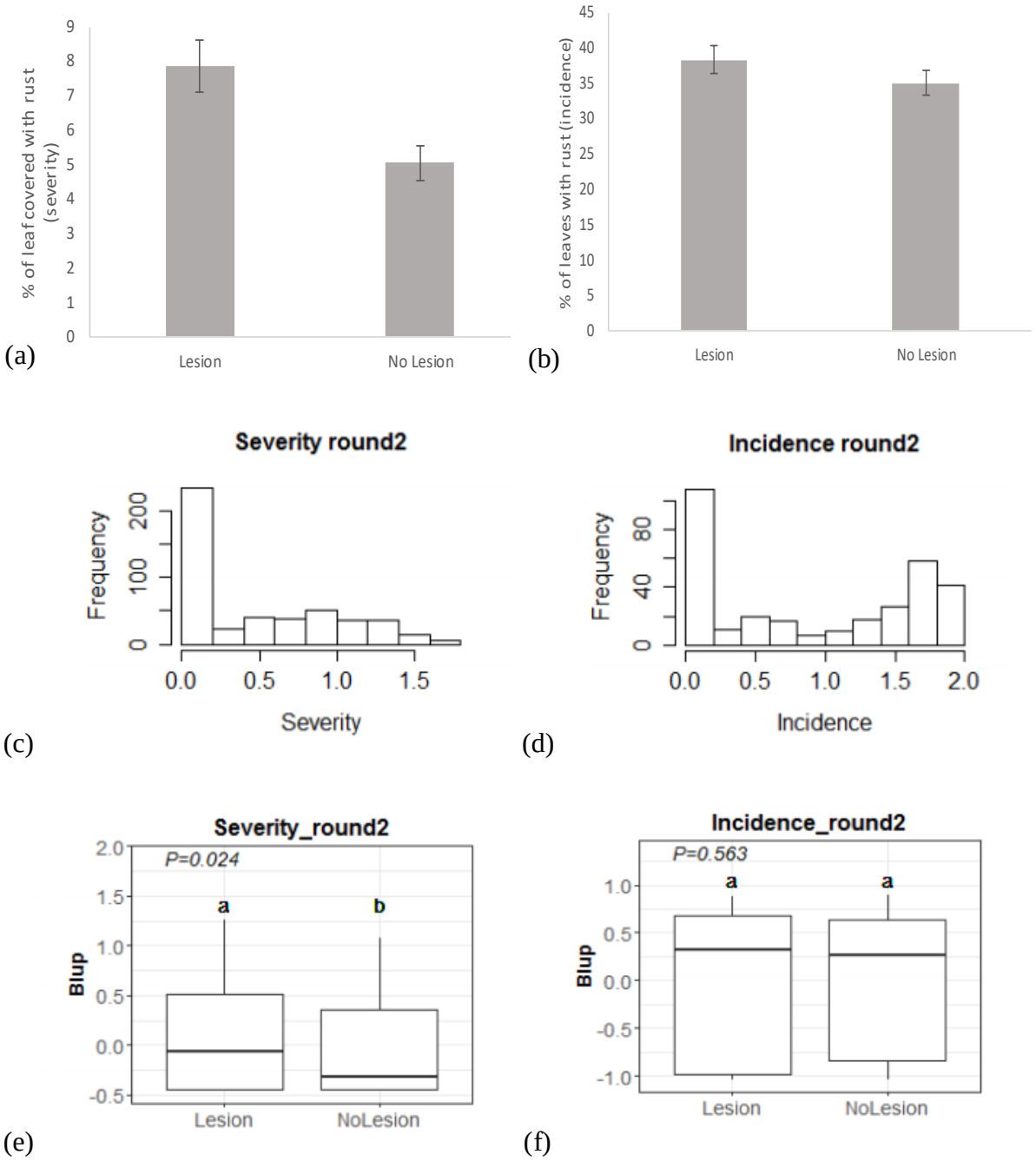
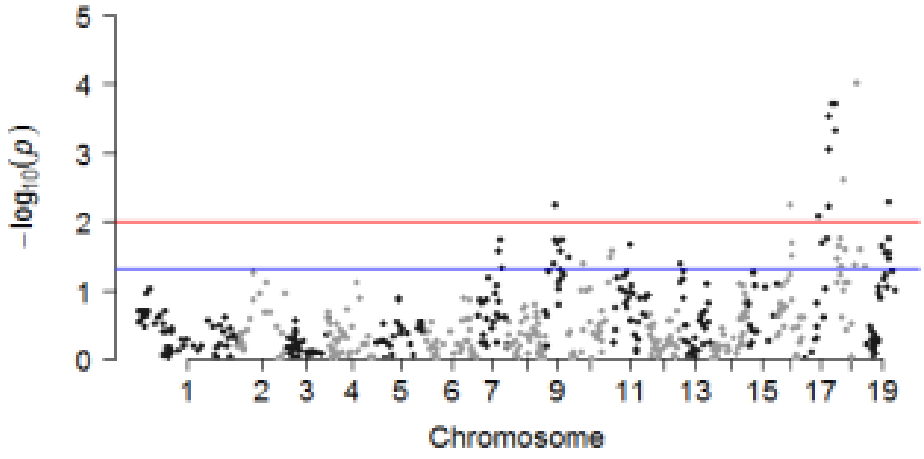
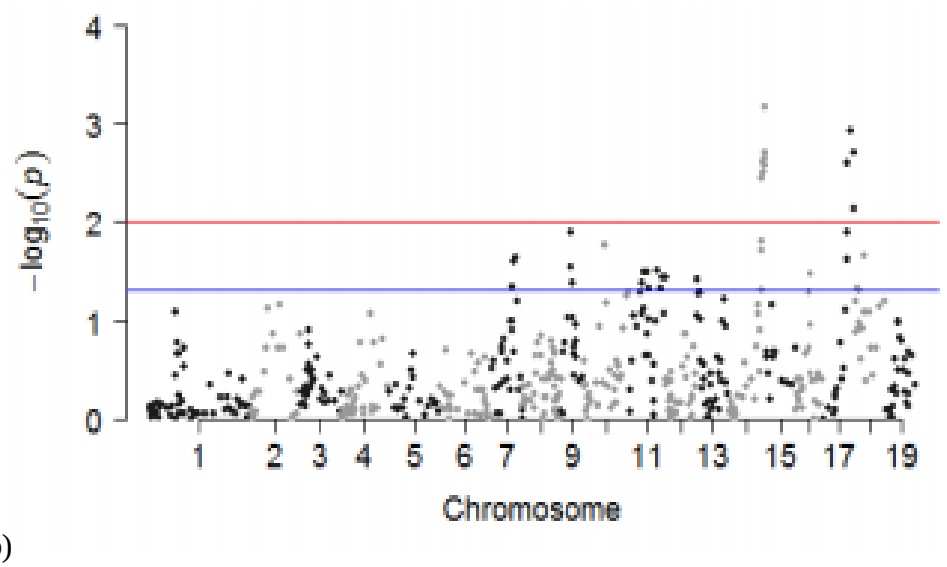


Figure 2.3. Severity and leaf incidence data from Inoculation 2. Panels (a) and (b) display average severity (percentage of leaf covered with rust) and leaf incidence (percentage of leaves on stem with rust) across all genotypes. Panels (c) and (d) display histograms showing severity and leaf incidence frequency. Best linear unbiased prediction (blup) was utilized in panels (e) and (f) to normalize data across replicates and populations to demonstrate the differences in severity and leaf incidence between genotypes with lesions and those without lesions.



(a)



(b)

Figure 2.4. Manhattan plots showing results from dosage QTL analysis for Inoculation 2 based on dosage information and phenotypic response to rust infection. Panels (a) and (b) display QTL peaks for severity and leaf incidence, respectively. Points above the red line indicate significance at $p<0.01$.

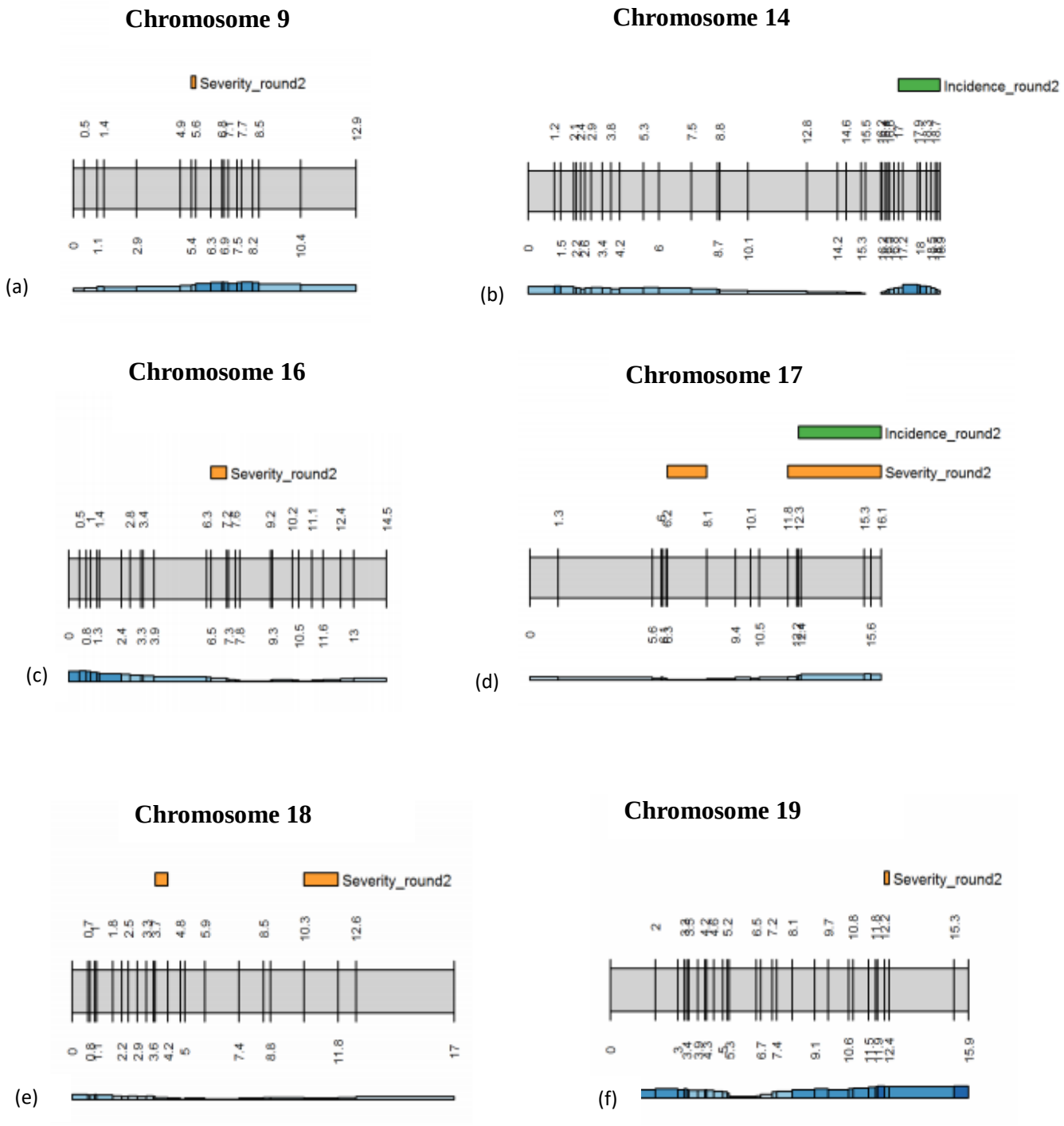
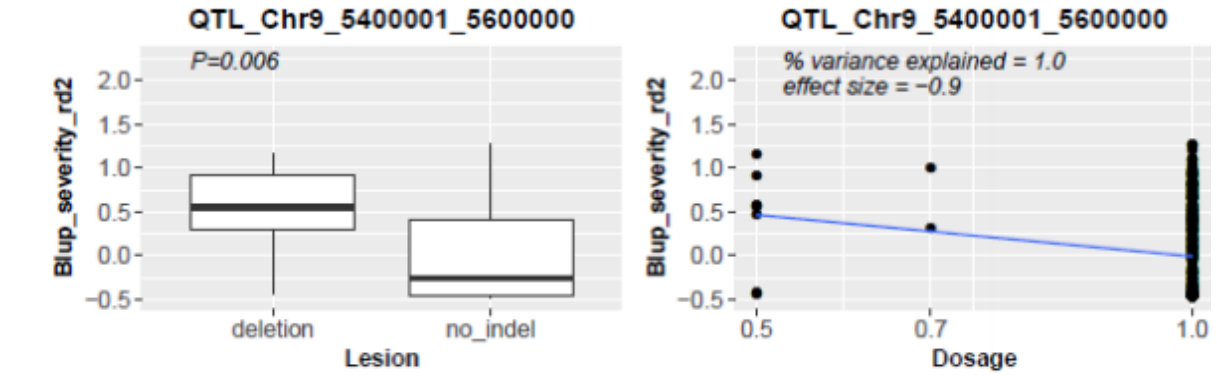
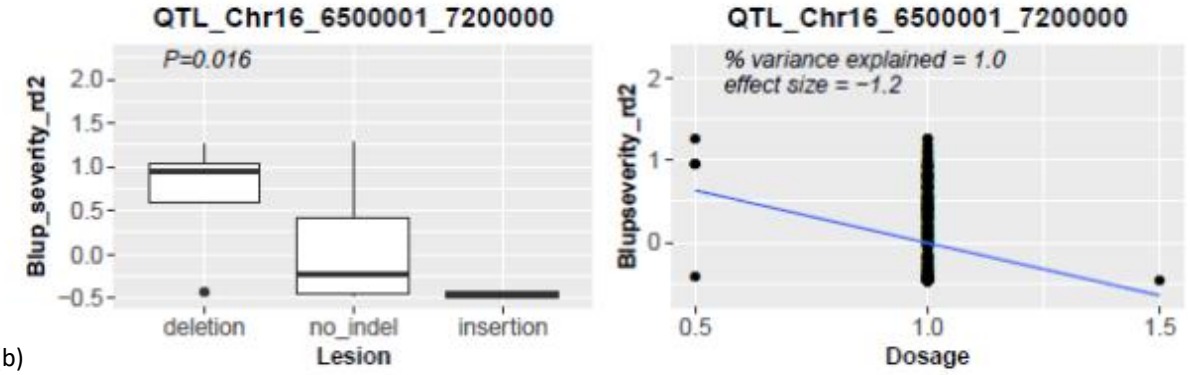


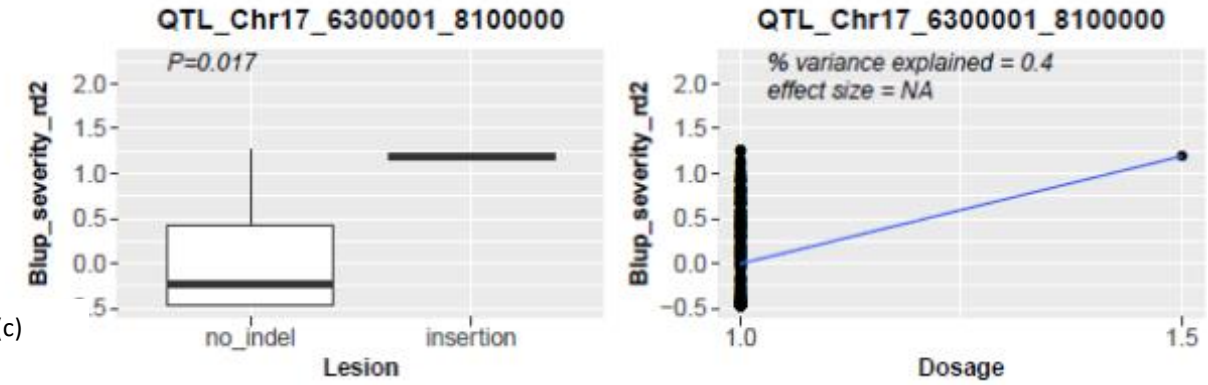
Figure 2.5. *P. trichocarpa* chromosome reference maps displaying location and relative length of significant severity and leaf incidence QTLs. Numbers represent megabase pairs.


QTL_Chr9_5400001_5600000
P=0.006
Blup_severity_rd2
2.0
1.5
1.0
0.5
0.0
−0.5
deletion
no_indel
Lesion
QTL_Chr9_5400001_5600000
% variance explained = 1.0
effect size = −0.9
Blup_severity_rd2
0.5
0.7
1.0
Dosage

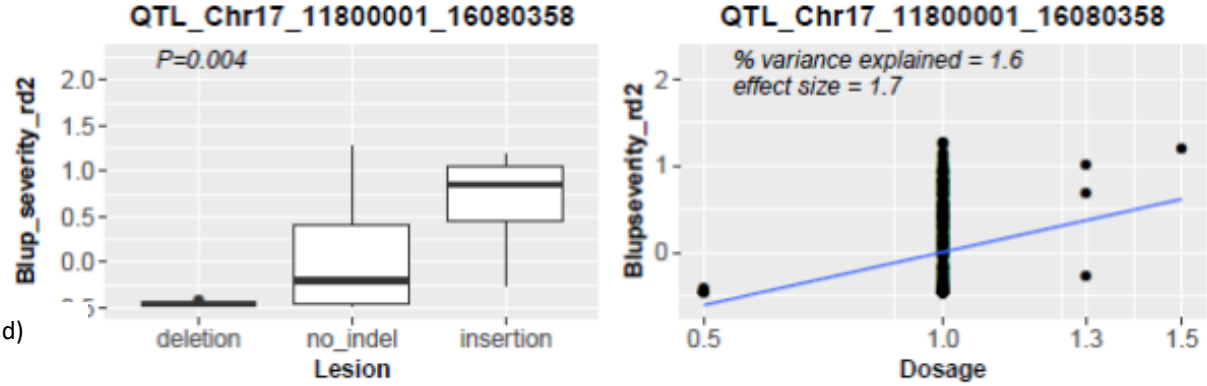
(a)


QTL_Chr16_6500001_7200000
P=0.016
Blup_severity_rd2
deletion
no_indel
insertion
Lesion
QTL_Chr16_6500001_7200000
% variance explained = 1.0
effect size = −1.2
Blupseverity_rd2
0.5
1.0
1.5
Dosage

(b)


QTL_Chr17_6300001_8100000
P=0.017
Blup_severity_rd2
no_indel
insertion
Lesion
QTL_Chr17_6300001_8100000
% variance explained = 0.4
effect size = NA
Blup_severity_rd2
1.0
1.5
Dosage

(c)


QTL_Chr17_11800001_16080358
P=0.004
Blup_severity_rd2
deletion
no_indel
insertion
Lesion
QTL_Chr17_11800001_16080358
% variance explained = 1.6
effect size = 1.7
Blupseverity_rd2
0.5
1.0
1.3
1.5
Dosage

(d)

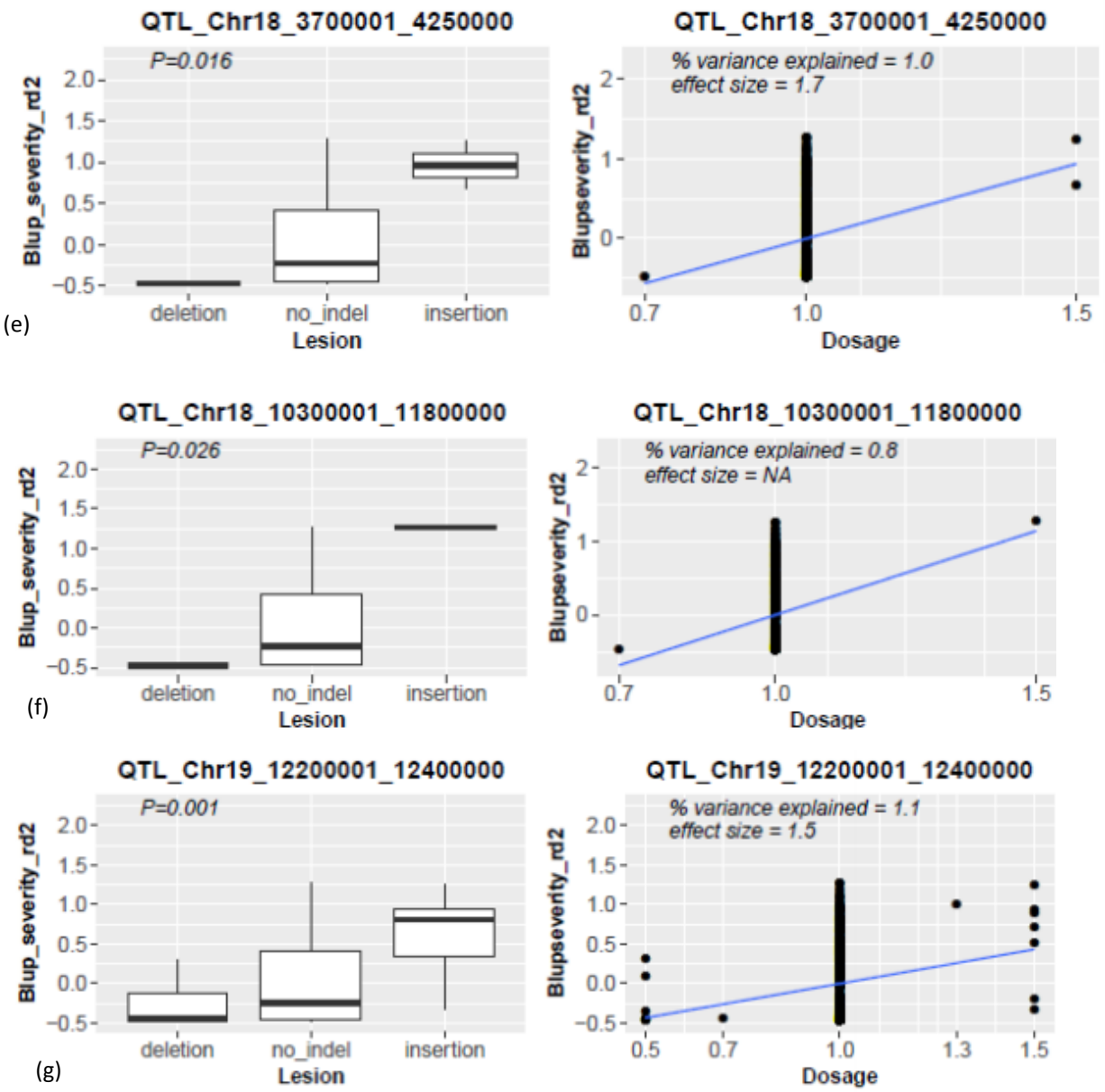


Figure 2.6. Effect of indel mutations on Inoculation 2 rust severity. Left-hand boxplots show means and extremes of the phenotypic BLUP values with the associated p-value of the one-way analysis of variance comparing the groups. Right-hand graphs show the linear relationship between dosage and phenotypic BLUP values with the associated r^2 and Cohen d effect size.

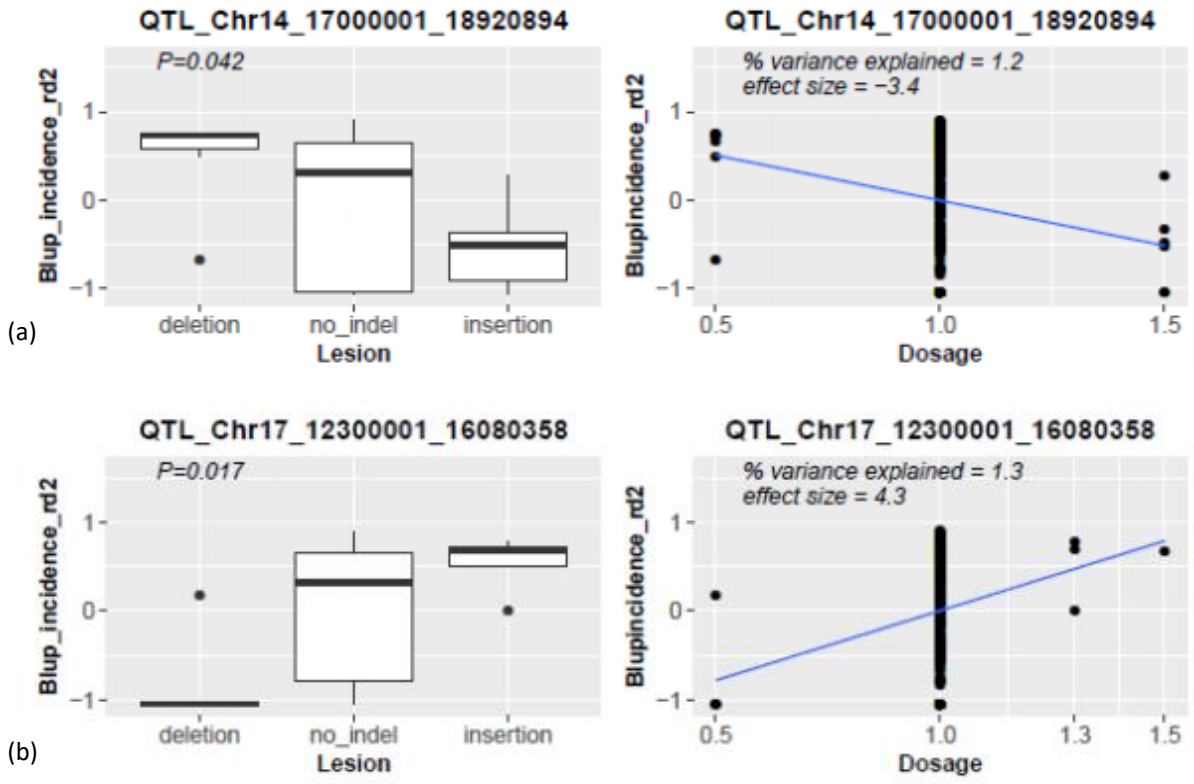


Figure 2.7. Effect of indel mutations on Inoculation 2 leaf incidence. Left-hand boxplots show means and extremes of the phenotypic BLUP values with the associated p-value of the one-way analysis of variance comparing the groups. Right-hand graphs show the linear relationship between dosage and phenotypic BLUP values with the associated R^2 and Cohen d effect size.

Successive inoculations yielded a higher density of rust on the leaves. There was a slight reduction in the number of resistant genotypes, indicating some "resistant" genotypes in the first inoculation were actually escapes. Only 20% of the genotypes were resistant in the second inoculation. Escapes were highly unlikely, as we did fifteen inoculations. Among the susceptible genotypes, those with lesions had an average of 7.82% ± 0.76% of leaf area covered with rust; genotypes without lesions had an average of 5.04% ± 0.49% of leaf area covered with rust (Figure 2.3a). Regarding leaf incidence, genotypes with lesions had an average of 38.3 ± 2.03 leaves displaying rust symptoms; genotypes without lesions had an average of 35.0 ± 1.84 leaves displaying rust symptoms (Figure 2.3b). Because there was still a high number of resistant genotypes (Figure 2.3c-d), data were again transformed before further statistical analysis. There was a significant difference in rust severity between genotypes containing lesions and those lacking lesions (p= 0.024) (Figure 2.3e). However, there was no significant difference in leaf incidence between genotypes containing lesions and those that do not (p= 0.563) (Figure 2.3f). QTL analysis using the permutation method yielded significant QTLs active in controlling both severity and leaf incidence (Figure 2.4a-b). Significant severity

QTLs were found on Chromosomes 9, 16, 17, 18, and 19 and explained 0.4-1.6% of phenotypic variation (Figures 2.5a, c-f, Figure 2.6). Deletion of the genomic region on Chromosomes 9 and 16 correlated with an increase in rust severity, while deletion of the genomic region on Chromosomes 17, 18, and 19 correlated with a decrease in rust severity (Figure 2.6). Significant leaf incidence QTLs were found only on Chromosomes 14 and 17 and explained 1.2-1.3% of phenotypic variation (Figures 2.5b, d, Figure 2.7). Deletion of the genomic region on Chromosome 14 resulted in a statistically significant higher leaf incidence, while deletion of the genomic region on Chromosome 17 resulted in significantly lower leaf incidence (Figure 2.7). A severity QTL on Chromosome 17 co-localized with a leaf incidence QTL on the same chromosomes (Figure 2.5d). The rest of the QTLs were found in distinct and separate chromosomal regions (Figure 2.5a-c, e-f).

Inoculation 3

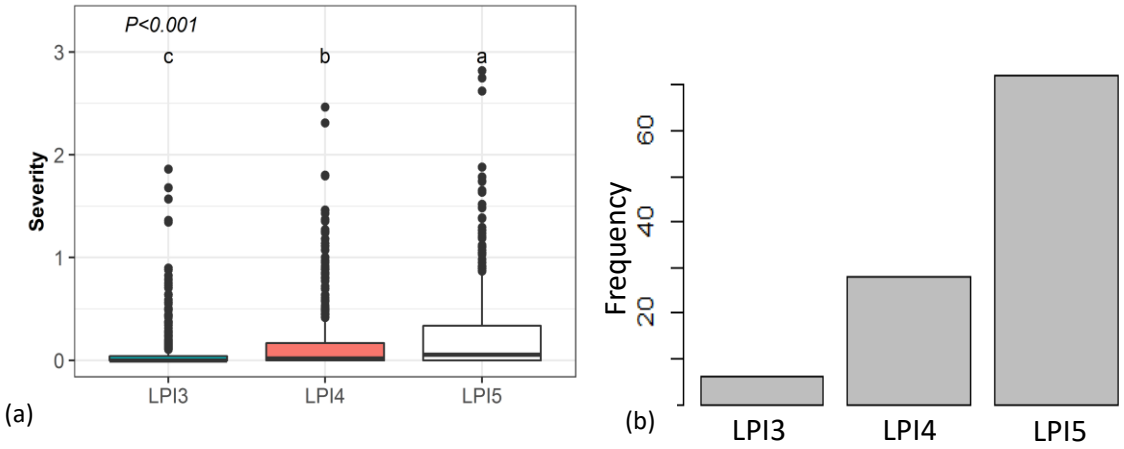


Figure 2.8. Rust severity data from Inoculation 3. Panel (a) displays rust severity results based on LPI as measured by uredinia/cm^2. Panel (b) shows the frequency with which each respective LPI displayed the highest rust severity.

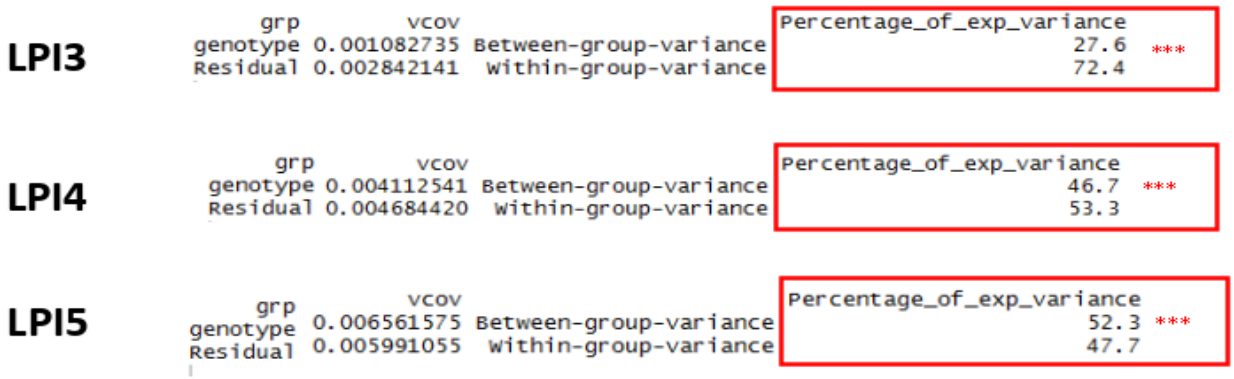


Figure 2.9. Comparison of between-group (genotype) and within-group (replicate) variations for each of the three tested LPI.

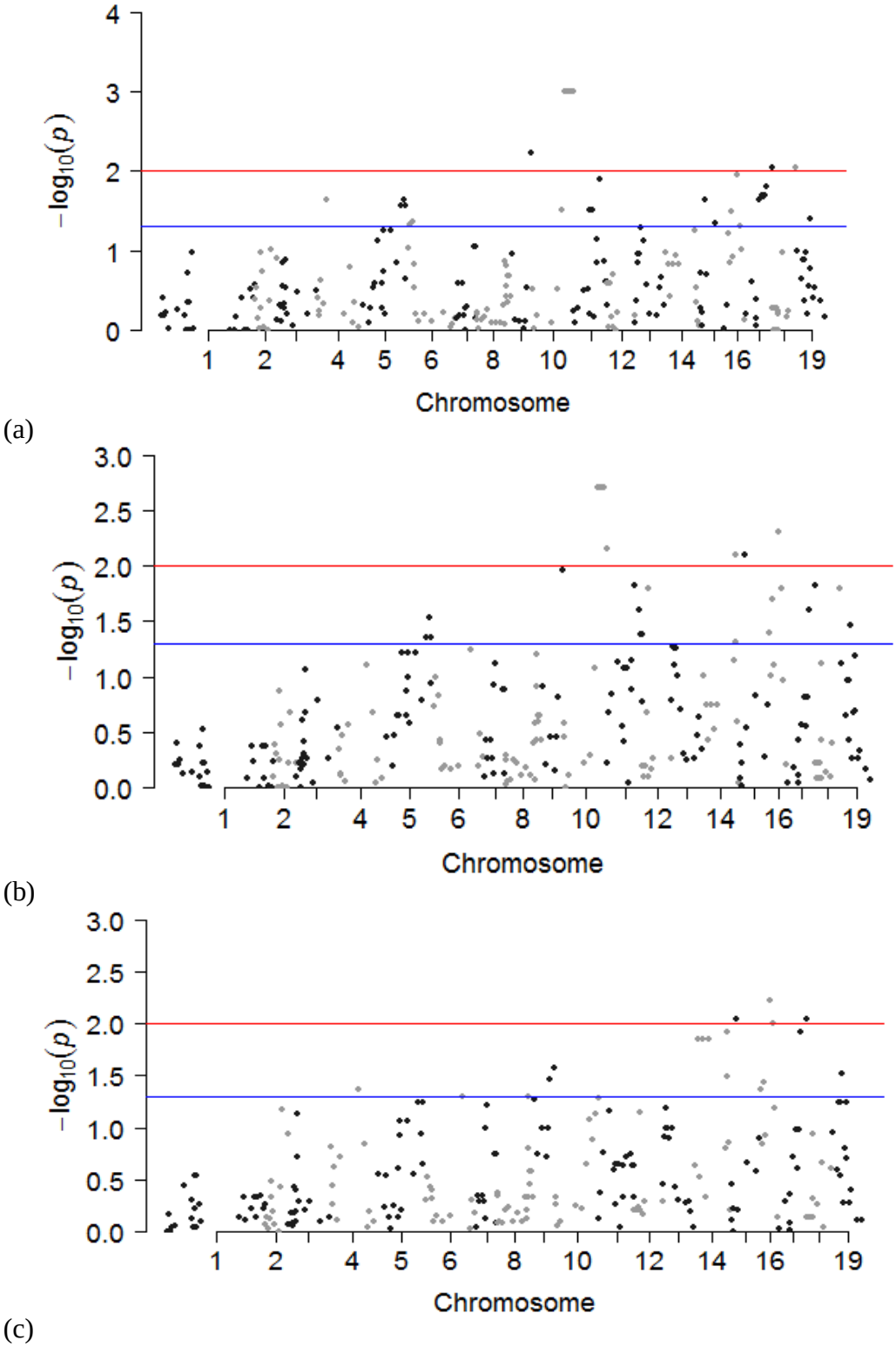


Figure 2.10. Manhattan plots showing results from dosage QTL analysis for Inoculation 3 based on dosage information and phenotypic response to rust infection. Panels show QTL peaks for severity based on LPI position; LPI 3 is shown in (a), LPI 4 in (b), and LPI 5 in (c). Points above the red line indicate significance at $p<0.01$.

Inoculation 3

Inoculation 3 demonstrated that there is a difference in rust severity depending on the leaf position. We inoculated LPIs 3, 4, and 5, and there was a significant difference between the rust infection of all three LPIs ($p<0.001$) (Figure 8a). LPI 5 was frequently the most susceptible LPI out of the three tested LPIs (Figure 8b). Since we had three replicates per genotype, we were able to calculate the within-group variance as well as the between-group variance. For LPI 3 and 4, the variance between the replicates of one genotype was larger than the variance between genotypes (Figure 9). However, for LPI 5, the variance between the replicates of one genotype was smaller than the variance between genotypes (Figure 9). Significant severity QTLs were found when data from each respective LPI was analyzed (Figure 10).

Discussion

In our study of *P. deltoides* × *P. nigra* irradiated hybrids, we found evidence of genes for both rust resistance and rust susceptibility that are sensitive to gene dosage. Genomic regions on Chromosomes 9 and 16 controlled rust severity in Inoculation 2 and contained dosage-dependent QTLs for rust resistance. As the dosage increased, rust severity decreased. Two loci on Chromosome 17, two locations on Chromosome 18, and one location on Chromosome 19 contained dosage-dependent severity QTLs for rust susceptibility. As dosage increased, rust severity increased as well. Only two QTLs were found that played a role in leaf incidence, indicating that dosage variation does not affect leaf incidence as strongly. Chromosome 14 contains a dosage-dependent leaf incidence QTL important in resistance to rust. As the dosage increased, rust severity decreased. On the other hand, Chromosome 17 contains a dosage-dependent leaf incidence QTL important in promoting susceptibility to rust. As the dosage decreased, rust severity increased. The exact mechanisms behind these reactions are not known. Potentially, in genotypes where deletions of a gene causes increase in rust compared to genotypes that have insertions, a missing dominant resistance gene that is present in one parent might be missing in the other parent. In genotypes where the opposite occurs, and deletion of a gene causes a decrease in rust compared to genotypes that have insertions, a suppressor gene sensitive to dosage from one parent may have been silencing a resistance gene from the second parent (Comai et al., unpublished).

Even though the QTLs displayed above were significant, the R-squared or "% variance explained" in each case was between only 0.5%-2% (Figure 2.6, left-hand graphs). At first this may seem contradictory, but a closer investigation into the analysis may solve the puzzle. R^2 values

simply report the variance of the points around the line. In each graph, there is significant variation around relative gene dosage of 1.0, thus contributing to the low R^2 value. The points around the 1.0 dosage marker represent the lines that do not contain lesions. However, they also represent mutant lines that contain indels, but just on another bin. Future tests may focus on removing some of the control lines to minimize variation and potentially increase the R^2 value. Despite the low R^2 values, there was a significant p-value in each case. The p-value represents the result of the Kendall rank correlation test. In the case of positive slopes and a significant p-value (for example, all three QTLs on Chromosome 17: Figure 2.6(d-f)), as gene dosage increases, rust severity increases as well. This indicates that the genes responsible for the phenotypic response are genes that are affected by dosage and code for a susceptible reaction to rust infection. In the case of negative slopes and a significant p-value (for example the QTL on Chromosome 9: Figure 2.6(a)), as gene dosage decreases, rust severity decreases. Therefore, the genes responsible for this phenotypic response are genes that are affected by dosage and code for rust resistance. Cohen d effect sizes were also calculated; an effect size of 0.8 is considered large, 0.5 is medium, and 0.2 is small (Fritz et al., 2012). In all cases, the effect size is over 0.8, indicating a high magnitude of the interaction between gene dosage and phenotypic response (Figure 2.6).

The third inoculation focused on Leaf Plastochron Index (LPI) (Erickson & Michelini, 1957). LPIs 3-5 have traditionally been used in poplar/rust studies (Newcombe, 1998, Stirling et al., 2001, Laurans & Pilate, 1999), and we wanted to see if there was a difference in rust severity based on leaf position. Our results demonstrated that there was a significant difference between rust infection amongst the three tested LPIs, with LPI 5 commonly the most severely infected (Figure 2.7(a-b)). Even though Inoculation 3 was performed on only a subset of the genotypes, significant QTLs were still seen based on the data from each of the three LPIs. This indicates that the signal will likely be even stronger when all the genotypes are considered. In late spring 2019, another inoculation will be run with two ramets of all the genotypes to test this hypothesis.

Quantitative resistance has been demonstrated to display race specificity (Chen et al., 2003, Talukder et al., 2004, & Caffier et al., 2014) raising the question of how exactly the mechanisms for qualitative (gene-for-gene) resistance and partial, quantitative resistance differ. It is still poorly understood if in some cases, quantitative resistance is simply due to eroded R genes that still display a weak defensive response. While we found significant QTLs in our pathosystem, further testing with different rust races would have to be conducted, as this study used only one isolate of *Melampsora ×sp. nov*.

There are several limits of our current focus and approach. At this point, we are unable to know for sure whether the weak QTL signal is due to noise from the varying control non-lesion lines or if these QTL are simply minor (quantitative) resistance genes. Additionally, there may be major or minor resistant genes that are not dosage dependent; it is difficult to detect such genes in mutant lines containing indels. Endophytes are another consideration. While endophytes in the greenhouse are likely to be restricted, there are undoubtedly greenhouse-associated fungi that came into contact with the poplar trees. These fungi can also be playing a role in response to rust infection. Another potential factor are thrips levels. An unfortunate greenhouse thrips outbreak could have altered the vigor of certain poplar genotypes; this stress could have potentially made the trees more susceptible to rust.

The durability of gene dosage-dependent resistant genes is also of critical importance. The ability of a pathogen to overcome qualitative resistance is well-documented, but pathogens can also adapt to quantitative resistance. Genotypes that display robust resistance should only be selected after being exposed to the pathogen for a sustained period of time, especially in environments that favor the development of disease (Johnson, 1984). Johnson (1984) states that an increase in pathogen aggression is a very common cause of eroded resistance. Our isolate of *M. larici-populina* was not very aggressive on our D×N population, as evidenced by the overall low severity of Inoculation 1 (Figure 2.2a); severity increased only with repeated inoculations. It is possible that this rust isolate may become more aggressive over time as it continues to rapidly reproduce and evolve. Extended exposure of poplar trees to this pathogen under field conditions would be helpful in determining the durability of our detected dosage-dependent resistance.

Future molecular directions will focus on RNAseq in both healthy and diseased tissues to further characterize transcriptional networks activated by rust infection and determine how such networks correlate with the significant QTLs. Also, the identification of candidate genes that underlie the QTLs is an important next step; this has broad implications for disease resistance in hybrid poplar. The identification of genes that are responsible for both rust susceptibility and resistance will be invaluable to breeders. Knockout of susceptibility genes will produce cultivars that can withstand pathogenic pressure. In addition, selective breeding and bioengineering of resistant genes will also greatly benefit the poplar industry.

Melampsora ×sp. nov.

M. larici-populina, native to Eurasia, was first reported in western North America in 1993 (Newcombe & Chastagner, 1993). After the first appearance in Washington was recorded, Pinon et

al. (1994) determined which pathotypes had been introduced. Strong founder effects have been demonstrated for this rust when it is introduced outside its native range (Barres et al., 2008). 17US07, the 2017 California *M. ×sp. nov.* isolate displaying *M. larici-populina* morphology, was identical to a 1995 *M. larici-populina* isolate from western Washington with the exception of one MLG. This isolate tested positive for the presence of both *M. larici-populina* and *M. occidentalis* DNA. Although the ratios were not 1:1, this isolate is still a hybrid and may even represent a second-generation hybrid. Therefore, the rust first discovered in western North America in 1993 was likely the same hybrid, not pure *M. larici-populina*. Given founder effects in other locations where *Melampsora* species have been introduced, it is not surprising that the North American population has remained constant genetically for almost 25 years. The hybrid rust *M. ×columbiana* (a hybrid between *M. medusae* and *M. occidentalis*) was discovered only three years after its introduction to the Pacific Northwest. Therefore, it seems likely *that M. larici-populina* hybridized with the native rust (*M. occidentalis*) rapidly after its introduction; the hybrid status just escaped detection, until now.

Single uredinia from California leaves sampled in Fall 2018 possessed spores with both *M. larici-populina* morphology and *M. occidentalis* morphology. However, it appears that only isolates with *M. larici-populina*-like morphology can infect our D×N population; the rust isolate from our greenhouse study (17US19) is genetically consistent with 17US07 and the rust strain from 1995 thought to be pure *M. larici-populina*, again differing at only one MPG. The ability of the California hybrid rust, displaying mostly *M. occidentalis* morphology to infect D×N 'Brabantica' also points to a hybrid origin. *M. occidentalis* is endemic to the Northwest and is most commonly found on its favored telial host, *Populus trichocarpa* (Jackson, 1917). It has not evolved with *P. deltoides, P. nigra*, or D×N hybrids, and it has never been reported to infect D×N trees. Therefore, if a rust with *M. occidentalis* morphology can infect D×N clones, it must have received virulence factors from another species of rust with which it hybridized.

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Chapter 3: Nematophagous *Pleurotus pulmonarius* consumes some nematode species but is itself consumed by others

Abstract

Pleurotus species are said to be nematophagous because they paralyze and consume some species of bacterial-feeding nematodes. It has never been clear whether that means all nematodes. Here we tested thirteen bacterial-feeding nematode species: seven species of family Rhabditidae, three species of Cephalobidae (one with three populations), two species of Panagrolaimidae, and one species of Diplogastridae. Nematodes interacted on water agar in Petri dishes with toxin-producing *Pleurotus pulmonarius*. Of the fifteen populations of the thirteen species, nine were susceptible (all individuals were paralyzed by *P. pulmonarius*) but six (four populations of two cephalobid species, one rhabditid, and one panagrolaimid) survived exposure to *P. pulmonarius*. The resistant six not only survived but multiplied their numbers on water agar in which the only food was *Pleurotus*. *Pleurotus pulmonarius* is nematophagous toward some nematodes, but it is also resisted and then consumed by others in three of the four families assayed. Species-specific interactions point to the need for studies of the host ranges for both 'nematophagous' fungi, and 'fungivorous' nematodes.

Introduction

Nematodes are roundworms, generally microscopic, that include parasites of animals and plants as well as beneficial species. Killing these parasites has become problematic as resistance has developed to many previously useful therapeutic chemicals (Clarke et al., 2019). Therefore, biocontrol agents of nematodes may provide less toxic and more sustainable control with more varied and complex mechanisms that are harder for nematodes to resist.

Nematophagy implies the consumption of nematodes. The predatory consumer could be another invertebrate such as a mite (Stirling et al., 2017) or bacteria such as *Bacillus thuringiensis* employing a toxin (Wei et al., 2003) or *Chryseobacterium nematophagum* producing enzymes (Page et al., 2019) or a fungus. But how do fungi manage this? Reviews of the subject frequently focus on the trapping and feeding mechanisms employed by nematophagous fungi (Nordbring-Hertz et al, 2006). Others focus on the diversity (Zhang & Hyde, 2014), and phylogenetic relationships (Thorn et al, 2000) of those 700 or so fungal species discovered thus far to be nematophagous. Traps, adhesive spores and specialized structures have been reported along with toxins. Nematophagous fungi are also sometimes called carnivores because they consume the 'meat' of the trapped or toxin-stunned

nematodes (Thorn & Barron, 1984). Some are termed predators whereas others are endo-parasitoids. The most recent proposal for mechanism-based classification of nematophagous fungi is for five groups: "nematode-trapping/predators, opportunistic or ovicidal, endoparasites, toxin-producing fungi and producers of special attack devices" (De Freitas Soares et al, 2018).

Few studies address the topic of host range in nematophagous fungi; host ranges of toxin-producing fungi appear to be particularly neglected. Host range of a nematophagous fungus should comprise all susceptible species of nematodes attacked and consumed by that fungus. Experimental determination of host range would require testing of many nematode species but all too often only a single or a few species were tested. There are exceptions, where numerous nematode species were used to determine host range. For example, Tzean & Liou (1993) employed 11 different nematode species across multiple feeding guilds to determine that nematophagous *Hyphoderma* species have specific nematode host ranges; some nematodes were not affected at all by the fungus. However, in the absence of experimental determinations, host range may be assumed to encompass more species of nematodes than is actually the case. In the case of *Pleurotus ostreatus*, its host range has been assumed to be broad since its toxin, *trans*-2-decenoic acid, derived from linoleic acid, has even been said to affect "not only nematodes, but also insects and other fungi" (De Freitas Soares et al, 2018).

We first began thinking about host range of nematophagous fungi when reading a research article on nematode feeding habits in which mention was made of *Pleurotus ostreatus* as a good host for a nematode, *Filenchus misellus* (Okada & Kadota, 2003). However, the medium employed in this research (Okada & Kadota, 2003) was not water agar on which nematophagy by *Pleurotus* is normally assayed. Instead, *P. ostreatus* was grown on potato dextrose agar (PDA), even though Barron & Thorn (1987) had found that the *Pleurotus* toxin is not as effective when grown on PDA; the toxin is most potent when the fungus is grown on a nutrient-limited media such as water agar. Since *F. misellus* had not been exposed to the toxin in its most potent form, definitive inferences could not be drawn.

Toxins are deleterious chemical compounds that are produced by, and that enhance the fitness of, living organisms. Typically, toxins are effective against some, but not all, other organisms. Species of white-rot fungi in *Pleurotus* possess a unique mechanism of toxin-assisted, nematode trapping which has been demonstrated on both water agar and wood (Barron & Thorn, 1987, Thorn & Tsuneda, 1992). Multiple studies have confirmed that bacterial-feeding nematodes belonging to various families in the order Rhabditida are susceptible to toxins produced by *Pleurotus* species (Barron & Thorn, 1987, Larsen & Nansen, 1991, Hibbett & Thorn, 1994, Kwok et al., 1992). When grown in a nitrogen-poor environment like wood, *P. ostreatus* will produce a toxin on aerial hyphae.

Instead of diffusing into the environment, the toxin remains as a droplet on the hyphae. In this manner, the toxin remains undetected by the unfortunate nematode until contact is made; the nematode is promptly paralyzed by the toxin. Hyphae will then colonize the nematode, and eventually digest it (Barron & Thorn, 1987). In 1992, this toxin of *P. ostreatus* was characterized and named trans-2-decenedioic acid. (Kwok et al., 1992).

Other species of *Pleurotus* produce toxins with nematode-stunning activity similar to that of trans-2-decenedioic acid. *Pleurotus pulmonarius*, the focus of our study of nematophagy, produces S-coriolic acid, linoleic acid, panisaldehyde, *p*-anisyl alcohol, 1-(4-methoxyphenyl)-1,2-propanediol and 2-hydroxy-(4′-methoxy)-propiophenone (Stadler et al., 1994). We used the standard assay on water agar to test the hypothesis that *P. pulmonarius* has a specific host range of nematodes it can paralyze. *Pleurotus*-resistant species were then further tested to confirm fungus-feeding ability by pairing them with *Rhizoctonia solani*.

Materials & Methods

Fungal culture

Pleurotus pulmonarius was isolated in early spring from a fresh basidiocarp collected on a *Picea abies* log in the University of Idaho Arboretum. *Pleurotus ostreatus* and *P. pulmonarius* are very similar, but *P. pulmonarius* grows on conifer wood in the Pacific Northwest and fruits in the spring (Trudell and Ammirati, 2009). Our isolate of *P. pulmonarius* was transferred to oatmeal agar where cultures were allowed to grow at room temperature (approximately 21 degrees C). For testing, 7-mm plugs were taken from growing cultures and transferred to plates containing 2% water agar (WA). These cultures were kept at room temperature and allowed to grow until the hyphae reached the edge of the plate (7 to 14 days). At that time, toxin production was noticeable.

Nematode culture

Nematode feeding habits are only partially known. For example, ‘bacterial-feeding’ nematodes can also graze on hyphae of at least some fungi. For our study we selected 13 species of bacterial-feeding nematodes from four families including Rhabditidae, known to include species susceptible to species of *Pleurotus*. All species of nematodes were grown and maintained on NGM agar with *Escherichia coli* OP50 as their food (Stiernagle, 1999). The following nematodes were then used in our *Pleurotus*-nematode interaction assay:

Family Rhabditidae

Oscheius dolichura (LKC50), *Oscheius myriophila* (DF5020), *Oscheius tipulae* (LKC57), *Caenorhabditis elegans* (N2), *Mesorhabditis inarimensis* (LKC51), *Poikilolaimus oxycercus.* (LKC64), and *Metarhabditis rainai* (LKC20)

Family Cephalobidae

Zeldia punctata (PS1192), *Acrobeloides varius* (LKC52), *Acrobeloides varius* (PS1959), *Acrobeloides varius* (LKC27), and *Acrobeloides* sp. cf *amurensis* (PS1146)

Family Panagrolaimidae

Panagrolaimus artyukhovskii (LKC44) and *Panagrellus redivivus* (PS1163)

Family Diplogastridae

Pristionchus aerivorus (LKC54)

***Pleurotus*-nematode interaction assay**

Twenty active individuals, varying in age, of each nematode species were transferred to respective WA plates containing live mycelium of *P. pulmonarius*. We checked for paralysis due to toxin immediately after transfer and then every hour for the next six hours. The cultures were also checked one and two weeks after the nematode transfer for survival and reproduction of nematodes. For the purpose of this study, we defined resistance as survival of some individuals after contact with *P. pulmonarius* on water agar, and their subsequent ability to reproduce. Susceptible nematode species were defined as ones in which all 20 individuals perished during the first few hours on the water agar plate containing the *Pleurotus* mycelium.

Phylogenetic analysis

DNA from nematode species was processed for the 18S rDNA marker (Carta and Li, 2018) or taken from GenBank. Taxa are phylogenetically disjunct in the tree containing only these taxa (Figure 3.1) so corresponding clades from a comprehensive large tree of 18S sequences (van Megen et al., 2009 and Blaxter and Koutsovoulos, 2015) is referenced and shown in Figure 3.2. Sequences were aligned with Clustal W (Thompson et al., 1994) and a Bayesian likelihood tree was constructed with the MRBAYES plugin (Huelsenbeck and Ronquist, 2001) in Geneious ver. 11.1.5 (Biomatters, Auckland, NZ) in Figure 3.1.

Fungal-feeding ability

In order to confirm fungus-feeding ability of the *Pleurotus*-resistant nematodes, all of which were known as bacterial feeders prior to this study, approximately 10 mixed-stage juvenile

nematodes were rinsed in sterile water from the bacterial plate and transferred by pipette to PDA plates containing 7- to 10-day-old mycelial cultures of *Rhizoctonia solani*. They were then observed for consumption of the fungus and production of nematode eggs.

Results

Pleurotus-nematode interaction assay

All 20 individuals of each of nine susceptible species were stunned or paralyzed by toxin in the first few hours after their introduction to *Pleurotus* plates. They were then consumed by *Pleurotus* hyphae. In the case of each of the six resistant populations of four species, a few individual nematodes appeared stunned during the first few hours. But other individuals were not, and they went on to reproduce and feed on *Pleurotus*. Of the fifteen populations of thirteen species, nine were susceptible. Six of seven rhabditid species were susceptible: *Oscheius dolichura* (LKC50), *Oscheius myriophila* (DF5020), *Oscheius tipulae* (LKC57), *Caenorhabditis elegans* (N2), *Mesorhabditis inarimensis* (LKC51), *Poikilolaimus oxycercus* (LKC64). Only one of five populations of three cephalobid species was susceptible: *Acrobeloides* sp. cf *amurensis* (PS1146). One of two panagrolaimid species was susceptible: *Panagrolaimus artyukhovskii* (LKC44). Finally, the only species of diplogastrid assayed was susceptible to *P. pulmonarius*: *Pristionchus aerivorus* (LKC54).

Six populations representing four species of nematode were resistant to *P. pulmonarius*: four of five cephalobid populations comprising two species, one of seven rhabditids, and one of two panagrolaimid species. It is evident from Figure 3.1 that *Pleurotus* resistance may have multiple origins among at least three families, including even the Rhabditidae, a family traditionally associated with *Pleurotus* susceptibility and which, here, was also mostly susceptible (six of seven species). Resistance was especially common among species of Cephalobidae, including multiple isolates of one species, *Acrobeloides varius*.

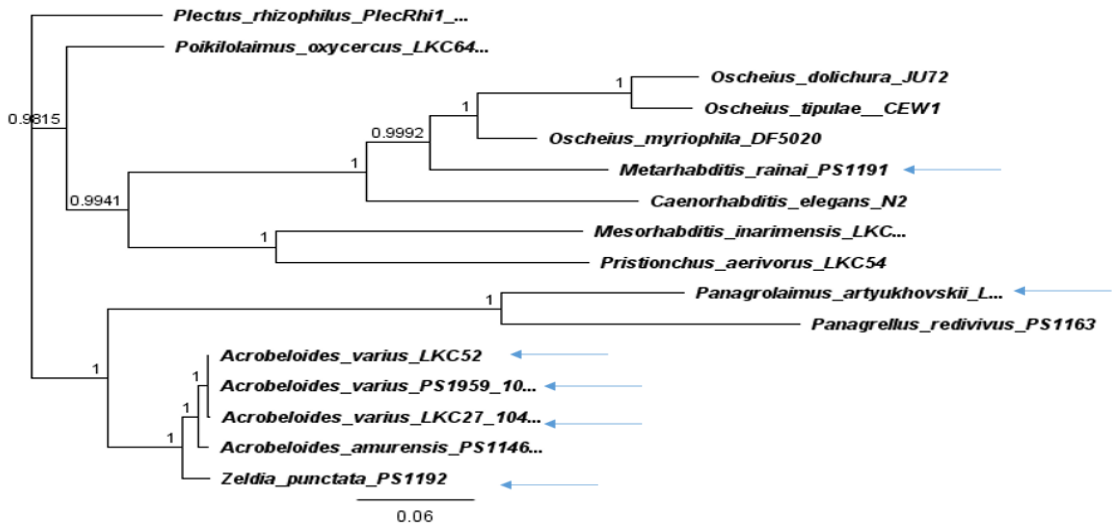

Figure 3.1. MrBayes Bayesian Likelihood phylogenetic tree of nematode populations. Based on a Clustal W alignment of 18S rDNA sequences as implemented in Geneious ver. 11.1.5. Arrows indicate taxa resistant to *Pleurotus* toxin.

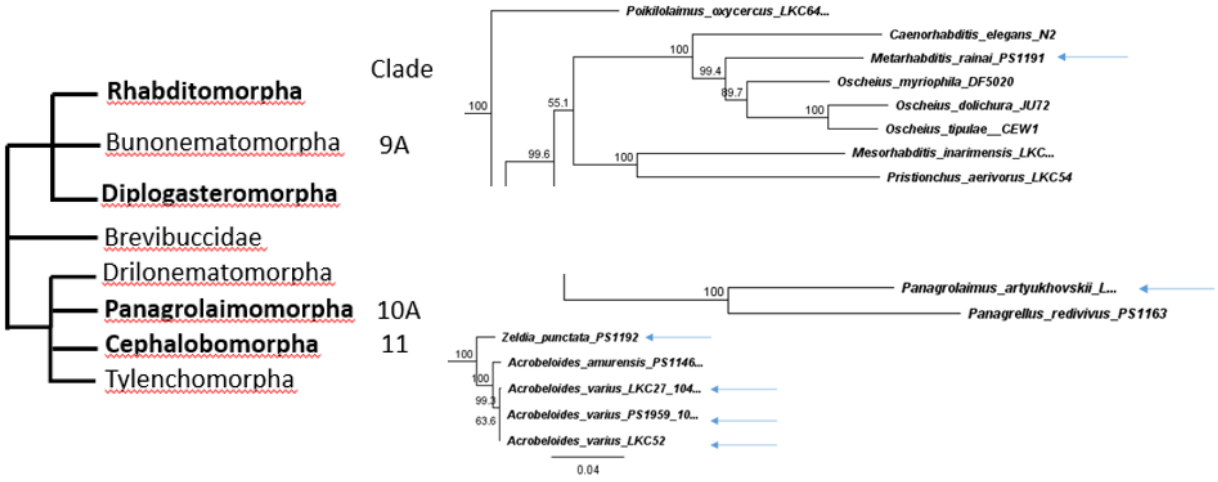

Figure 3.2. Disjunct NJ distance trees in phylogenetic context of van Megen et al., 2009. Arrows indicate taxa resistant to *Pleurotus* toxin.

Feeding ability

The ability of the *Pleurotus*-resistant, bacterial-feeding populations to multiply on another fungus, *Rhizoctonia solani*, was confirmed. This was not completely clear after two weeks. However, after three weeks of culturing on *R. solani*, all six of the *Pleurotus*-resistant nematode populations had produced eggs and multiplied their numbers.

Discussion

Our findings show that *P. pulmonarius* is more specialized within Nematoda than previously believed. In other words, nematophagous species of fungi have host ranges, a term applied by Barron (1978), analogous to the host ranges of parasitic fungi (Carnegie & Lidbetter, 2012), or the tree hosts of mycorrhizal fungi (Marx, 1977) or the range of mycorrhizal fungi parasitized by mycoheterotrophic plants (Merckx et al, 2012). The term applies not only to nematophagous fungi but also to fungivorous nematodes that might consume *P. pulmonarius* and *R. solani*, as here, but would likely be unable to consume others.

It seems likely that other toxin-producing nematophagous fungi, including other species in Pleurotaceae, might be similarly specialized if tested in the manner of this study. It is entirely possible that there are nematophagous fungi that can attack the six *Pleurotus*-resistant species of this study. Conversely, it seems possible that the nine *Pleurotus*-susceptible nematodes of this study would feed on fungi other than *P. pulmonarius*. *Pleurotus pulmonarius* itself was either the consumer or the consumed. It will be interesting to conduct further research to see whether this zero-sum game might be more widely operational among interacting fungi and nematodes.

In this study only bacterial-feeding nematodes were assayed because they had previously been used to show that *Pleurotus* species were nematophagous. Evidently, many bacterial-feeding nematodes can survive on fungus as the sole food source. An earlier study in which that was demonstrated involved a bacterial-feeding *Chiloplacus* species that was maintained on a fungal culture (i.e., *Phoma*; Procter, 1986). Overall, research has focused little on bacterial-feeding nematodes' ability to consume fungi, indicating a gap in knowledge regarding feeding behavior and potential host range of nematodes. Therefore, there may be a significantly higher number of nematodes that could be considered fungivorous. Future research on resistance or susceptibility to nematophagous *Pleurotus* might involve nematode trophic groups or feeding guilds other than the bacterial-feeding group tested here. In particular, nematodes that are parasites of vertebrates and plants can be targeted by nematophagous fungi in biological control efforts (Waller and Faedo, 1993).

Resistance to toxins, presumably the reported S-coriolic acid, linoleic acid, panisaldehyde, *p*-anisyl alcohol, 1-(4-methoxyphenyl)-1,2-propanediol and 2-hydroxy-(4′-methoxy)-propiophenone, allowed six populations of nematodes to graze on *P. pulmonarius*. We do note that some of the original individual nematodes in each of the six species were paralyzed whereas others were resistant. Age-related resistance could factor into this observation; we used individuals of varying

life stages in our assay. The mechanism for resistance to these toxins has yet to be determined, but we can propose the following. First, the toxins may simply be lacking recognition or binding sites in resistant nematodes. This concept has been demonstrated in endoparastic nematophagous fungi, where differential trapping is noted. For example, Jansson et al. (1985) showed that conidia of endoparasitic, nematophagous *Meria coniospora* can only attach and infect some species of nematodes. Their study agrees with prior work that suggests sialic acid on the nematode cuticle proves to be an important recognition and attachment factor for the fungus; reduction of sialic acid by the presence of sialic-acid specific lectin reduced conidial attachment (Jansson et al., 1984).

A second hypothesis is that resistant nematodes may have evolved a mechanism to detoxify toxins before paralysis. Thirdly, members of the microbiome of resistant nematodes could hypothetically offer protection from *Pleurotus* toxins. With respect to the third hypothesis, Dirksen et al. (2016) found that three *Pseudomonas* species in the microbiome of *C. elegans* did exhibit antifungal activity towards pathogenic fungi. Thorough understanding of resistance will also have to integrate the fact that *Pleurotus* species also kill some species of bacteria (Thorn and Tsuneda, 1992).

The soil bacterium *Burkholderia cepacia* also produces a diffusible paralytic toxin that contributes to killing *Caenorhabditis elegans* (Köthe et al., 2003). Several strains were tested among a similar phylogenetic spectrum of bacterial feeding nematodes as in this study. *Zeldia punctata* and *Pristionchus pacificus* were especially resistant among the tested nematodes to the toxic effects of *B. cepacia* (Carta, 2000), in line with their resistance to *Pleurotus* toxin in this work. Another cephalobid nematode, *Acrobeloides maximus*, was attracted to and fed on more bacterial genera than *C. elegans*. This was interpreted as an adaptation to a less enriched ecosystem (Tahseen & Clark, 2014) since cephalobid nematodes are early stage colonizers (Ferris et al. 2001). Perhaps similar resistance mechanisms are working in nematodes that have resistance to those bacterial toxins and to these fungal toxins.

Barron (1978) noted that “highly specific host/parasite associations exist although the nature and reasons for this specificity are not currently clear.” Examples are evidently to be found among the zero-sum interactions between bacterial-feeding nematodes and toxin-producing, nematophagous fungi and their varied modes of action (Liang et al., 2019). More extensive testing of host ranges of both nematophagous fungi and fungivorous nematodes, as well as mechanisms for nematode resistance (Kitchen et al., 2019), should shed light on the physiology and ecology of interactions needed for targeted, integrated biocontrol.

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