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Differential Infection and Colonisation of Needle Age Classes by *Cronartium ribicola*

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ABSTRACT

White pine blister rust (WPBR) is a disease on North American five-needle white pine trees caused by the non-native fungal pathogen *Cronartium ribicola* that is causing widespread decline and mortality of *Pinus flexilis* (limber pine) in high elevation western forests. Elucidating the infection process is important for developing solutions for managing the disease. As an obligate biotroph, *C. ribicola* infects pine needles through stomatal pores and parasitizes needle tissue before growing into the stem, causing stem cankers. *Pinus flexilis* retains 8–9 years of needles on shoots, and it remains unclear whether infection and fungal colonisation, as well as the quantities of epicuticular wax, differ between needles of various age classes or between trees with and without major gene resistance to WPBR. To assess the relationship between quantity of *C. ribicola* DNA in *P. flexilis* needles, needle age, and resistance status, *P. flexilis* shoots from mature resistant and susceptible field trees were artificially inoculated with *C. ribicola*, where dispersal of fungal inoculum was applied evenly across all needle age classes. The quantity of epicuticular wax by needle age class and tree resistance status was explored. The results demonstrate that all needle age classes of *P. flexilis* are susceptible to *C. ribicola* infection. A significant positive correlation between needle age class and *C. ribicola* DNA quantity, regardless of tree resistance status, was noted. Additionally, epicuticular wax weight increased significantly with needle age, and wax did not appear to hinder needle colonisation by *C. ribicola*.

1 | Introduction

Elucidating the infection process of plant pathogens is critical for developing effective disease management strategies. Variations in microclimate, along with anatomical and physiological susceptibility of foliage within the canopy, can influence pathogen infection (Costes et al. 2013; Kraus et al. 2018; Pangga et al. 2013). One source of variation within the canopy of evergreens, such as pines and other conifers, is the age class distribution of needles (Ewers and Schmid 1981; Reich et al. 1996; Schoettle 1990; Schoettle and Fahey 1994). Some pathogens preferentially infect young or older foliage while others can infect and cause disease in needles of all ages (Mafia et al. 2009; Woods et al. 2005). Differential susceptibility of needle cohorts can significantly affect the target area of a mature canopy and

the likelihood of infection. Pines with short needle lifespans have a greater proportion of the crown composed of current or 1-year-old needles than those with longer needle lifespans (Schoettle 1994). Likewise, species with rapid needle turnover may reduce pathogen persistence by shedding infected needles before fungal growth penetrates branch tissue. Conversely, greater needle longevity may provide a longer opportunity for latent infections in needles to cause disease before being shed.

Biotrophic pathogens such as *Cronartium ribicola*, the causal agent of white pine blister rust (WPBR), infect their hosts either by direct penetration or through stomatal pores. Amongst those that enter through stomata, many species preferentially infect younger leaf tissue (Mapuranga et al. 2022). *Cronartium ribicola* enters through stomatal pores and parasitizes needle tissue

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before growing into the stem, causing stem cankers. Previous studies have shown *C. ribicola* tends to infect current-year needles in some species including eastern white pine (*P. strobus*) and western white pine (*P. monticola*), although findings across species are mixed (Duarte et al. 2025; Geils et al. 2010; Hudgins et al. 2005; Hunt and Jensen 2000; Snell 1936; Woo et al. 2004). Other factors such as tree age and shoot age can also alter infectability (Mielke 1943; Pierson and Buchanan 1938). Most research within the WPBR pathosystem has focused on pine species with relatively short needle lifespans (2–4 years), and it remains unclear whether these findings extend to other pine hosts that routinely retain needles for 8–15 years or longer (Ewers and Schmid 1981). For example, *P. flexilis* retains needles on its shoots for 6 to 10 years (Schoettle and Rochelle 2000), and differential susceptibility across different needle age classes could play a significant role in disease progression. If younger needle age classes exhibit higher susceptibility to colonisation, their placement in the outermost canopy could enhance the likelihood of spore deposition and subsequent infection. Alternatively, if older needles are more vulnerable, infections may initiate closer to the main stem, potentially speeding up the process of canker formation and accelerating tree mortality. Understanding the dynamics of pathogen infection across the needle age class distribution for species with long-lived needles is necessary for accurately dating disease introductions and dynamics, especially for non-native invasive pathogens such as *C. ribicola* (Arsdel et al. 2006; Kearns et al. 2009; Schoettle et al. 2011).

White pine blister rust poses a severe threat to five-needle white pine species across North America. Since its introduction in the early 1900s, WPBR has led to significant tree decline and mortality (Brar et al. 2015; Burns et al. 2023; Schoettle et al. 2022). *Pinus albicaulis* (whitebark pine) and *P. flexilis* populations have been particularly devastated by this disease, with *P. flexilis* population decline comparable to that observed in whitebark pine a decade prior to its federal designation as a threatened species under the Endangered Species Act (Goeking and Windmuller-Campione 2021).

Natural resistance to WPBR in *P. flexilis* populations can occur via major gene resistance (MGR), specifically the *Cr4* gene (Schoettle et al. 2014). In addition to discrepancies by needle age, it is not yet understood how *C. ribicola* colonisation of needles differs between *Cr4*-resistant and susceptible trees. Observations of needle lesions in trees with MGR demonstrate that initial needle infection does occur, but the timing and mechanism of colonisation cessation remain unknown. Investigating these dynamics is crucial for understanding the role of MGR in limiting WPBR progression.

Beyond the role of MGR, it is important to consider other constitutive defence strategies in pine trees. Pine needle surfaces are protected by a cuticle composed of epicuticular wax, a hydrophobic barrier that prevents microbial penetration (Evert et al. 2006). Studies have shown wax levels decrease with needle age in *P. sylvestris* (Scots pine), potentially due to environmental factors such as wind abrasion, heavy precipitation, or atmospheric pollutants (Cape et al. 1989; Turunen et al. 1997). Notably, *P. monticola* resistant to WPBR has been

found to possess higher quantities of epicuticular wax relative to susceptible trees (Noshad et al. 2023; Smith et al. 2006), yet it remains unclear whether increased wax levels significantly increase needle resistance to *C. ribicola* infection (Woo et al. 2001).

Despite extensive research on *C. ribicola* infection, critical gaps remain in understanding early stages of colonisation within needle tissue. It remains unclear whether infection and fungal colonisation, as well as the quantities of epicuticular wax, differ between needles of various age classes or between trees with and without MGR to WPBR. This study aims to address key gaps in our understanding of early *C. ribicola* infection in *P. flexilis* by examining fungal colonisation patterns and needle wax quantity by needle age and tree resistance status. Specifically, our objectives are to: (1) determine whether *C. ribicola* colonisation varies across needle age classes 21 days post-inoculation, (2) assess whether epicuticular wax levels differ by needle age class or resistance status and (3) evaluate the potential role of epicuticular wax in the infection dynamics of *C. ribicola*. The findings will contribute to a deeper understanding of factors that can influence the colonisation of obligate biotrophs like *C. ribicola* and inform conservation strategies for at-risk pine populations.

2 | Methods

2.1 | Plant Material

Pinus flexilis shoots were collected in the Colorado Rocky Mountains (approximately 9 km NE of Nederland) in a stand with no evidence of WPBR from two healthy, mature trees, one resistant (*Cr4*) to WPBR (R1; 8.4 cm DBH) and one susceptible (*cr4*) to WPBR (S1; 23.3 cm DBH) based on previous studies (Schoettle et al. 2014). All stems from both trees were collected from full-sun, south-facing shoots. To prevent drying and to maintain an intact xylem water column, shoots remained under water during and after pruning. Each shoot was inserted through a soft plastic lid with a pre-cut hole, re-cut underwater approximately 1 cm away from the initial cut, and placed into individual 60 mL plastic tubes filled with water. Parafilm was applied around each shoot at the tube lid opening to ensure a tight seal and prevent water leakage and evaporation.

2.2 | Inoculation With *C. ribicola*

In preparation for shoot inoculation, *C. ribicola* was initially grown on potted *Ribes nigrum* plants to obtain basidiospore inoculum. To do this, aeciospores of *C. ribicola* collected from multiple *P. flexilis* trees were used to inoculate *R. nigrum* grown in a growth chamber (Conviron CMP6050) set to a 12-h photo period (400 µmol light), 18°C/23°C and 75% relative humidity (RH). Plants, in 2-gal pots, were approximately 91.4 cm tall with many branches prior to inoculation. They were fertilised with granular fertiliser (Osmocote Plus 14-14-14) according to the manufacturer's instructions every 2 weeks and watered daily. *Ribes* plants (12 ca.) were inoculated with a suspension of 1×10^5

spores/ml *C. ribicola* aeciospores in Tween-20 (0.03% v/v). Inoculum was applied to the underside of each leaf until fully saturated with an 80 mL finger-pump sprayer and leaf tops were sprayed with tap water to increase humidity. Plants were placed in plastic bags and returned to the growth chamber set to 18°C at 80% RH in the dark for 66 h. Bags were removed, and plants were watered to full saturation and placed back in the growth chamber at 23°C and 18°C with a 12 h photoperiod (400 µmol/m²/s photosynthetically active radiation) to stimulate uredia formation. Fifty days post-inoculation of the *Ribes*, the growth chamber with infected *Ribes* was set to 15°C at 75% RH, with a day/night photoperiod of 12 h with light levels at 400/0 µmol/m²/s for 21 days to promote teliospore and subsequent basidiospore formation.

Twelve *P. flexilis* shoots were inoculated in a single dew chamber (Percival Model I36D; Perry, ID) at the same height while laying horizontal to ensure even distribution of inoculum from *R. nigrum* leaves with *C. ribicola* basidiospores supported on a wire mesh above the shoots (Figure S1a). The dew chamber was set to 16°C air temperature and 100% RH (29°C water temperature, 11°C wall temperature). Shoots were inoculated over a 5-day period and were horizontally rotated after the first 24 h. Two shoots used for control, one from each source tree, were left vertical on a lab bench out of direct sunlight for 5 days (Figure S1b).

To quantify inoculum distribution, six glass slides with a layer of rubber cement glue were placed in two rows in the centre, left and right of the dew chamber around and above the pine shoots. Slides were put in the chamber immediately after *Ribes* leaves were added and changed every 24 h for 5 days. When slides were removed, they were stained with lactophenol blue, and three photos (0.5 mm² each) were taken randomly per slide at 100× magnification with a microscope digital camera (Olympus SC180). In total, 90 photos were analysed. Total spore count and the number of germinating spores were counted per photograph to calculate the average spore counts and density as well as germination rate (germinated spores/total spores) per area. A final estimate of total spores per cm² deposited across the 5-day inoculation period was calculated. Across the 5-day inoculation period, an estimated 16,286 basidiospores per cm² were deposited, and an estimated 9651 per cm² germinated. Average spore count (15.47–18) and percent germination (49%–83%) were calculated for each slide position per day. There was no evidence of a significant relationship between slide position and average spore count per slide, while accounting for date ($F=0.04$, $p=0.99$), indicating that basidiospore drop was approximately evenly distributed throughout the dew chamber.

Post-inoculation, all shoots were placed upright in containers on a laboratory bench for 21 days to provide time for *C. ribicola* colonisation of infected needles. The shoots were placed next to a humidifier, grow lights were placed over the pines on a 12-h light cycle, and a layer of soft plastic was placed over the grow lights to maintain humidity. Water was added as needed to each shoot container, and water usage was measured. By 21 days, all shoot foliage remained green, and water usage and chlorophyll fluorescence measurements indicated that shoots were still physiologically active.

2.3 | Quantitative Comparisons of *C. ribicola* in Needle Tissue

Quantity of *C. ribicola* in *P. flexilis* needle tissue was compared by needle age class and tree resistance status following artificial inoculations. Three weeks post-inoculation, shoots were destructively sampled. Each annual cohort was aged using bud scale scars (Ewers and Schmid 1981; Schoettle 1990). While still attached to the stem, needles of each age class were dark-adapted for 30 min with dark clips, and chlorophyll fluorescence was measured using a chlorophyll fluorometer (Opti-Sciences F_v/F_m Meter, USA) (Figure S1c). Freshly excised needles from each cohort were then weighed.

Needle surfaces were cleaned and disinfected prior to processing for molecular detection of *C. ribicola* to eliminate any external *C. ribicola* DNA. All needles per cohort per shoot were placed in a 0.2% Tween-20 solution, vortexed at 500 rpm for 10 min, followed by a one-minute rinse in 70% ethanol. Needles were dried by dabbing onto a clean paper towel, placed on ice, then finely cut (~6 mm) with sterilised scissors and placed into a new, sterile tube. Tubes were shaken to homogenise needle cuttings, and 60 mg of needles were weighed from each needle cohort from each shoot for DNA extraction. To grind needle tissue, mortar and pestles were first sterilised using 90% ethanol and flamed, then wiped with 3% hydrogen peroxide. Liquid nitrogen was poured into each mortar with the pestle placed inside to cool. Pre-weighed needles were frozen in liquid nitrogen and ground with mortar and pestle until powdered. DNA was extracted using a Synergy Plant DNA Extraction Kit (OPS Diagnostics, Lebanon, NJ) following manufacturer protocols with the addition of bead beating each sample for 4 min in step 2. DNA quantity and quality were assessed for all samples using a spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific).

Real-Time quantitative PCR was performed following methods described previously (Bertram 2021) using PrimeTime Gene Expression 2× Master Mix (IDT, Coralville, IA). Two primer sets were used, one that targets *C. ribicola* gene *Crib190* (Bergeron et al. 2019), and a newly developed qPCR assay was designed that targets the pine host gene *Agp6* (Table 1) (Syring et al. 2005). Reactions were conducted in a 96-well plate with 10 µL Master Mix, 0.50 µL forward primer (100 µM), 0.50 µL reverse primer (100 µM), 0.25 µL probe (100 µM), 8.75 µL molecular grade water, and 2 µL sample DNA extract per reaction. Seven targeted DNA standards were run in duplicate on each plate for *C. ribicola* (ten-fold change from 11.5 ng/µL to 0.0115 pg/µL), and for *P. flexilis* (five-fold change from 118 ng/µL to 7.5 pg/µL), and reactions specific to each species were run on separate plates in parallel using real-time thermocycler machines (CFX Connect Real-Time PCR Detection Sybranch, Bio-Rad, US). Initial DNA denaturation was set at 95°C for 15 min followed by 50 cycles of denaturation at 95°C for 15 s and annealing at 58°C for 60 s. Mean reaction efficiency was 0.83 (±0.12). The average quantitation cycle (C_q) number was used to create standard curves for the DNA on each plate. Conversion of C_q value to absolute quantity of DNA was performed using CFX Manager Software (v3.1, Bio-Rad, US). DNA was then standardised for each sample using Equation (1), where absolute quantity of *C. ribicola* DNA was

TABLE 1 | Forward and reverse primers and probe (IDT, Coralville, IA) of *Agp6* (Syring et al. 2005) developed specifically for *Pinus flexilis* used to standardise quantified *Cronartium ribicola* DNA in *P. flexilis* needles following qPCR.

Assay	Targeted taxon	Primer, TaqMan probe	Sequence	Tm	Amplicon length	GenBank accession no.
Agp6	<i>Pinus flexilis</i>	Agp6F	5'-CTGCTAAACCACCCACAAC-3'	62.4°C	134 bp	AF101785
		Agp6R	5'-CTTGGTGGGAGCAACGG-3'	62.4°C		
		Agp6T	5'-FAM/TCTCAACTC/ZEN/CGAAGCCTCCCAC/IBFQ-3'	67.4°C		

divided by the absolute quantity of *P. flexilis* DNA. Equation 1 as Standardised *C. ribicola* DNA quantity = *C. ribicola* DNA quantity / *P. flexilis* DNA quantity.

2.4 | Wax Quantity Experiment

Quantity of epicuticular wax was examined in *P. flexilis* needles by age class and tree resistance status to WPBR. *Pinus flexilis* shoots from three susceptible (*cr4*) and two resistant (*Cr4*) trees were collected from Pilot Hill, WY. The shoots were dissected into age cohorts as described above, and all needles per cohort were excised and weighed (fresh). Cohorts were organised such that each tube contained needles of only one age class from one shoot, ranging from 0 to 10 year old needles. Needles were cut into approximately 1 cm segments using scissors sterilised with 70% ethanol, returned to their respective tubes, and gently shaken to homogenise. Wax quantity was measured following previous studies (Noshad et al. 2023; Smith et al. 2006). A total of 1 g subsample of each age cohort per shoot was added into pre-weighed 10 mL borosilicate glass tubes and 3 mL 100% chloroform was added to each tube to fully submerge the needles for two minutes. Needles were then removed using a microspatula and tweezers that were sterilised between samples with 100% ethanol. Needles were discarded, and tubes remained in the fume hood until all chloroform had evaporated, approximately 96 h. Each tube was then re-weighed, and the dry weight of epicuticular wax was calculated by subtracting the initial tube weight from the final tube weight. Needle wax was standardised by dividing the dry weight of wax by the total weight of needles per sample. Nine blank tubes were used that contained 3 mL 100% chloroform with no needles; sterilised collection tools were submerged in three of them to assess possible contamination. All weight measurements were carried out on an analytical scale (Torbal AGCN120) with a standard deviation rating of 0.0001 g.

2.5 | Statistical Analyses of Inoculation Data

To assess an effect on needle stress, a linear mixed model was fit (Equation 2), with F_v/F_m predicted by the interaction between needle age class (categorical) and tree resistance status while accounting for whether the tree was in the inoculation or control group and stem number as a random effect, followed by an ANOVA test. Equation 2 as $F_v/F_m \sim \text{Needle Age Class} \times \text{Tree} + \text{Experiment} + (1|\text{Stem})$. Effect size was calculated using partial

eta-squared. Spearman's correlation was used to evaluate the relationship between F_v/F_m and standardised *C. ribicola* DNA. To assess overall differences in standardised *C. ribicola* DNA concentrations, two Kruskal-Wallis non-parametric tests were conducted, with one test predicting differences by age class and the other by tree resistance status. Following a significant result, a Dunn's test with Benjamini-Hochberg adjustment for small sample size was performed to identify specific pairwise differences amongst age classes while controlling for multiple comparisons. Residual normality for all models was assessed using Shapiro-Wilks tests, and linearity was assessed using residual vs. fitted plots. Difference in mean accumulative shoot water usage per shoot needle mass (g/g) was examined using a Student's *t*-test. All analyses were performed with a significance level (α) set to 0.05 in R Statistical Software (v3.3.4; R Core Team 2023).

2.6 | Statistical Analyses of Epicuticular Wax Data

Wax weight was calculated by the change in glass weight (g) divided by the total weight of needle mass (g) subsampled. To assess the effects of age class and susceptibility on wax weight, a generalised linear regression model (GLM) with a Gaussian family distribution was fit (Equation 3). Wax weight (g) was modelled as a function of age class (categorical) and tree resistant status with Equation 3 as Wax weight ~ Needle Age Class + Tree Resistance Status. Model fit was assessed using Akaike Information Criterion (AIC) and ANOVA assumptions were checked using a Shapiro-wilk test and standardised vs. fitted residuals plot. Once all assumptions were met, an Analysis of Variance (ANOVA) test was used, followed by a Tukey HSD. Further, Spearman's rank correlation was calculated to assess direction and strength of correlation. All analyses were performed in R Statistical Software (v3.3.4; R Core Team 2023) with a significance level (α) set to 0.05.

3 | Results

3.1 | Quantitative Comparisons of *C. ribicola* Colonisation

To better understand the dynamics of *C. ribicola* colonisation, the relationship between needle age class and the mean quantity of standardised *C. ribicola* DNA following artificial inoculation was examined (Figure 1). In all inoculated branches

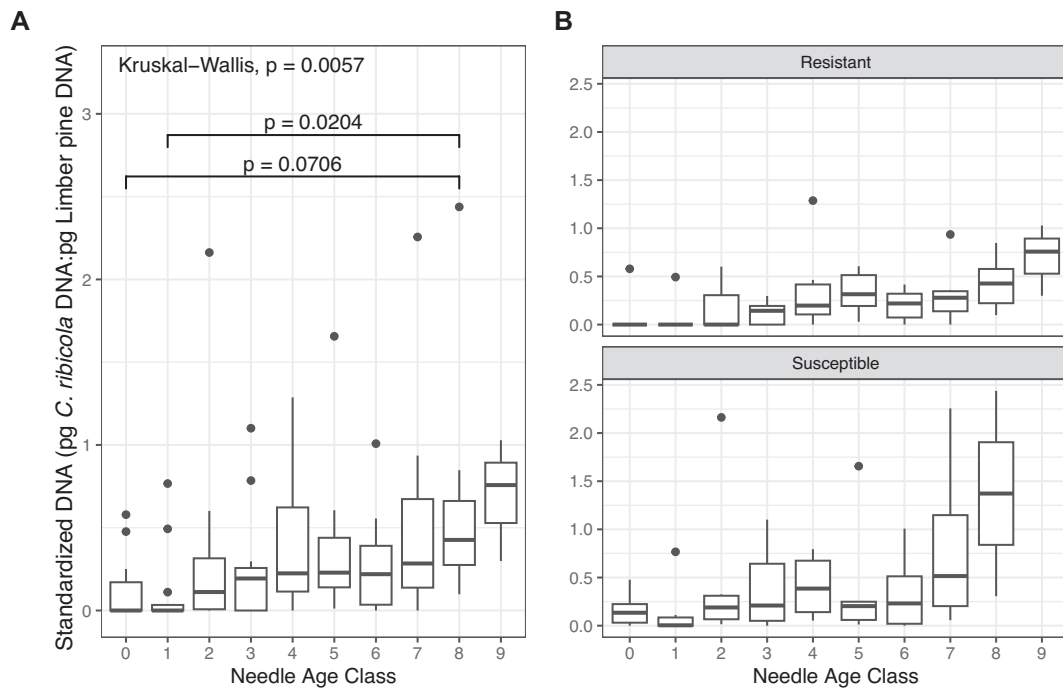


FIGURE 1 | Standardised DNA (pg *C. ribicola* DNA:Pg. *P. flexilis* DNA) by age class per extraction three weeks after artificial inoculation of branches collected from one susceptible and one resistant (*Cr4*) mature field tree with six biological replicates each. Older needles exhibited significantly higher quantities of *C. ribicola* DNA than younger needles ($X^2 = 23.23$, $p = 0.006$) (A), while there was no significant difference in DNA quantity between needles sourced from the susceptible and resistant trees ($X^2 = 0.707$, $p = 0.401$) (B).

from both susceptible and resistant trees, there was a significant relationship between needle age class and the mean quantity of standardised *C. ribicola* DNA (pg *C. ribicola*:pg *P. flexilis*) ($X^2 = 23.23$, $p = 0.006$). Pairwise comparisons indicated that older needles contained significantly higher levels of *C. ribicola* DNA compared to current or 1-year old needles (Table 2). There was no difference in mean *C. ribicola* DNA by tree resistance status ($X^2 = 0.707$, $p = 0.401$).

Chlorophyll fluorescence measurements were used to assess the physiological condition of the needle cohorts (Figure 2). The interaction between needle age class and tree resistance status was analysed and revealed a significant interaction with a large effect size ($\eta_p^2 = 0.15$, $X^2 = 15.768$, $p = 0.046$), indicating that the effect of age class on F_v/F_m varied depending on the source tree. Mean F_v/F_m values of the uninoculated control stems were not significantly different from inoculated stems ($X^2 = 0.007$, $p = 0.935$), suggesting that a change in F_v/F_m values is not detected at 21 days post-inoculation. Age class showed a large effect size ($\eta_p^2 = 0.20$) and explained 20% of the variance in F_v/F_m , though the influence of tree alone was negligible ($\eta_p^2 = 0.008$). Further, no significant correlation between F_v/F_m and standardised quantity of *C. ribicola* DNA (Spearman's $\rho = 0.048$, $p = 0.633$) was found, suggesting that incidence and quantity of *C. ribicola* infection do not significantly affect needle stress in the first three weeks post-inoculation, and photochemical efficiency varies by needle age class depending on the source tree. Lastly, per shoot on average, accumulative water use by needle mass (g/g) was not significantly different between uninoculated shoots (1.436, ± 0.448) and inoculated shoots (1.780, ± 0.895) after 21-days post-inoculation ($t(17.148) = 2.000$, $p = 0.401$).

TABLE 2 | Pairwise comparisons of mean quantity of standardised *C. ribicola* DNA (pg *C. ribicola*:Pg. *P. flexilis* DNA) in pine needles of different age classes (in years) following artificial inoculation.

Age classes	Z	p	p adjusted
0-4	-2.206	0.014	0.068
1-4	-2.613	0.004	0.04
0-5	-2.075	0.019	0.085
1-5	-2.482	0.007	0.042
1-6	-1.882	0.03	0.104
0-7	-2.344	0.01	0.054
1-7	-2.732	0.003	0.035
0-8	-2.954	0.002	0.035
1-8	-3.318	0	0.02
2-8	-1.85	0.032	0.103
3-8	-1.949	0.026	0.105
0-9	-2.612	0.005	0.034
1-9	-2.869	0.002	0.031
2-9	-1.838	0.033	0.099
3-9	-1.909	0.028	0.105
6-9	-1.679	0.047	0.131

Note: Comparisons indicate that older needles contained significantly higher levels of *C. ribicola* DNA compared to current or 1-year old needles. Significant results using raw p-value from Dunn's test for multiple comparisons are shown, as well as Benjamini-Hochberg-adjusted p-values.

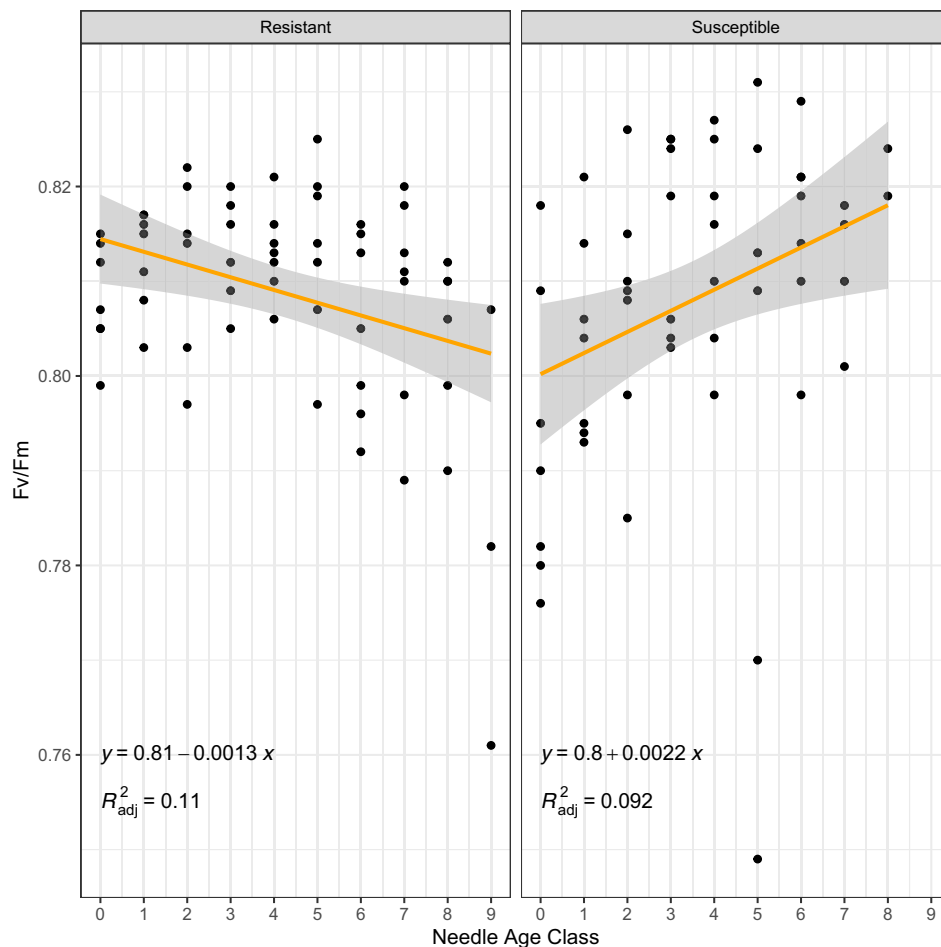


FIGURE 2 | Chlorophyll fluorescence (F_v/F_m) by needle age class from two mature *P. flexilis* trees by WPBR resistance status 3 weeks post-inoculation with *C. ribicola*. There is a statistically significant interaction between needle age class and source tree resistance status on F_v/F_m when accounting for whether the stem was inoculated or control ($X^2 = 15.779$, $p = 0.046$).

3.2 | Variation in Wax Quantity

To explore variations in epicuticular wax quantity, the linear relationship between needle age class and epicuticular wax weight was explored while controlling for tree resistance status (Figure 3). The analysis revealed a significant relationship between age class and wax weight ($F = 13.123$, $p < 0.0001$). Wax weight increased with needle age, as indicated by a positive correlation ($r = 0.54$, $p < 0.0001$, $n = 152$). The mean wax mass (g_{wax}/g_{needle}) sourced from resistant trees and susceptible trees was not significantly different ($t(147.19) = 1.560$, $p = 0.121$).

4 | Discussion

This study demonstrates that *C. ribicola* can infect and colonise all needle age classes in *P. flexilis*. Colonisation was more extensive in older needles regardless of whether the needles were sourced from a tree with or without *Cr4*-resistance. Colonisation of *C. ribicola* was measured across all needle age classes using molecular markers and quantitative PCR, marking one of the first completed studies using this technology on this pathosystem. These findings challenge long-held assumptions about needle susceptibility and provide new insights into the biology of *C. ribicola* infection.

It is commonly observed that biotrophic pathogens preferentially infect and colonise younger tissue compared to older tissue due to lower levels of defence, such as a thin cuticle and relatively lower phenolic content (Farber and Mundt 2017; Li and Xu 2002; Mapuranga et al. 2022). However, previous literature presents conflicting results regarding which pine needle age class is most susceptible to *C. ribicola*. In previous studies on *P. monticola* and *P. strobus*, inoculated young seedlings demonstrated high susceptibility in current-year needles (Hunt 1991; Patton 1961) and in young trees and shoots (Arsdel 1961; Patton 1970). In contrast, studies on *P. monticola* of naturally inoculated older trees (>5 years) exhibited lower infection on current-year needles (Hunt and Jensen 2000; Pierson and Buchanan 1938). It is therefore suggested that as trees mature, their needle cohorts exhibit decreasing susceptibility to *C. ribicola* colonisation, though all age classes remain susceptible.

Molecular tools used in this study revealed greater *C. ribicola* colonisation in older needles of mature *P. flexilis*, regardless of whether the needles were sourced from a susceptible or resistant tree. Our results align with previous research that suggested that white pines with MGR to WPBR are colonised at the same rate as susceptible trees during the early stages of infection (Stone et al. 2011). This provides additional evidence that the *Cr4*-mediated resistance mechanism in *P. flexilis* occurs after

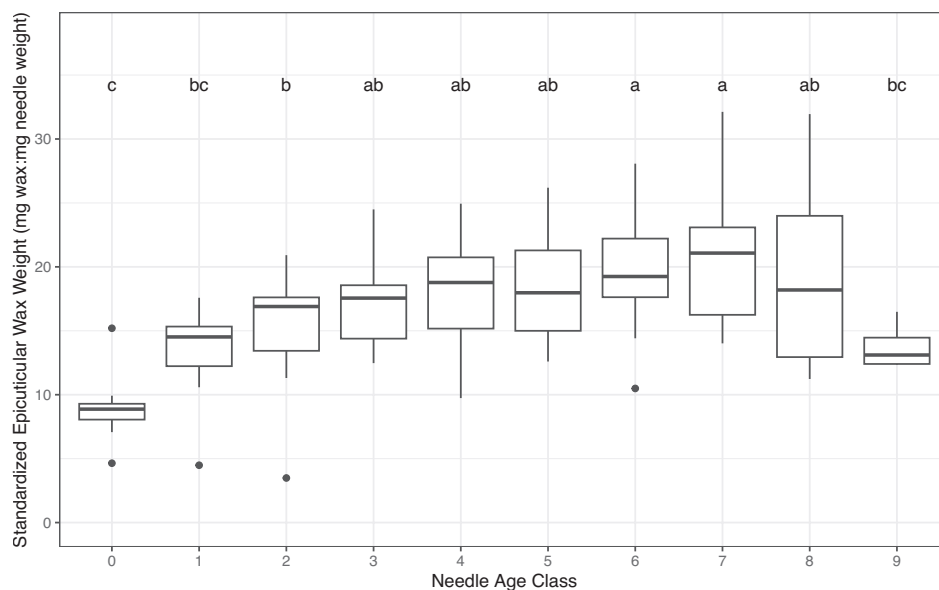


FIGURE 3 | Epicuticular wax weight (mg) exhibits a significant linear relationship with needle age class ($F = 13.1226$, $p < 0.0001$) after accounting for tree resistance status. Brackets represent Bonferroni-adjusted p -values following pairwise comparisons that revealed significantly higher mean wax weight between the age class with the highest mean wax weight at 7 years and the oldest age class at 9 years, relative to the current year's needle.

initial needle colonisation. In *P. strobus*, for example, *C. ribicola* growth was halted in resistant individuals approximately seven weeks post-infection due to increased production of phenolic compounds (Jurgens et al. 2003). These results suggest that both resistant and susceptible trees may experience similar early colonisation by *C. ribicola*, with resistance mechanisms diverging later in the process.

Epicuticular wax, a physical barrier to fungal penetration, was also evaluated in this study. Mean wax quantities were similar in needles sourced from both susceptible and resistant *P. flexilis* field trees. Conversely, previous literature suggests trees resistant to WPBR exhibit higher epicuticular wax quantity than susceptible trees, such as in *P. strobus* (Jurgens et al. 2003; Smith et al. 2006) and *P. monticola* (Noshad et al. 2023). Increased epicuticular wax can act as a physical barrier to stomatal entry, leading to decreased penetration of a fungal germ tube and thus decreased levels of infection (Patton and Spear 1980; Smith et al. 2006). Interestingly, the results presented here showed that older needles, which had relatively more epicuticular wax, were more heavily colonised by *C. ribicola*. This suggests that wax quantity alone does not play a significant role in WPBR resistance, aligning with earlier research on *P. monticola* and *P. strobus* that propose wax is not a major mechanism of needle resistance (Patton 1970; Woo et al. 2001). Differences in inoculum load may contribute to discrepancies from past work, and further research incorporating a range of inoculum loads is necessary to refine the understanding of this relationship.

A significant interaction was observed between needle age class and source tree on F_v/F_m values of inoculated needles, indicating that photochemical efficiency is influenced by both factors. With increasing needle age class, the resistant tree exhibited a negative trend and the susceptible tree exhibited a positive trend. While small sample sizes limit the ability to draw conclusions about the role of resistance status, these findings indicate the relationship between needle age class and F_v/F_m values

varied by source tree. Additionally, a significant difference in mean F_v/F_m values was not observed between inoculated and uninoculated shoots at 21 days post-inoculation, suggesting that *C. ribicola* inoculation may not cause notable changes in needle stress 3 weeks following inoculation. Studies in other rust pathosystems have reported significant decreases in F_v/F_m values following inoculation, including *Puccinia coronata* on *Avena sativa* (oat) (Scholes and Rolfe 1996) and *Hemileia vastatrix* on *Coffea arabica* (coffee) (Honorato Júnior et al. 2015). However, (Bertram 2021) found no change in F_v/F_m values of *P. flexilis* needles in the first 16 weeks following inoculation with *C. ribicola*, but observed a significant decrease after 18 weeks. Therefore, 21 days post-inoculation was likely too early to detect a similar effect. Chlorophyll fluorescence and water uptake values indicated that all stems remained alive and needles were viable at 21 days post-inoculation (Figure S1).

In this study, nine-year-old *P. flexilis* needles were particularly susceptible to *C. ribicola* colonisation. At this age, the highest median quantity of *C. ribicola* DNA (standardised) was observed as well as a marked decrease in both needle photochemical efficiency and epicuticular wax quantity. These physiological changes suggest the oldest needles are undergoing senescence, a process that may create a favourable environment for biotrophic colonisation. Senescing tissues often experience increased levels of soluble sugars and nitrogen compounds as chlorophyll degrades and nutrients are mobilised for reabsorption (Lei et al. 2023; Thomas and Ougham 2014). Chloroplast degradation, in particular, is a major source of remobilised nitrogen, as these organelles store a significant portion of total leaf nitrogen in proteins such as Rubisco (Hörtensteiner and Feller 2002). This release of nitrogen-rich compounds may facilitate pathogen establishment, particularly for rust obligate biotrophs like *C. ribicola*, which have been shown to exhibit impaired nitrogen assimilation pathways thus relying fully on their host for reduced nitrogen (Duplessis et al. 2011). Furthermore, Maupetit et al. (2018) observed *Melampsora larici-populina* was more

aggressive on older *Populus deltoides* × *P. nigra* ‘Robusta’ poplar leaves and proposed this was due to increased accumulation of phenolic content relative to young leaves, as they observed no clear correlation between pathogen success and leaf sugar, total C, or N content. Further research into the metabolic and structural changes in ageing needles could provide deeper insights into host susceptibility dynamics.

Age-related differences in needle susceptibility have significant implications for our understanding of disease spread, particularly in pine hosts that can maintain needles for over a decade. Tracking disease spread relies on accurately determining the timing of initial infection, which is often inferred by estimating canker age. This approach provides crucial insights into infection timing and subsequent pathogen progression. However, estimating the timing of initial infection from the location of a canker on a shoot has proved challenging as it requires assumptions of the age of leaves at infection and the length of the latent period between infection and visual disease development (Kearns et al. 2009; Schoettle et al. 2011). Canker ageing is typically inferred from its location relative to young needles, under the assumption that only those needles are susceptible. In hosts with long-lived needles, this approach becomes more complex. Prolonged needle retention can introduce ambiguity to canker ageing as needles may experience prolonged exposure to infection or reinfection over multiple seasons, potentially increasing the margin of error by several years (Schoettle et al. 2011). Accurately determining the temporal progression from needle infection to visible canker formation is critical for epidemiological modelling. Kearns et al. (2009) developed a method to estimate canker age on *P. flexilis* based on canker dimensions and expansion rate. However, no clear method currently exists to estimate the timing of initial infection or length of the latency period. The results presented here suggest that because all needle age classes are susceptible to infection further research is needed to develop a method for estimating the timing of initial infection and the duration of the latency period.

This study offers a unique perspective by utilising mature field trees grown under natural conditions rather than seedlings grown under controlled nursery settings. Discrepancies from prior research could stem from variations in tree age, white pine species, environmental conditions, natural versus artificial inoculations, or genetic makeup of tree families used. While the sample size in this study is limited, it provides valuable insights into differential susceptibility by needle age, especially in the context of WPBR. Future research with larger sample sizes will be important to confirm these findings and further investigate factors that influence needle susceptibility.

5 | Conclusion

This study advances our understanding of *C. ribicola* infection dynamics in *P. flexilis* by demonstrating that all needle age classes can be colonised, challenging the long-standing assumption that younger needles are the most susceptible. Molecular tools provided novel insights, showing that colonisation rates do not differ between susceptible and resistant trees during early infection stages. Furthermore, epicuticular wax quantity did not appear to impede infection by *C. ribicola* in *P. flexilis* under the

artificial inoculation conditions used. Instead, resistance mechanisms including *Cr4* likely act post-colonisation, emphasising the importance of physiological and biochemical defences. By utilising branches from mature field trees, this study highlights the need to consider natural environmental variability when evaluating host-pathogen interactions. These findings contribute to the understanding of early infection by obligate biotrophic fungal pathogens like *C. ribicola* in white pines and inform future management strategies aimed at improving resistance in at-risk pine populations.

Author Contributions

Ashley E. Miller: investigation, methodology, writing original draft. **Anna W. Schoettle:** investigation, methodology, supervision, reviewing and editing draft. **Jorge R. Ibarra Caballero:** investigation, methodology. **Tori Teegardin:** investigation, methodology. **Jane E. Stewart:** methodology, supervision, reviewing and editing draft.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Limber pine shoots were collected from mature field trees and inoculated with *Cronartium ribicola*. Images are of shoots at 0 (A) and 21 days post-inoculation (C), and immediately prior to inoculation (B). Needles showed minimal browning and shoots were still taking up water at 21 days post-inoculation.