

Anatomical and cellular responses of *Pinus monticola* stem tissues to invasion by *Cronartium ribicola*

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Summary

White pine blister rust (*Cronartium ribicola*) causes extensive damage to white pines and their associated ecosystems across North America. The anatomical and cellular characteristics of *C. ribicola* colonization in *Pinus monticola* branch and stem tissues were studied as a basis for understanding host tree reactions that may be related to resistance. Samples examined showed typical fusiform swelling and some had produced aecia. The reaction of phloem and xylem tissues was compared with non-infected tissue using light and electron microscopy. Cortical parenchyma and phloem polyphenolic parenchyma cells underwent mitotic division, cell swelling, and ca sixfold greater accumulation of phenolic compounds in colonized *vs.* control stems. In the cortex and secondary phloem, haustoria were common in parenchymatous cells, and hyphae were abundant in the intercellular spaces, but cell death was rare, unless aecia had ruptured the stem cortex. Hyphae were also common in xylem rays, tracheids and between tracheids. Disease-induced changes in the cambial zone included development of cambium-derived xylem traumatic resin ducts. Results demonstrate that diverse host defence responses were activated in the bark of apparently susceptible trees, but lack of mechanical damage by *C. ribicola* to the phenol-containing host cells and the resin duct system allowed extensive colonization and development of aecia despite elicitation of these stem defences. Interactions between *P. monticola* and *C. ribicola* are discussed and compared with other conifer–fungus pathosystems.

1 Introduction

The fungus *Cronartium ribicola* J. C. Fisch. is the causal agent for white pine blister rust (WPBR), a disease of five-needled pines (McDONALD and HOFF 2001). The pathogen infects the tree through needle stomata, and if not checked in needles, it grows into the short shoots and eventually into the stems where it can cause cankering and tree death. Although this macrocyclic rust fungus has been in North America for <100 years, it has spread rapidly and caused extensive damage to five-needled pines. Much of the current research has focused on genetic resistance against *C. ribicola* in economically important white pines (*Pinus monticola* Dougl., *P. strobus* L., and *P. lambertiana* Dougl.; McDONALD et al. 2004). Several broad physiological responses to *C. ribicola* have also been characterized in some resistant *P. monticola* and include the premature shedding of infected needles, inhibition of fungal colonization into the short shoots from infected needles, and general bark reactions that slow canker growth and isolate or limit fungal spread (HOFF and McDONALD 1971; HOFF et al. 1980, 2001; HUNT 1997, 2002).

Resistance to *C. ribicola* is evident in some *P. monticola* trees; however, the resistance mechanisms are poorly understood. The best-known resistance mechanism is ‘major gene resistance’ (MGR), whose effects are apparent even shortly after needle colonization

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(KINLOCH et al. 1999). In MGR-phenotype *P. monticola*, the dominant disease-resistance gene *Cr2* prevents rust colonization from reaching the stem (KINLOCH et al. 1999). Observed responses of *P. monticola* *Cr2* phenotypes include hypersensitive host cell death and the deposition of host defence compounds in needles (KINLOCH et al. 1999). In eastern white pine (*P. strobus* L.), phenolics accumulate in needles after infection (BOYER and ISAAC 1964; BOYER 1964). Phenolic deposition occurs more rapidly around mycelial masses in needles of resistant lines, and can occur without significant host cell death (JURGENSEN et al. 2003). BOYER (1964) and BOYER and ISAAC (1964) found that vacuole fragmentation and release of phenolics into the cytoplasm and walls occurred after infection in first-year but not second-year needles, and speculated that this may be associated with greater resistance of first-year needles to blister rust. The importance of phenolics in resistance to fungal pathogens and general defence has been studied for decades (FARKAS and KIRÁLY 1962; HUNTER 1974; NICHOLSON and HAMMERSCHMIDT 1992; BECKMAN 2000), and qualitative alterations in phenolic compounds have been reported in *Pinaceae* species following fungal inoculations (BRIGNOLAS et al. 1995; LIEUTIER et al. 1996; BOIS and LIEUTIER 1997; BONELLO et al. 2003; NAGY et al. 2004). Hypersensitive and phenolic responses could presumably act together to kill fungal hyphae in needles of *P. monticola* seedlings carrying *Cr2*, but the recovery of living rust cultures from resistant needle spots from the similar *Cr1* resistance phenotype in sugar pine (*P. lambertiana* Dougl.) suggests that some hyphae may be only inactivated (KINLOCH and DUPPER 1996).

Studies on processes occurring in the stem during *C. ribicola* infection include few details on host responses (KREBILL 1968; WELCH and MARTIN 1974, 1975). A fine-scale characterization of anatomical and cellular changes that occur in bark and wood tissues in response to blister rust is important for understanding mechanisms of stem colonization or resistance after *C. ribicola* has breached the needle defences. It is of specific interest to determine how *C. ribicola* copes with constitutive chemical defence of the bark relative to what is known for necrotrophic fungal pathogens, and if similar induced defences are elicited. Constitutive defences in stems of *Pinaceae* include stored chemicals, such as resin and phenolics (HUDGINS et al. 2003a, 2004; FRANCESCHI et al. 2005). Resins are stored in ducts, canals, blisters, or resin cells (TRAPP and CROTEAU 2001), while phenolics are stored in vacuoles of phloem polyphenolic parenchyma (PP) cells (KREKLING et al. 2000). These compounds are released when the cells or structures (i.e. canals) are damaged by an invading organism, and can then kill the organism if the amount of stored material is great enough or toxic enough. Inducible defences can result in an increase of existing chemical compounds, as well as produce new defence compounds and cellular or tissue reactions. The intensity of these reactions can lead to compartmentalization or killing of the invasive organism, thus imparting resistance. Constitutive and inducible defences of conifer bark have been extensively studied with respect to other fungal pathogens, such as blue stain fungi (FRANCESCHI et al. 2000; KROKENE et al. 2000, 2001, 2003; KREKLING et al. 2004), and these studies can provide valuable comparisons for understanding the aetiology of WPBR in stems of five-needled pines.

Polyphenolic parenchyma cells make up the majority of living tissue in the bark of conifers (KREKLING et al. 2000) and play a prominent role in defence against necrotrophic pathogens. Aside from their stored phenolics, PP cells show enhanced activity during attack that can be seen as substantial increases in phenolic contents (FRANCESCHI et al. 1998, 2000; EVENSEN et al. 2000; NAGY et al. 2000, 2004; KREKLING et al. 2004), involvement in wound periderm and wound callus formation (FRANCESCHI et al. 2000; NAGY et al. 2000), and synthesis of signalling compounds (HUDGINS and FRANCESCHI 2004). There is evidence that stem phenolics are involved in defence of *P. monticola* against fungal pathogens, and these phenolics are almost certainly primarily in PP cells. ENTRY et al. (1992) found a negative correlation between phloem phenolic concentrations and *P. monticola* sapling mortality rates caused by the root-rot pathogen *Armillaria ostoyae* (Romagn.) Herink, and *P. monticola*

stem wounding-induced accumulation of phenolics in PP cells at sites both surrounding and distant from the wound (HUDGINS et al. 2003a). However, the interaction between *C. ribicola* and PP cells of the stem bark has not been characterized.

Another group of potential defensive compounds, the terpenoid resins, can be seen outside older cankers, but it is unclear whether *C. ribicola* colonization disrupts constitutive resin structures or induces traumatic resin duct (TD) formation, either of which could impact the success of colonization. It is well known that abiotic or biotic tissue damage can induce the formation of secondary resin (TRAPP and CROTEAU 2001), much of which is produced in new ducts, referred to as TDs (REID et al. 1967; FAHN and ZAMSKI 1970; WERNER and ILLMAN 1994; ALFARO 1995; NAGY et al. 2000; LEV-YADUN 2002). The induced resin may also be more fungistatic than primary resin because of altered terpene content and addition of phenolics (SCHUCK 1982; PAINE et al. 1987; NAGY et al. 2000; KROKENE et al. 2003). It is of specific interest to determine the mechanism by which *C. ribicola* contends with resin defence in the stem during colonization, and whether it induces secondary resin accumulation, as this may be related to relative resistance of trees.

Fungal pathogens that invade conifers are diverse in both their methods of initial ingress and their interactions with the host following successful establishment. For example, some blue stain fungi (e.g. *Ceratomyces polonica*) are vectored by insects and subsequently cause host mortality through rapid fungal colonization, destruction of the cambium and phloem, and disruption of flow in the xylem of the sapwood (PAINE et al. 1997). In contrast, *C. ribicola* is an obligate parasite that enters through needles and spreads into branches and stems, generally resulting in mortality of its hosts after a much longer period of time. However, the details of the interactions between *C. ribicola* and stem tissues in five-needled pines are not well characterized. To successfully colonize the stem of the tree, *C. ribicola* must avoid the constitutive defences of the bark and the inducible defences, if they are elicited, or prevent elicitation of inducible defences. Thus, information on the process of stem infection is important to determine potential resistance mechanisms against colonization (MCDONALD et al. 2004).

The purpose of this study was to elucidate the basic anatomical and cellular features of *C. ribicola* colonization of *P. monticola* stem tissue. The interaction of the fungus with various tissues of the bark was studied to determine whether constitutive defences were avoided or whether the fungus is generally resistant to these defences. In addition, the potential for the fungus to elicit common visually identifiable inducible defences was studied. The results provide baseline information on the mechanism of colonization of stem tissues by *C. ribicola*, and demonstrate that trees examined have the usual responses seen with necrotrophic fungal invasion. However, the patterns of *C. ribicola* colonization and host utilization minimize its contact with constitutive defences and the consequences of induced defences.

2 Materials and methods

2.1 Field collection of *Pinus monticola* stem and branches

Tissue was collected from branches and main stems (boles) of *C. ribicola*-infected *P. monticola* trees from a naturally infected stand near Elk River, Idaho (lat. 46°48'N; long. 116°09'W) on 24 July 2003. From branches, entire cankers were collected, cut in half and placed in a fixative solution as described below. Branches were 3–7 years old and approximately 1–3 cm in diameter. Control branches were collected from infected trees and consisted of non-infected, intact branch segments from the same whorls and at similar positions. Infected bark and sapwood were excised from the aelial, mid-canker, and peripheral regions of stem cankers as described by WELCH and MARTIN (1974). For bole cankers, control tissue consisted of samples excised from non-infected bark on the opposite

side of the bole from the canker. Observations indicated the samples were free of visible fungal hyphae and showed the same anatomy as uninfected boles or stems. Phloem and xylem tissues were collected from beneath the central collapsed region and from the living peripheral regions of cankers. Trees with bole cankers were between 14 and 17 years old and 10–15 cm d.b.h., and represent natural regeneration in a stand where 80 years of exposure to rust had caused about 95% mortality in the parental generation (data on file at Forestry Sciences Laboratory, Moscow, ID, USA). A total of six infected trees were chosen to supply tissues from three branch and three bole cankers and corresponding control tissues. Although the presence of aelial cankers indicates that trees were susceptible to WPBR, past selection processes may have affected the relative susceptibility of these trees. The results presented are representative of observations across all trees, as reactions were generally consistent from tree to tree.

2.2 Sampling and microscopy

Samples collected in the field were placed in fixative solution [2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde buffered in 50 mM L-piperazine-*N*-*N'*-bis(2-ethane sulfonic) acid, pH 7.2]. In the laboratory, samples were cut into 2.5 × 2.5 mm cross-section blocks and further fixed for 24 h at 4°C, dehydrated with ethanol, and infiltrated and embedded with L.R. White acrylic resin (London Resin Company Ltd, Reading, Berkshire, UK) as previously described (HUDGINS et al. 2003b). For light microscopy, sections (1 µm thick) were stained with Stevenel's blue (DEL CERRO et al. 1980) and photographed with a Leitz Aristoplan photomicroscope. For examination of starch, sections were stained with the periodic acid-Schiff's (PAS) reagent procedure as previously described (NAGY et al. 2000), which stains starch and cells walls, or with 3% (w/v) iodine potassium iodide (IKI), which only stains starch. Use of IKI allowed a distinction between fungal walls and plant starch granules in infected cells, because these structures could be of similar size in thin sections and they could not always be distinguished by the PAS staining procedure. To better visualize fungal structures, some sections were stained with 0.1% (w/v) aq. acridine orange, and viewed in epifluorescence mode with excitation by blue light of 450–490 nm. Hyphae and haustoria fluoresced differently than plant tissues.

Branch and stem samples for scanning electron microscope (SEM) were fixed and dehydrated as described for light microscopy. Following dehydration, samples were critical-point dried with CO₂ using a Samdri-PVT-3D critical point dryer (Tousimis Research Corp., Rockville, MD, USA) and then sputter coated with gold. The sections were examined with a Hitachi S-570 SEM (Hitachi Ltd., Tokyo, Japan) at 20 keV accelerating potential.

Samples for TEM were fixed for 48 h at 4°C in 2% (v/v) paraformaldehyde, 2.5% (v/v) glutaraldehyde, and 1% (w/v) tannic acid in 50 mM phosphate buffer, pH 7.2. Blocks were post-fixed for 24 h in 2% aq. (w/v) osmium tetroxide and 1% (w/v) tannic acid at 4°C, dehydrated with acetone, then infiltrated and embedded in Spurr's Epoxy Resin (Ted Pella Inc., Redding, CA, USA). Cross-sections, 80–100 nm thick, were mounted on Formvar-coated, nickel grids and stained with Sato's lead citrate (HANAICHI et al. 1986) and 2% aq. (w/v) uranyl acetate. Images were collected using a Jeol JEM-1200 EX TEM (Jeol USA, Inc., Peabody, MA, USA).

2.3 Image and statistical analysis

Scion Image 1.62 imaging software (Scion Corporation, <http://www.scioncorp.com>) was used to make measurements from micrographs. A tangential row of PP cells is formed each year, and thus appears as a 'growth ring' in the secondary phloem. PP cells from the most recent 3 years were counted and measured to determine changes in abundance and

distribution of phloem phenolic content. The constitutive and induced resin ducts in the most recent year of xylem were also analysed. The area with phenolic staining ($\mu\text{m}^2/\text{mm}^2$), number of resin ducts per mm^2 , area of resin duct lumens (μm^2), and the number of parenchyma cells surrounding the xylem resin ducts of healthy and infected branches were determined. Averages were calculated based on three replicates from three branches, and differences were assessed between control and *C. ribicola*-infected tissue using two-sample *t*-tests with unequal variances and constant *p*-values (<0.05).

3 Results

3.1 Anatomy of *Pinus monticola* pine stem

The anatomy of non-infected branches from the same whorl and at the same distance (age) from the stem as branches with infected regions and main stem tissue controls from the opposite side from infected regions provided a basis for identification of fungal-induced changes. Anatomy of the bark and xylem of *P. monticola* branches and stems was similar to that of other species of the *Pinaceae* in its highly organized pattern of tissues. The periderm was relatively thin in the trees examined and characterized by several layers of dead, flattened, suberized cells (phellem) overlying the phellogen, and a couple of layers of cells that often contained densely stained phenolic bodies (Fig. 1a, inset). The layers varied in thickness from sample to sample. Beneath the periderm, the cortex contained large and numerous intercellular spaces, large vacuolate parenchyma cells, and a single tangential row of axial cortical resin ducts that exhibited considerable variation in lumen size (Fig. 1a). These ducts contained primary (constitutive) resin as identified by staining with copper acetate, which detects resin acids (not shown). Scattered sclerenchyma (stone cells) was

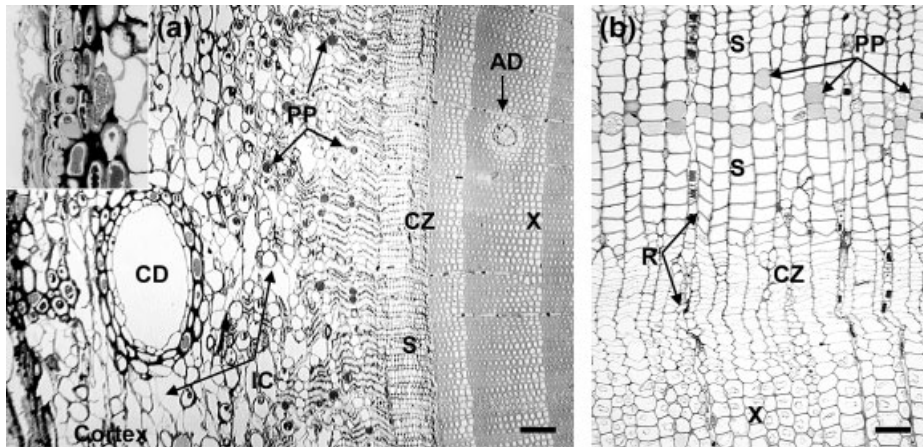


Fig. 1. Branch and stem cross-sections showing normal anatomy of *Pinus monticola*. (a) Anatomy of branch bark, cambial zone, and xylem. Image shows a large cortical duct (CD) in the cortex and axial polyphenolic parenchyma cells (PP) scattered in secondary phloem separated by sieve cells (S). Notice large intercellular cortical spaces (IC). A single constitutive axial resin duct (AD) is seen in the previous year's xylem (X). Inset shows high magnification of periderm layer. Bar = 150 μm . (b) Stem cross-section shows secondary phloem with an annual row of PP cells separated by sieve cells. Cambial zone (CZ) contains columns of thin-walled rectangular-shaped cells about 10 cells deep, separated by occasional columns of radial ray parenchyma cells (R) that extend into xylem. Bar = 50 μm .

observed in cortical tissue and older secondary phloem. Crystals of calcium oxalate were found distributed in the secondary phloem and were intracellular in crystalliferous parenchyma as previously described by HUDGINS et al. (2003b).

The secondary phloem is composed of three primary cell types in the *Pinaceae*: PP, sieve cells, and ray parenchyma (ABBE and CRAFTS 1939; GRILLOS and SMITH 1959; KREKLING et al. 2000). Axial PP cells were usually found in distinct tangential rows in younger secondary phloem (Fig. 1a,b), but these rows become less organized in older phloem. PP cells had a circular cross-sectional profile, contained vacuolar phenolic bodies, and occurred in distinct rows separated by 5–15 rows of sieve cells and associated albuminous cells. Sieve cells had consistently thick rectangular cell walls, but became compressed and disorganized towards the periphery of the bark where they are no longer conductive. Ray parenchyma cells were continuous between the phloem and xylem (Fig. 1b). At the time of sampling, the cambial zone was active. The centripetal extensions of radial ray parenchyma interrupted the thin-walled, rectangular-shaped cells of the cambial zone that give rise to other xylem and phloem cell types every 5–10 rows of cells (Fig. 1b). In the xylem, scattered axial resin ducts could occasionally be found in all years of growth (Fig. 1a). Anatomy was similar in branches and stems (Fig. 1a,b).

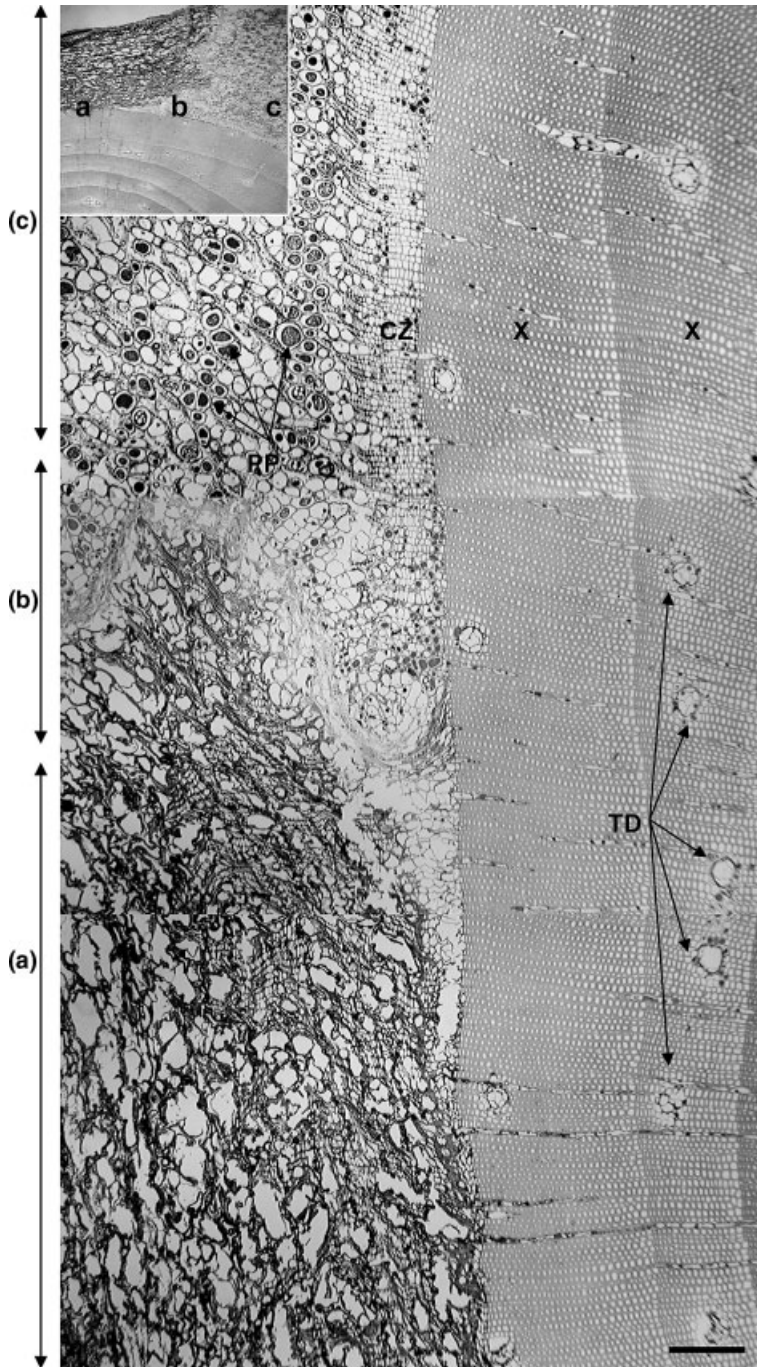
3.2 Canker anatomy and pathway of *Cronartium ribicola* growth in bark and wood

Figure 2 shows the overall anatomy of a canker. The centre of the canker consists of a sunken region of dead collapsed cortex, secondary phloem, and cambial zone. A wound periderm is present at the boundary between this region and surrounding regions of fusiform swelling. The raised appearance of the latter could be attributed to both increased cell numbers in the bark and swelling of living bark cells, primarily the PP cells of the secondary phloem. Axial xylem resin ducts (traumatic ducts) are of increased number in the xylem underlying the canker (Fig. 2).

3.2.1 Periderm and cortex

In sections of *P. monticola* branch and stem displaying fusiform swelling typical of *C. ribicola* infection, hyphae were found to be associated with most host cell types. SEM images indicated fungal hyphae were 2–5 µm in diameter and formed an anastomosing network (Fig. 3a). As viewed in tissue sections, the fungus was particularly abundant just beneath the periderm in this region. Hyphae at this location appeared to be surrounded by or embedded in an extracellular substance, and the periderm was intact (Fig. 3b). Samples that included only fusiform swelling, without a necrotic lesion, also contained aecia (Fig. 3b). Aecia were found developing beneath the periderm and primarily within the cortical regions. In the cortex, hyphae were abundant, with the hyphae predominantly in intercellular spaces (Fig. 3c). The majority of the cortex was intact, but often enlarged in these infected areas, and cells of the cortex frequently had enlarged nuclei (Fig. 3b,c). Hyphae were also seen in living cortical parenchyma cells (indicated by an intact cytoplasm and nucleus) and infrequently within dead parenchyma cells. The lumen of cortical resin

Fig. 2. General anatomy of a branch canker. Inset shows a lower magnification cross-section of a branch canker, divided into three regions for description. (a) This sunken region contains dead collapsed cortex, secondary phloem, and cambial zone (CZ). (b) Wound periderm was produced at the margin between necrotic and living tissue. (c) In this region, infection of the cortex by *Cronartium ribicola* has not been delimited by wound periderm. Macroscopic fusiform swelling, increased cell division in the bark, and swelling of living bark cells are evident. Cortical and polyphenolic parenchyma cells (PP) of the secondary phloem that are infected contain large quantities of phenolics. In both years of xylem that can be seen here, axial xylem traumatic ducts (TD) have higher linear density in the xylem (X) underlying the canker than in control branches. Bar = 250 µm



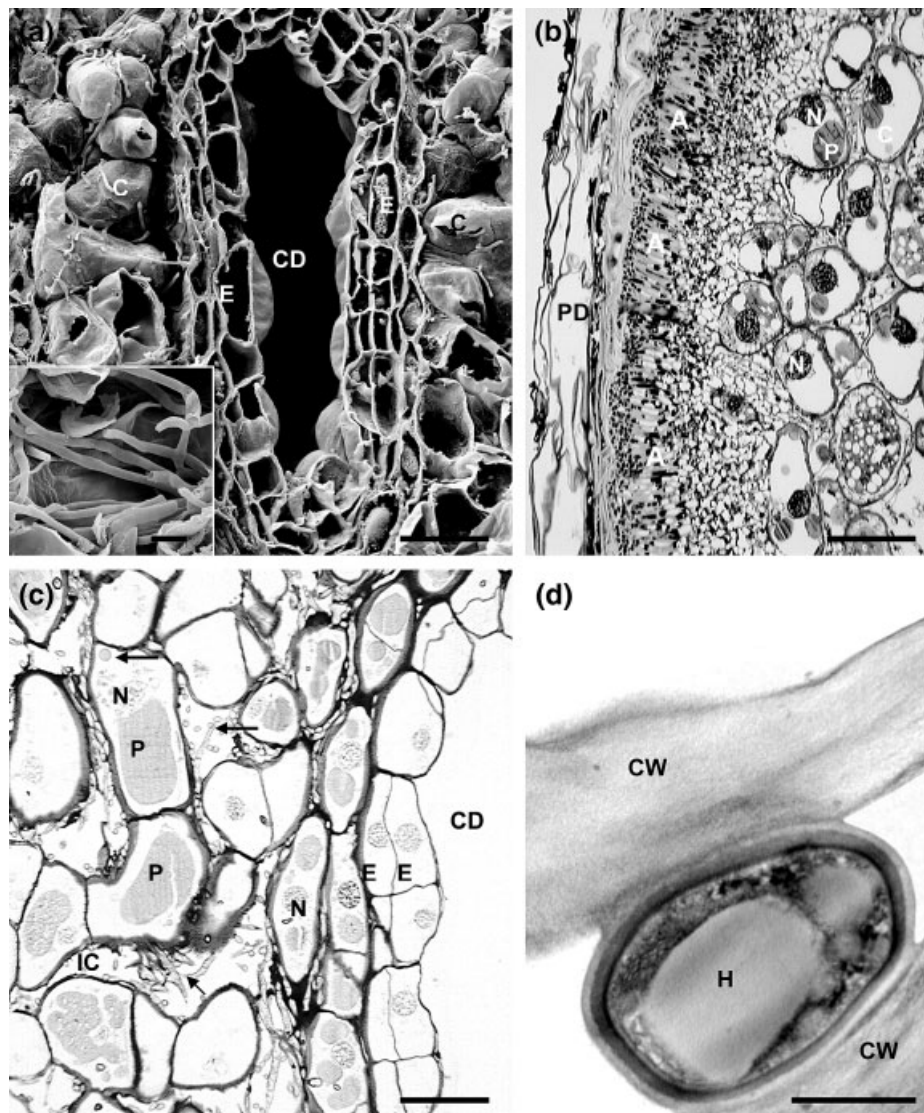


Fig. 3. Location of *Cronartium ribicola* in bark and wood of infected stems, and features of cellular responses. (a) SEM image of hyphae located in secondary phloem cortex (C) and around a cortical duct (CD), but not internal to the CD epithelial cells (E) Bar = 75 μm . SEM analysis indicated fungal hyphae were 2–5 μm in diameter in the cortex and form an anatomizing network (inset). Bar = 8 μm . (b) *Cronartium ribicola* was abundant below the periderm (PD), and aecia (A) were found developing below the periderm in some samples. Cortical cells (C) generally contained enlarged nuclei (N) and phenolic bodies (P). Bar = 100 μm . (c) In the cortex, hyphae (arrows) are abundant in intercellular spaces (IC) and are also found in parenchyma cells. In contrast, lumens of cortical resin ducts (CD) rarely contained hyphae, and epithelial cells (E) surrounding the ducts were rarely invaded by fungal haustoria. Bar = 50 μm . (d) TEM image showing extracellular hyphae (H) affixed to host cell walls (CW). Bar = 1 μm .

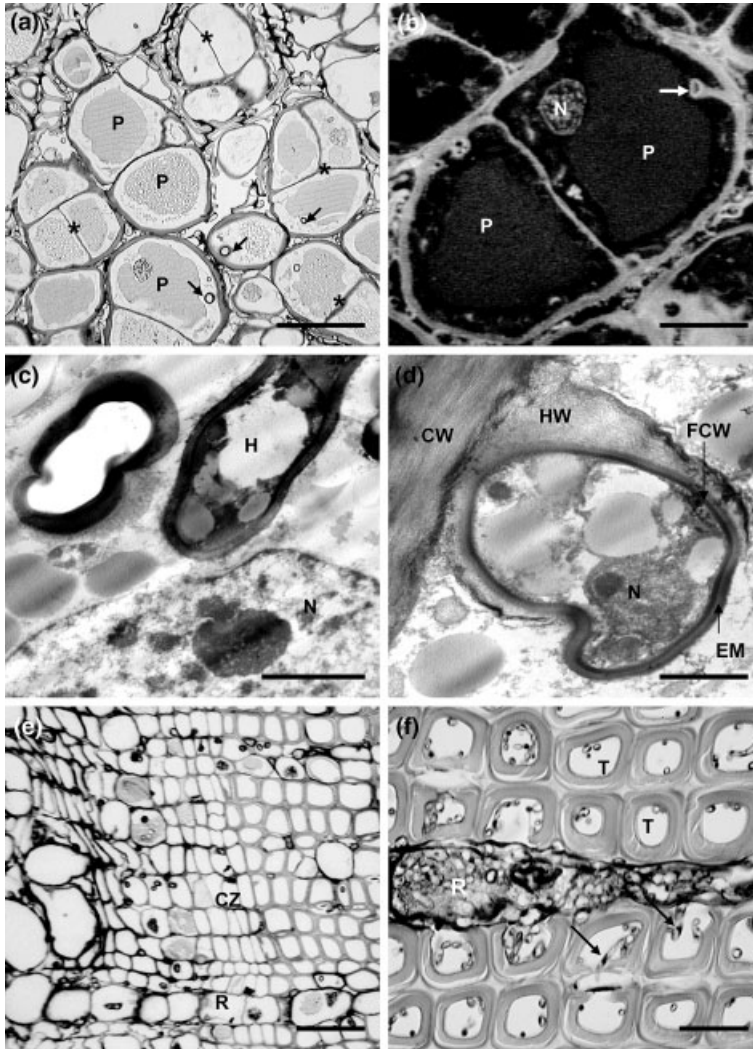


Fig. 4. Interaction of *Cronartium ribicola* with *Pinus monticola* vascular tissue. (a) In the secondary phloem, *C. ribicola* (arrows) was found to penetrate living host cells that accumulated large phenolic bodies (P) and commonly underwent increased cell division post-infection (new walls marked by asterisk). Bar = 100 μ m. (b) Staining with acridine orange revealed host vacuole invagination adjacent to a *C. ribicola* haustorium in a parenchyma cell that had undergone a division. Bar = 25 μ m. (c) TEM of *C. ribicola* haustorium (H) located near a host nucleus (N) in phloem parenchyma cell. Bar = 2 μ m. (d) TEM of fungal haustorium that entered through an original cell wall (CW) of a phloem parenchyma cell, prior to division. The haustorium contains a fungal cell wall (FCW), extra-haustorial matrix (EM), and additional host cell wall (HW) material. Bar = 2 μ m. (e) Cambial zone (CZ) cells occasionally contained *C. ribicola* haustoria but the number of hyphae was generally highest around rays. Bar = 50 μ m. (f) Ray parenchyma (R) cells commonly contained an abundance of hyphae, and the spread of *C. ribicola* into the xylem appeared to occur predominately through the xylem rays. Hyphae also spread through bordered pits into adjacent tracheids (T) (arrows). Bar = 50 μ m

ducts in living tissue rarely contained hyphae, and fungal haustoria were rare in the epithelial cells surrounding the ducts (Fig. 3c). Hyphae or haustoria may have induced the epithelial cell proliferation or division observed in fungus-infected tissues (Fig. 3a,c). Hyphae were often affixed to host cell walls and to other hyphae as has been previously reported by WELCH and MARTIN (1975). Adherence was associated with a gel-like wall substance with lower staining intensity than that of the capsular fungal sheath or host cell walls (Fig. 3d).

3.2.2 Secondary phloem

Hyphae were abundant in the intercellular spaces surrounding PP cells of the secondary phloem (Fig. 4a). Among PP cells with observable haustoria, the host cell nucleus and vacuoles remained distinct and intact in most cases, indicating that cell death had not occurred (Fig. 4a–c). This observation is consistent with historical observations that as the haustoria developed within the host cell, the cytoplasm and nucleus appeared to be ‘indented’ but without fungal penetration of the plasma membrane (Fig. 4c; COLLEY 1918). Haustoria contained both a fungal cell wall and an extrahaustorial matrix, as shown in Fig. 4d, similar to descriptions of *Vicia faba*–*Uromyces fabae* by HAHN and MENDGEN (1997). Hyphae spread through the intercellular spaces along rays and among PP cells, and a number of hyphae were found within living phloem ray cells near the cambium (Fig. 4e). Hyphae were less commonly found within the lumen of active sieve cells in the current year’s phloem.

3.2.3 Cambial zone and xylem

Cambium cells adjacent to rays were occasionally penetrated by fungal haustoria, but most of the cambial zone cells appeared to be free of haustoria (Fig. 4e). Xylem rays commonly contained an abundance of hyphae growing through parenchyma cells (Figs 4f and 6a), and spread of hyphae into the xylem appeared to occur predominately through xylem rays. Hyphae appeared to penetrate tracheids by growing from adjacent ray parenchyma cells (Fig. 4f). Tracheids next to ray parenchyma were more likely to have hyphae in their lumen than tracheids further away from rays (Fig. 4f). Light microscopy cross-sections indicated that intracellular hyphae in tracheids had spread into adjacent tracheids through bordered pits (Fig. 4f). Hyphae of *C. ribicola* were occasionally found extending into 3- to 4-year-old xylem rays, intercellular spaces, and tracheid lumens of the sapwood. In general, hyphal growth in the xylem was more restricted and less abundant than in phloem tissue, although radial columns of tracheids occasionally contained hyphae extending into multiple years of wood. The proportion of tracheids with hyphae varied considerably, and appeared to be related to the overall intensity of the infection in the sampled section. Hyphae and haustoria were also found in parenchyma cells that surround axial xylem resin ducts (Fig. 6b). Xylem rays are often closely associated with xylem resin ducts and appear to provide the predominant pathway for fungal advance into the region of the ducts.

3.3 Structure of *Cronartium ribicola* hyphae and haustoria

Hyphae and haustoria were most abundant in the secondary phloem of infected stems. Examples of haustoria/hyphae penetrating the walls of living cells were common, as clearly indicated by the intact cytoplasm, vacuole, and nucleus of the penetrated cells (Fig. 4a–d). The haustoria in living PP cells had multiple short branches and relatively thick walls. In contrast, hyphae in ray cells tended to be less branched and had thinner walls. Haustoria were seen throughout the cytoplasm and vacuole and were even found penetrating phenolic bodies of the PP cells, although in such cases the haustoria appeared to be lined with a layer

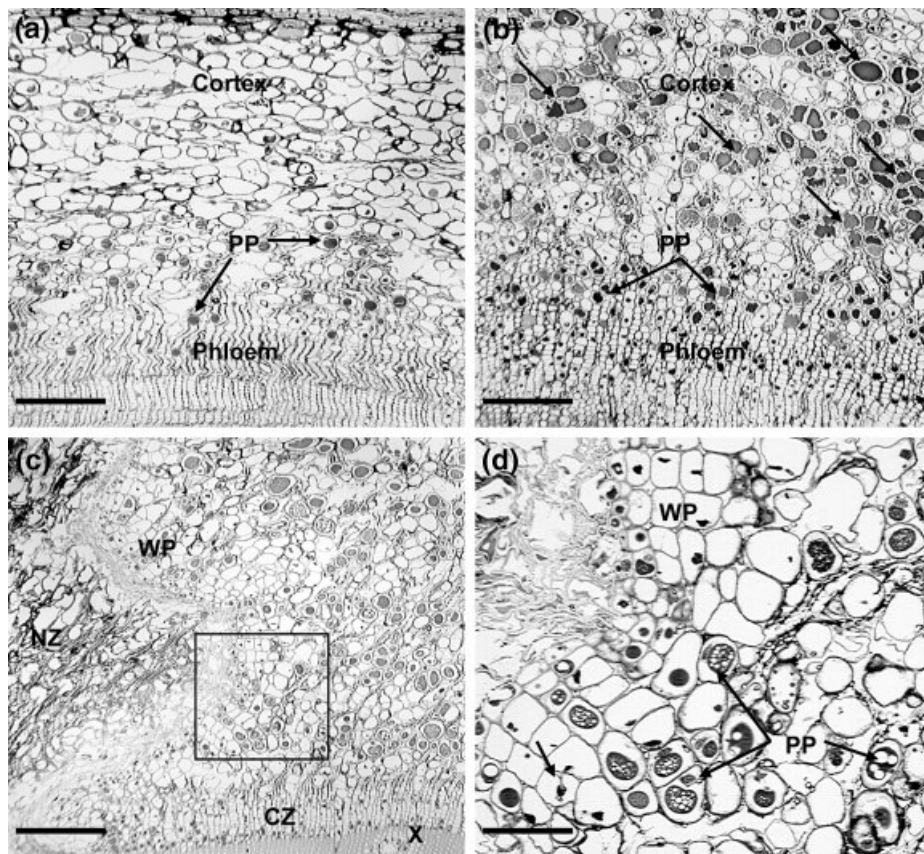


Fig. 5. Phloem responses in control and *Cronartium ribicola*-infected tissue. (a) Control tissue. (b) Cortical parenchyma (arrows) and polyphenolic parenchyma cells (PP) showing increased cell division and phenolic accumulation following invasion by *C. ribicola*. Bar = 250 μ m. (c) Sample with acacia production and lesion formation, showing a necrotic zone (NZ) delimited by wound periderm (WP) that extends to the cambial zone (CZ). Bar = 250 μ m. (d) High-magnification image of box in (c). An occasional haustorium (arrow) was observed in the meristematic cells of the wound phellogen, which establishes the necrophylatic wound periderm; PP cells are seen with large, phenolic-rich vacuoles. Bar = 100 μ m

of host cytoplasm. The walls of the haustoria were distinctly different from hyphal walls in adjacent intercellular spaces. Haustorial walls were thicker and more robust than hyphal walls in adjacent intercellular spaces. In addition, haustorial walls fluoresced reddish orange when stained with acridine orange, in contrast to the greenish fluorescence of hyphae (not shown). In the PP cells of the phloem and in parenchyma cells surrounding TDs that contained haustoria, additional internal host cell-wall layers were laid down around the fungal haustoria (Fig. 4d). As viewed at the TEM level, the host wall material often extended half the length of the haustoria (Fig. 4d).

3.4 Phloem responses to WPBR

Comparisons of cortical parenchyma and PP cells of branches and stems infected by *C. ribicola* vs. control tissue revealed substantial morphological differences (Fig. 5a vs.

Table 1. Image analysis of *C. ribicola*-infected tissue compared with non-infected branch and stem tissue. Analysis includes the most current 3 years of phloem and xylem. Sections were from three different saplings displaying fusiform swelling. Polyphenolic parenchyma (PP) cell vacuoles show a 6.3-fold increase in total phenolic area. Traumatic ducts (TDs) increase in the number of ducts per linear mm of xylem (5.75-fold increase), lumen area (8.45-fold increase), and the number of parenchyma cells surrounding the TDs

	Non-invaded	Invaded
PP cell phenolic area	92 ± 23	579 ± 160
No. of TDs per mm	0.77 ± 0.21	4.42 ± 0.83
TD lumen area	233 ± 43	1969 ± 315
No. of parenchyma cells	6 ± 2.7	24 ± 9.3

Fig. 5b,c). Similar differences were found between adjacent cells that contained or lacked haustoria. Cortical cells and PP cells were enlarged throughout infected tissues, which is an indication of PP cell activation (FRANCESCHI et al. 2000; HUDGINS et al. 2003a). Also, extensive accumulation of phenolics was seen in PP cell and cortical cell vacuoles (Fig. 5b,c). Image analysis indicated a sixfold greater total visible phenolic content in cells of infected tissue compared with control tissue (Table 1). Phenolic accumulation was not induced in the ray parenchyma. In addition to activation of PP cells existing at the time of colonization, new PP cells were produced in scattered locations, often disrupting the regular patterns of sieve cells (see Fig. 5b,c).

A particularly interesting response was the induction of cortical and PP cell division in infected stems. Although PP cells of some conifers may remain living for up to 100 years (KREKLING et al. 2000), they do not generally divide after the initial division of the cambial layer that produces them, except in the outermost layers of older stems (near the periderm). In infected tissue, thin cell walls and 'balls' of cells that differ from the typical line of cells indicated that many of the cortical and PP cells had recently undergone two or three additional divisions (Figs 4a and 5b,d). This process accounts for observed increases in phloem and cortex diameter. The combination of cortical and PP cell division, accumulation of phenolics in enlarged vacuoles, and overall cell enlargement thus appears to produce the characteristic fusiform swelling seen in the stems and branches at the macro level.

In branches that contained only fusiform swelling without wound periderm, cell death was rare. The wound periderm, when present, compartmentalized an exterior region of necrotic tissue. Necrotic regions having well-defined, established wound periderm were similar in branches and stems: in both, cell death appeared to be correlated with production of aecia (Figs 2 and 5c). *Cronartium ribicola* could be found in necrotic regions that had been excluded by wound periderm, but the rust in this area primarily consisted of hyphal fragments embedded in host secondary metabolites (resin and phenolics). Haustoria were present but less common in meristematic cells of the wound phellogen that established the necrophylatic wound periderm than in cambial and parenchymatous tissues (Fig. 5d).

3.5 Xylem responses to WPBR

The most dramatic anatomical change seen in the xylem of infected tissue was the presence of axial TDs (Fig. 6c,d vs. Fig. 6e,f). Non-infected tissues had only a few scattered constitutive axial resin ducts (Fig. 6c), while *C. ribicola*-infected stems and branches contained tangential rows of axial xylem TDs (Fig. 6e) that in most samples occurred into multiple years of xylem. Image analysis showed almost six times the

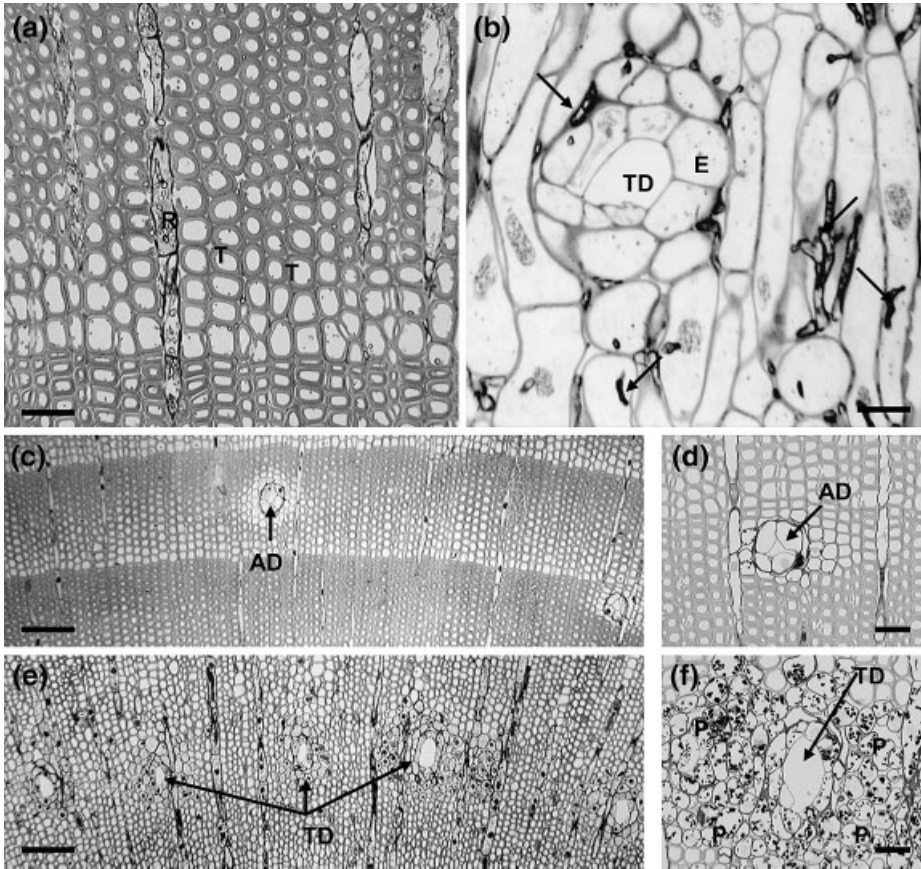


Fig. 6. Xylem changes following infection with *Cronartium ribicola*. (a) *Cronartium ribicola* was abundant in the xylem ray parenchyma (R) and was also seen in some tracheids (T). Image shows 2 years of wood with hyphae in tracheids. Bar = 100 μ m. (b) High magnification radial section of traumatic resin ducts (TD). Hyphae were established around the epithelial cells (E) surrounding the xylem resin ducts, and haustoria were found in parenchyma cells without apparent host cell death (arrows). Bar = 50 μ m. (c) Non-infected cross-section of xylem with normal axial resin ducts (AD). Bar = 50 μ m. (d) PAS staining showing only small quantities of starch in parenchyma cells and epithelial cells surrounding constitutive axial resin ducts. Bar = 100 μ m. (e) Infected tissues developed tangential rows of axial xylem traumatic resin ducts that often occurred into several years of xylem. Bar = 200 μ m. (f) PAS staining showing massive accumulation of starch in parenchyma cells (P) and epithelial cells surrounding traumatic axial resin ducts of infected stem, and significantly higher numbers of parenchyma cells (see Table 1). Bar = 100 μ m

number and 8.5 times the lumen area of xylem TDs in infected branch and stem regions than in uninfected branch and stems (Table 1). TDs in the infected tissues also had four times as many parenchyma cells surrounding them than were found in the constitutive xylem ducts of uninfected stems (Table 1). Xylem ray parenchyma cells that contained an abundance of hyphae appeared wider than uninfected cells of the same type in control tissue. KREBILL (1968) also noted this phenomenon with infections of several canker rust fungi on various pine species.

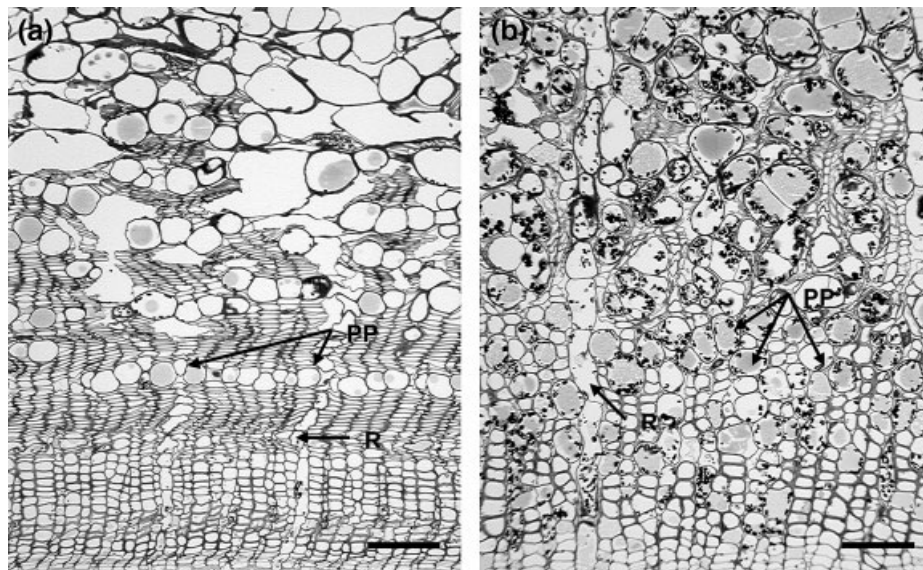


Fig. 7. Starch accumulation in phloem of control and *Cronartium ribicola*-infected branch tissue. Bars = 200 μ m. (a) PAS staining of control tissue shows only small granules of starch within polyphenolic parenchyma (PP) and ray parenchyma (R). (b) Tissue infected with *C. ribicola* stained with PAS indicates large quantities of starch. In the phloem, the predominant sites for starch accumulation were PP and ray parenchyma cells

3.6 Starch distribution

We evaluated the modification of starch reserves and starch distribution in tissues infected with *C. ribicola*. Although PAS and IKI staining indicated small quantities of starch in PP cells and ray parenchyma of control tissue at the time of sampling (Fig. 7a), there was massive accumulation of starch in the same cell types in infected tissue from branches and stems (Fig. 7b). Also striking was the abundance of starch in TD secretory epithelial cells, parenchyma cells that surrounded these, and ray parenchyma cells immediately adjacent to the TDs in the xylem of infected tissues (Fig. 6d). The abundant starch granules occurred only in infected tissue and were not observed in constitutive branch or stem axial xylem duct-associated secretory cells from the same sampling date (Fig. 6b). Because fungal haustoria were also stained in the PAS reaction and we were uncertain of the chemical nature of their staining, we stained sections sequential to those used for the PAS reaction with IKI (not shown). These tests confirmed that the host granules stained in the PAS reaction were composed of starch.

4 Discussion

Virulent fungal pathogens elicit strong inducible defences in conifer bark. The inducible defences involve the activation of cells within the infected tissues which leads to a series of events that can kill or wall off fungal pathogens and limit their growth (FRANCESCHI et al. 2005). In this study, it is shown that colonization of stem bark tissues by the obligate biotrophic fungus *C. ribicola* can induce some of the same reactions within bark that were seen in Norway spruce (*Picea abies* L. Karst) colonized by the virulent, necrotrophic blue stain fungus, *Ceratocystis polonica*. Despite activation of these defences, neither *C. ribicola*

nor the host cells of the bark were killed early in the development of susceptible interactions. Thus, *C. ribicola* displays an ability to circumvent constitutive stem defences as well as the inducible defences up-regulated by infection. Anatomical and ultrastructural observations presented here demonstrate both the responses of stem tissues to *C. ribicola* infection and the manner in which the fungus colonizes the stem while avoiding the host's defences.

Rust fungi, such as *C. ribicola*, require living host cells to obtain their nutrients (HEATH 1997), and as shown here, their infection mechanisms are quite different from necrotrophic fungi, such as beetle-vectored blue stain fungi that rapidly kill host tissues (PAINE et al. 1997). The primary colonization by *C. ribicola* is through passive growth in intercellular spaces and the apoplast of cortical tissue and secondary phloem without killing host cells. Nutrients are obtained by hyphal and haustorial penetration of most types of living secondary phloem cells. Micrographs of haustoria and hyphae that show these structures surrounded by plant wall and plasma membrane indicate that apparent ingress into host cells is technically extracellular, and not intracellular. In the infected tissues of *P. monticola* examined, TEM analysis clearly revealed that host cells laid down secondary wall thickenings around the haustorial neck at its contact with the host cell wall, and that the host cell plasma membrane expanded to accommodate intrusion of the haustorium into the body of the cell. This mechanism of growth of the haustorium elicits defence response similar to that seen with necrotrophic fungi, including activation of PP cells and intracellular accumulation of phenolics. However, because the hyphae and haustoria never penetrate the plant cell plasma membrane, they are not exposed to the phenolics and other toxic compounds stored or produced during induction. During *C. ribicola* infection of needles, it was found that mesophyll vacuoles fragmented, and the phenolic material could be released to the cytoplasm or walls of the cells (BOYER and ISAAC 1964; BOYER 1964). Fragmentation of the vacuoles of infected bark PP cells was not seen in this study; however, significant amounts of phenolics accumulated in these cells, changing the overall appearance of the phenolic bodies.

It was surprising to see that *C. ribicola* infection also induced reprogramming of the cambial zone to produce TDs. However, the accumulated resin produced by the TDs and the constitutive resin ducts did not directly affect the fungus as the hyphae did not breach the epithelial cell lining. In fact, it appeared that hyphae avoided the duct epithelium layer in general. Previous studies have also shown that pycniospores are produced between the living phelloderm tissue and the dead outer layer of the phellem. This pycniospore production and even their release by rupture of the phellem causes no serious damage to the living phloem (COLLEY 1918; HIRT 1964). In this manner, *C. ribicola* can colonize and develop within the bark and effect cross fertilization among pycnia while avoiding the release of constitutive and induced intracellular defence chemicals (i.e. resins, phenolics), until aeciospore development disrupts living cells within the bark. This process may contrast with other interactions within WPBR pathosystems, where resin flow could potentially result from specific host resistance mechanisms, elicitation from variant pathogen genotypes, or interactions with other organisms.

Both physical wounding and infection by a pathogenic fungus have been shown to induce PP cell swelling and phenolic accumulation in the bark of Norway spruce and other conifer species (FRANCESCHI et al. 2000; HUDGINS et al. 2003a; KROKENE et al. 2003; KREKLING et al. 2004; NAGY et al. 2004). In this study, PP cells underwent cellular expansion and accumulated large quantities of vacuolar phenolics in response to *C. ribicola* infection. In addition, *C. ribicola* infection induced PP cell division. This latter response is not generally seen in tissues after wounding or infection by necrotrophic fungi, except where callus or wound periderm is produced. Extensive division of PP cells is partly responsible for the fusiform swelling of infected stems. It is not known what signalling events promote this response, but fungal or plant-induced cytokinin synthesis and accumulation is one possibility. Cytokinins are involved in promoting cell division and differentiation (MOK and MOK 2001), and have been previously reported to accumulate in slash pine (*P. elliotii*

Engelm. var. *elliottii*) and loblolly pine (*P. taeda* L.) during gall formation in stem responses to inoculations with *C. quercuum* f. sp. *fusiforme* (AMMON et al. 1990).

The study's finding that hyphae of *C. ribicola* grow in tracheid lumens has potential significance for understanding patterns of host colonization among different conifer rust phylogenetic lineages, especially in regard to relationships between *C. ribicola*, *C. occidentale* (a native North American stem rust fungus causing pinyon blister rust), and other native *Cronartium* species that cause limb rusts. KREBILL (1968) found *C. occidentale* to have hyphae in tracheids, but found no *C. ribicola* hyphae in tracheids, although hyphae were found between ray parenchyma cells and between tracheids. This is in stark contrast to this report and that of SCHMID (1954). Our observation was that although *C. ribicola* hyphae in infected stems did occur between ray parenchyma cells and between tracheids, hyphae were also quite common in tracheid lumens, especially in those tracheids adjacent to ray parenchyma. The patterns of colonization by both *C. ribicola* in susceptible *P. monticola* and *C. occidentale* in pinyon pine (*P. edulis* Engelm.) are quite different from the limb rusts. The latter rusts grow longitudinally for great distances within the lumens of tracheids of older sapwood in tree boles, but are lacking in the most recently formed layers of tracheids. These fungi re-emerge from the inner sapwood in smaller branches, colonizing branch phloem through ray parenchyma that traverse the non-colonized outer tracheid layers (PETERSON and SHURTLEFF 1965). Sporulation then occurs on branches.

As *C. ribicola* and *C. occidentale* have alternate hosts in the same genus (*Ribes*) and both cause blister rusts in the *Haploxyylon* or soft pine subgenus of *Pinus*, these rusts have been considered by some to be closely related. In a comparison of phylogenetic trees from rDNA internal transcribed spacer (ITS) sequencing and earlier isozyme data, VOGLER and BRUNS (1998) found that isozyme data placed *C. occidentale* closer to *C. ribicola* and away from the limb rusts, whereas ITS sequencing indicated that *C. occidentale* was closer to limb rusts, and somewhat distant from *C. ribicola*. The difficulty in interpreting whether patterns of colonization observed for *C. ribicola* in *P. monticola* may in some way correspond to molecularly determined phylogenetic relationships is complicated by the relatively short time since *C. ribicola* and North American five-needled pines have been in contact since the introduction of the pathogen. Colonization might be expected to be more extensive in highly susceptible individuals, such as in hosts that lack co-adapted levels of resistance that are built up after longer associations between host and pathogen. At some locations where *P. monticola* and sugar pine (*P. lambertiana* Douglas) have had the longest contact with the introduced *C. ribicola*, many of the infected but surviving pines have cankers with scant sporulation. The cankers may be long and linear, and fluted to sunken, or of apparently regular dimensions, but with no observable sporulation scars or swelling. These might indicate that as further natural selection occurs in North American five-needled pines, there may be greater diversity in patterns of colonization and sporulation in stem phloem and xylem. Thus, the patterns of colonization shortly after introduction might not be indicative of stabilized interactions, and not useful for understanding phylogenetic relationships until a new stable state is reached that incorporates effects of artificial or natural selection for resistance and phenotypic plasticity.

The hypersensitive response (HR) is a defence response to rust fungi that indicates one form of resistant (incompatible) reaction (KINLOCH and LITTLEFIELD 1977; JEWELL 1989; KINLOCH and WALKINSHAW 1989). The HR in branch or stem may cause cells surrounding the zone of infection to undergo rapid necrosis and subsequently a necrophylactic periderm is thought to isolate the pathogen (KINLOCH and WALKINSHAW 1989). In the current study, samples that displayed only fusiform swellings were heavily infected with *C. ribicola*, but did not show visible signs of the HR. However, the periderm is eventually ruptured during aeciospore formation, and the cortex and secondary phloem become exposed and desiccated

(COLLEY 1918; HIRT 1964; WICKER and WOO 1973). These damaged tissues did contain wound-induced cell death, and a subsequent necrophylactic periderm had been established, indicating that adjacent PP cells surrounding the lesion were capable of repairing damaged tissue. However, there has been no examination to identify whether the rapid oxidative pathways that are characteristic of HR occur in the stems.

STRUCKMEYER and RIKER (1951) reported that phloem wound periderm formation in resistant eastern white pines provided a barrier that could 'cork out' the fungus, and limit it from further spread into healthy tissues. In the branches and stems examined in our study, fungal hyphae had grown well beyond margins of the lesions. Lesions contained a wound periderm, but were not arrested from further colonization of the stem. In contrast to these stems, when 'corking out' is operating as a mechanism of resistance, patches of bark at the base of needles can be 'corked out' and the pathogen can be compartmentalized and eliminated from further colonization. When such resistance is expressed as very small 'corked-out' patches, the expression of this host response may be initiated in the needles.

In bark infected by blue stain fungi, starch disappears (NAGY et al. 2000), while in phloem and xylem in *C. ribicola*-infected tissues, starch increases, even after a number of metabolically demanding processes, such as cell division, phenolic accumulation, and secondary resin synthesis have occurred. These observations indicate that the WPBR fungus or host is signalling considerable changes in the translocation and partitioning patterns of carbohydrates. Carbohydrate concentrations in the host may reflect patterns of demands and periods of metabolism and dormancy of both the host and the obligate parasite *C. ribicola*. For example, with *C. ribicola*-infected *P. monticola* bark, MARTIN (1987) found that fungal metabolism is relatively greater than host metabolism during February. This is a period of early aeciospore development that coincides with late host dormancy. During aeciospore production, this difference in metabolic phase between pathogen and host favours the pathogen by allowing access to metabolic pools stored by the host prior to dormancy.

It is well established that resistance against *C. ribicola* in five-needled pines has a distinct genetic and heritable component (KINLOCH et al. 1999; EKRAMODDOULLAH and HUNT 2002; LIU and EKRAMODDOULLAH 2004). However, resistance may be conferred by various mechanisms. Trees that show a HR in needles (KINLOCH et al. 1999), or slow canker growth and bark reactions in stems (HUNT 1997) provide evidence for several different types of phenotypic resistance. The samples we examined showed characteristic bark defence reactions, but not an effective level of resistance against *C. ribicola*. This study demonstrates that *C. ribicola* is able to avoid contact with major constitutive and inducible defences of white pine stem tissues. It does not appear to elicit a HR in stem tissues; rather, the fungus induces a wound response only indirectly, once the periderm is destroyed. However, it would be interesting to determine whether *C. ribicola* has lower rates of successful infection and colonization of five-needled pines after induced responses have been up-regulated prior to rust exposure. In previous experiments with *P. monticola*, we found that methyl jasmonate (MJ) is a potent elicitor of general conifer defence after exogenous application, whereupon it activates phenolic accumulation and induces resins to be exuded into intercellular spaces in the absence of wounding (HUDGINS et al. 2003a). Similarly, FRANCESCHI et al. (2002) found that application of MJ to 30-year-old Norway spruce prior to inoculation with the fungal pathogen *Ceratocystis polonica* reduced the fungal-induced lesion length by more than half. Our next objective will be to determine whether pretreatment with MJ is capable of protecting *P. monticola* from *C. ribicola* infection and whether treatment of previously infected stems with MJ can generate increased host resistance to WPBR. These approaches will provide further insight into how *C. ribicola* avoids/tolerates the defence mechanisms expressed in the stem of five-needled pines.

Dedication

This paper is dedicated to the memory of Dr Vincent R. Franceschi (1953–2005). Vince was a premier scientist who contributed immeasurably to diverse areas of plant sciences through his teaching, research, and countless other activities. Of special note is his pioneering work on defence responses of woody plants, which provided a critical basis for ongoing studies in this area. Vince will be greatly missed by his family, friends, colleagues and students.

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Résumé

Réponses anatomiques et cellulaires des tissus de Pinus monticola à la colonisation par Cronartium ribicola

La rouille vésiculeuse des pins à cinq feuilles (*Cronartium ribicola* J. C. Fisch.) cause des dégâts importants aux pins blancs et aux écosystèmes associés dans toute l'Amérique du Nord. Les caractéristiques anatomiques et cellulaires de la colonisation par *C. ribicola* des tissus des branches et troncs de *Pinus monticola* Dougl. ont été étudiées dans la perspective de comprendre les réactions des arbres associées à la résistance. Les échantillons étudiés montraient un renflement fusiforme typique et quelques-uns produisaient des écies. La réaction des tissus du phloème et du xylème a été comparée avec des tissus non infectés, par microscopie optique et électronique. Les cellules parenchymateuses corticales et polyphénoliques du phloème présentent des divisions mitotiques, un gonflement cellulaire, et une accumulation de phénols environ six fois plus forte dans les tiges colonisées par rapport aux tiges témoins. Dans le cortex et le phloème secondaire, de nombreux haustoria sont observés dans les cellules parenchymateuses et des hyphes abondants dans les espaces inter-cellulaires; mais la mortalité cellulaire est rare, sauf dans le cas où des écies ont éclaté le cortex de la tige. De nombreux hyphes sont également observés dans les rayons du xylème, les trachéides, et entre les trachéides. Le développement de canaux résinifères traumatiques dérivés du cambium fait partie des changements induits par la maladie dans la zone cambiale. Ces résultats montrent que différentes réponses de défense des hôtes ont été activées dans l'écorce des arbres apparemment sensibles mais l'absence de dégât mécanique aux cellules de l'hôte contenant des phénols et au système conducteur de la résine par *C. ribicola* permet une colonisation importante et le développement d'écies malgré l'éllicitation de ces défenses. L'interaction *P. monticola*–*C. ribicola* est discutée et comparée avec d'autres pathosystèmes conifère-champignon.

Zusammenfassung

Anatomische und zelluläre Reaktionen von Geweben im Stamm von Pinus monticola auf eine Infektion durch Cronartium ribicola

Der Weisskiefern-Blasenrost (*Cronartium ribicola*) verursacht grosse Schäden an fünfnadeligen Kiefern und den mit ihnen assoziierten Ökosystemen. Um Hinweise auf mögliche Resistenzmechanismen zu erhalten, wurden anatomische und zytologische Merkmale in von *C. ribicola* infiziertem Stamm- und Astgewebe von *P. monticola* untersucht. Die Proben wiesen typische fusiforme Anschwellungen auf und einige hatten Äzidien gebildet. Die Reaktionen des infizierten Phloems und Xylems wurde mit denjenigen von nicht infizierten Geweben licht- und rasterelektronenmikroskopisch verglichen. Im Cortexparenchym und in polyphenolhaltigen Phloemparenchymzellen wurden Mitosen, das Anschwellen von Zellen und eine um ca. sechsfache erhöhte Akkumulation phenolischer Substanzen beobachtet. Im Kortex und im sekundären Phloem traten zahlreiche Haustorien in den Parenchymzellen auf und in den Interzellularen waren viele Pilzhypen vorhanden; die Zellen starben aber nur selten ab, ausser wenn die Äzidien das Kortexgewebe zerrissen hatten. Auch in den Xylemstrahlen, in den Tracheiden und zwischen den Tracheiden kamen häufig Hypen vor. Im Kambialbereich entwickelten sich traumatische Harzkanäle. Die Befunde zeigen, dass in der Rinde von offenbar anfälligen Bäumen verschiedene Abwehrreaktionen aktiviert wurden. Die phenolhaltigen Zellen und das Harzkanalsystem wurde jedoch durch *C. ribicola* nicht mechanisch

geschädigt und der Pilz konnte deshalb das Wirtsgewebe gut besiedeln und äzidien bilden. Die Interaktionen zwischen *P. monticola* und *C. ribicola* werden diskutiert und mit anderen Koniferen-Pilz-Pathosystemen verglichen.

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