Traumatic resin canals as markers of infection events in Douglas-fir roots infected with Armillaria root disease

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Summary

The timing and sequence of root infections caused by root disease pathogens can be difficult to establish, but knowledge of these events provides essential data for epidemiological studies of disease. In response to an infection, traumatic resin canals (TRCs) are formed in the roots of many conifers when penetration to the cambium occurs. TRCs may therefore be used to determine the timing of infections events. Juvenile Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) tree roots at coastal and interior sites in British Columbia were wounded at various times of the year using a hole punch and were inoculated with an isolate of Armillaria ostoyae (Romagn.) Herink, and root sections were taken to determine the timing and extent of TRC formation. Douglas-fir at three interior sites aged 20-32 years were also pulled from the soil with roots intact and a sample of the naturally infected trees was used to determine the extent of the TRCs in infected roots, uninfected roots on infected trees, and in the lower stem. Wounds made in March and October had poor or no TRC formation while the summer wounds responded strongly and were associated with resin soaking in adjacent tracheids. Roots wounded in October did not respond until the following year in all trees except one. Trees from every field site produced TRCs and resin soaked tracheids at all times of the year in response to the fungus. The most striking difference between wounding and fungal inoculation was the multiple bands of TRCs produced in response to the fungus. TRCs at natural A. osfoyae infections in the interior sites were found 92% of time in roots at the stem junction and 74% of the time in the stem at soil line. TRCs were produced in uninfected roots on infected trees but disappeared with increasing distance from the initiating lesion. TRCs can be used to time yearly and seasonal root infections when they can be traced from an identified lesion.

includes tangential bands of parenchyma, abnormally shaped tracheids, and resin ducts; parenchyma cells rich in polyphenols were the first to form followed by a layer of compacted tracheids and then the resin ducts (Tippett and Shigo 1980).

The objectives of this study were to determine: 1) the extent and distribution of vertical TRCs caused by natural infections within infected and neighboring uninfected roots and the lower stem; 2) the timing and position of the vertical xylem TRCs in wounded roots, at both coastal and interior sites, and compare this to the TRCs induced by Armillaria ostoyae at inoculated roots; and 3) the feasibility of determining the number and timing of root infections at the stump top level. The removal of large root systems is difficult and expensive and analyses at the stump top level would greatly reduce the cost and effort.

2 Materials and Methods

2.1 Naturally infected roots

Douglas-fir roots naturally infected by A. ostoyae were sampled at three stands in the ICH zone of BC near Nakusp (tree age 32), Enderby (age 24) and Barriere (age 20). Trees were carefully removed from the soil with roots in late fall by an excavator with a clamshell attachment, and laid on the soil through the winter. In early spring the following year, the soil was removed from the root system by hand, and about 150 stumps per site were randomly selected and brought back to Victoria, BC for processing. Stem cross-sections were taken from each tree at soil line (0 meters), 0.3 m, and 1.3 m, and marked with respect to the root system and brought back to Victoria, BC. After processing these stumps, a randomly selected sub-sample of completely intact root systems was made among the three sites (n=39 trees total). Primary roots (>10 mm dia.) originating at the root collar were mapped before dissection. The proximal and distal diameter, length, horizontal azimuth (in plan) of the root with respect to the bole, and distance and diameter of roots at each infection were recorded. The primary roots were cut at the root collar and cross-sectioned every 20 cm from the collar. A transverse section was taken through the lesion. The root and stem pieces were dried and sanded. TRCs were traced from the lesion using a light microscope (50X) and the number of annual rings from present was counted to the first TRCs. For each stump, the extent of TRC was traced proximally and distally along the infected root, up the stem, and down adjacent roots using the root and stem sections. All lesions on each tree were aged; however, the occurrence of TRCs was determined only for the oldest lesion. The oldest infection was used so that only one infection occurred on the root system at that time in the case where a tree received multiple infections subsequently.

branch became colonized with mycelium or rhizomorphs were formed. The blocks were then placed in moist vermiculite and transported to the study site.

At study sites a hole was carefully excavated alongside each candidate tree to expose a lateral primary root. Blocks were placed in the mineral soil 30 cm distal from the stem with the branch end touching the root. The block was positioned so that its top was a few centimeters below the level of the mineral soil. The mineral soil was backfilled and packed around the block and the duff was replaced on top without mixing with the mineral soil.

The inoculated Douglas-fir trees were located at one site each in the Coastal Douglas-fir biogeoclimatic zone (CDF) near Victoria, BC (49^0N 124^0W), and in the Interior Cedar-Hemlock biogeoclimatic zone (ICH) at Meadow Creek, BC (50^0N 117^0W). The wounded and inoculated roots were excavated and a section was removed with a chainsaw and transported to Victoria. A 25-mm-thick cross-section was cut proximal to the wound or lesion using a bandsaw. These sections were cut again into 15 by 30 mm blocks centered on the wound or fungal lesion in order to fit into a microtome. The samples were submerged in FAA (formaldehyde, 95% ethanol, glacial acetic acid) for 24 hrs and then transferred to 70% ethanol for 24 hours. The samples were sectioned at 60 µ with a microtome, stained with Safranin O in 50% ethanol for 30 seconds, dehydrated in 70%, 95%, and 100% ethanol for 2 minutes each, then transferred to 100% Hemo De® for 15 minutes and slide mounted using Permount®. The slides were viewed with a light or fluorescence microscope at 10-100 X, and the year of response, the number of tracheids prior to the TRCs, and the cross-sectional areas of the TRCs were measured.

3 Results

3.1 TRCs caused by natural infections

3.1.1 Infected stump roots

Natural infections were examined from Douglas-fir roots in three plantations. The occurrence of TRCs induced by the fungus in infected root sections depended on whether the section occurred proximal or distal to the lesion ($p=0.003$; Table 2). TRCs were found 92% of the time in sections proximal to the lesion versus 57% in sections distal to the lesion. As the distance from the lesion to the root section increased, the incidence of TRCs decreased ($p<0.0001$ Table 2); however, TRCs were present 80% of the time for distances up to 80 cm away. At the root collar of infected roots, 92% of the sections had TRCs arising from a lesion on that root. TRCs could be traced proximally along an infected root from a lesion as far as 110 cm away. Tracheids

Table 3. Maximum Likelihood Analysis of Variance Table showing the effect of sample and lesion position on the occurrence of traumatic resin canals in uninfected roots of trees naturally infected by *A.* ostoyae.

Parameter	d.f.	χ^2	P
Intercept	1	0.03	0.8537
L2L[1]	1	15.58	<0.0001
Distance'	1	80.55	<0.0001
Azimuth[3]	1	6.40	0.0114
Site	2	10.52	0.0052
Likelihood Ratio	951	498.66	1.0000

[1] L2L = the distance from the lesion to the root collar on the infected root.

'Distance= the distance from the root collar on the uninfected root.

[3]Azimuth= degrees from the infected root in plan.

3.13 Stem

The stem sections at 0, 0.3 and 1.3 m were examined for the presence of TRCs induced from an associated root lesion. Stem disk position was the most important variable ($p<0.0001$ Table 4) with 74% of the 0 m stem disks containing TRCs induced by an infected root, 46% at 0.3 m and 2% at 1.3 m. When the initiating lesion was further from the stem, the TRCs occurred less frequently in the stem ($p=0.0013$ Table 4). TRCs in all stem disks were always located above the infected root, and did not cover the disk circumference unless the bole was almost girdled, or most of the roots were infected.

Table *4.* Maximum Likelihood Analysis of variance Table showing the effect **of** sample and lesion position on the occurrence of traumatic resin canals in stems naturally infected by *A.* osfoyae.

Parameter	d.f.	χ^2	P
Interceot	1	17.02	<0.0001
L2L[1]	1	10.36	0.0013
Section'	1	19.87	<0.0001
Site	2	4.04	0.1328
Likelihood Ratio	67	51.83	0.9140

[1] L2L = the distance from the lesion to the root collar.

[2] Section= the stem section position taken at Om, 0.3 m and 1.3 m.

The 0 m stem disks of healthy and naturally infected trees were examined for TRCs to try to predict the incidence of infected trees at the Nakusp site. When trees were separated into healthy and infected classes based on the presence of TRCs in the stem disk, there was a 4 %

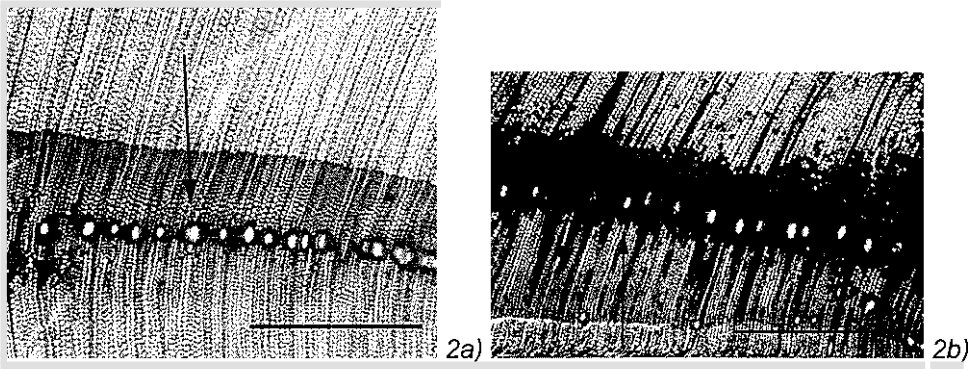

Fig. 2. Cross-section of a summer (June) interior Douglas-fir root wound (a) showing traumatic resin canals but with reduced resin soaking of the adjacent tracheids, in contrast to a summer (June) wounded coastal root ***(b).*** *Bar=1 mm.*

By the end of July, coastal trees produced tangentially arranged TRCs in every case in the same year they were wounded; however, the dark resinous material in the surrounding tracheids was less prevalent than in June. The interior trees showed identical results. The average number of tracheids to the TRC was 112 for the coastal trees and 121 for interior trees.

3.23 Fall wounding

Coastal trees wounded in October formed TRCs in the year following wounding in all cases except two, which produced no TRCs at all. The TRCs occurred after an average of 26 tracheids were formed the following year and were loosely tangentially arranged similar to the roots wounded in March (Fig. 3). Dark resinous material was absent from the adjacent tracheids in almost all samples. All interior trees, except one, TRCs were loosely tangential arranged in the year following wounding. An average of 10 tracheids were formed before the TRCs appeared and the adjacent tracheids had little resin soaking associated with them. One interior tree produced TRCs the same year as wounded. In this tree TRCs were tangentially arranged, but with low canal density and little resin soaking in adjacent tracheids.

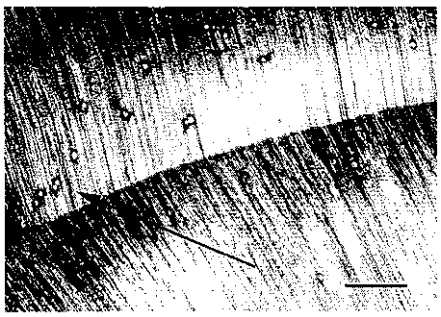

Fig. 3. Cross-section of an Il (October) interior Douglas-fir root wound that produced weak and scattered traumatic canals and no resin soaking the year following wounding. Bar=1 mm.

the TRCs, five of the seven coastal lesions induced TRCs soon after cambial awakening, and two after August. For interior trees the range was 2-82 (mean = 32±31 S.D.). Four of ten fungal lesions on interior trees induced TRCs near to cambial awakening and the remainder induced TRCs sometime between June and July.

4 Discussion

Naturally occurring TRCs induced in Douglas-fir roots by *Armillaria* osfoyae could be detected more than 1 m away from lesions. The frequency decreased proximally, distally, and tangentially away from the lesion. The TRCs at lesions were found more frequently in the proximal direction, agreeing with others (Fahn and Zamski 1970, Tippett et al. 1982). TRC induction in adjacent roots from a neighboring infected root on the same tree has not been quantified before. Our results suggests that long distance signaling needed to induce the TRCs at such great distances from the site of stimulation is mostly localized to the infected root in question. This helps to minimize overlap of TRCs from lesions on opposing roots on the same tree. In this study, natural fungal infections on older trees stimulated TRCs more frequently in uninfected roots adjacent to infected roots. In infected roots, the age effect was not important as found by Bannan (1936). The "optimal defense hypothesis" (Hamilton et al. 2001) predicts that expression of host defense is a function of host fitness. The fitness benefit of maintaining canopy position in young stands probably overrides an extensive defense response initially, but this is probably reversed as stands age because of the increasing incidence of infection and the cost of root disease to host fitness.

Mechanical wounding in the early spring or late fall produced weak TRC response and resin soaking in adjacent tracheids. With one exception, trees in the interior did not respond to the fall wounding until the following year, as found by others (Bannan 1936, Fahn et al. 1979). The one interior tree that responded to the October wound that year was apparently still active or cambial reawakening had occurred; this is possible (Denne 1977, Fahn et al. 1979) and may allow a tree to detect and respond to the fungus earlier. Observations on a larger sample of natural lesions in the interior roots indicated that TRCs occurred mostly during the summer, but a few were also found early and late in the annual ring suggesting fungal colonization in the fall or spring. TRCs located early in the annual growth ring may result from a fall infection after tree dormancy the year before.

The variation in timing of the TRCs induced by fungal inoculations probably relates to a variety of site factors affecting rhizomorph growth, attachment, and penetration of the host. It is interesting that the interior sites where the disease is more prevalent had higher infection rates

(Fahn and Zamski 1970). The pattern of TRC induction probably indicates successive disturbances over the growing season and may be indicative of a biotic agent. On the coast, multiple bands of TRCs at fungal lesions within an annual ring were rare, probably because of quick containment of the fungal advance, unlike the interior lesions that spread for a longer time (Cruickshank et al. 1997).

In this study, many TRCs were seen in the first 10 years in the lower stem sections of the interior trees removed from the soil, but without the associated resin soaking. These were probably induced by snow press causing stress on the lower stem similar to wind stress (Bannan 1933, Fahn and Zamski 1970, Thompson and Sifton 1925). While the lack of resin soaking might be possible with low impact abiotic stimuli, wounds deeper into the xylem do produce resin soaking; thus, it would be difficult to separate TRCs caused by a wound from a fungal infection in the 0 m disks. Further, fungal lesions did not induce TRCs in the 0 m stem disk when the lesion was some distance from the stem, nor was the 0 m disk reliable for dating the age of infections on roots. Interference from other stimuli was also found to be a problem when dating fire scars (McBride 1983).

TRCs can be used reliably to date lesions by tracing the TRCs from a lesion caused by a known agent such as *A.* ostoyae. Removal of the infected root should be sufficient to properly date the infection on that root; however, often overlapping TRCs from more than one event can only be separated by observing the whole stump and lower stem disk. Lesions that have spread from the original penetration site will not cause a problem for dating, as the TRCs will still be present in the ring from the original penetration. Dating the timing of infection events can be done at least within a 6-month window when the TRCs occur in the early wood. It is suggested that root systems be removed from the soil so that responses to multiple infection events may be recorded.

In the case where it is not practical to remove root systems and trace an infection event from a lesion, then the stump top disk or preferably the root collar disk or both must be used. In this case, TRCs combined with reduction in annual ring width and or with double bands of TRCs, or TRCs produced successively over several years, may be indicative of fungal induction. Fungal infections cause a dramatic loss in annual ring width locally in roots (Shigo 1979, Tippett and Shigo 1981) and up the stem over the infected root (Cruickshank 2002). This reduction is probably related to the shift in energy needed for defense compounds, which have a high cost for the tree (Entry et al. 1991, Bendz-Hellgren and Stenlid 1995). Reduction in annual ring width was not visibly noticed in wounded roots in this study.

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