

Article

Xylem Traumatic Resin Duct Formation in Response to Stem Fungal Inoculation in Douglas-Fir and Lodgepole Pine

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Abstract: Xylem traumatic resin ducts (TRDs) in Douglas-fir form in response to mechanical injury, fire, and root pathogens, but it is unknown if these form at the stem in response to bark-beetle-associated fungi. Meanwhile, TRDs are rarely documented in lodgepole pine. In the southern Rocky Mountains, TRD formation in the two species from sterile (Control) and fungal inoculation treatments (Aggressive, Weak (Douglas-fir only)) were compared; predicting the following: (1) both trees would produce TRDs in response to fungal treatments; (2) in Douglas-fir, Aggressive treatment would promote denser and larger TRDs than Weak or Control treatments; and (3) interspecifically, Douglas-fir would produce a higher density of TRDs than lodgepole pine in Aggressive treatments. Two months post-treatment, the position of TRDs indicated these were only induced on all Douglas-fir treatments. Aggressive and Weak treatments had similar responses, except a second TRD line formed in two Douglas-fir Aggressive treatments. Douglas-fir produced >7× more resin ducts that were twice the size of those in lodgepole pine. Douglas-fir's stronger induced response indicates better resistance traits against bark beetle fungal associate colonization. Understanding the characteristics of TRD produced in reaction to specific damage in Douglas-fir can improve past disturbance reconstructions and explain interspecific tree response differences conducive to bark beetle resistance.

Keywords: bark-beetle-associated fungi; induced defense; inoculation response; resistance mechanism; traumatic resin duct



Citation: Mercado, J.E.; Walker, R.T.; Franklin, S.; Kay, S.L.; Ortiz-Santana, B.; Gomez, S.K. Xylem Traumatic Resin Duct Formation in Response to Stem Fungal Inoculation in Douglas-Fir and Lodgepole Pine. *Forests* **2023**, *14*, 502. <https://doi.org/10.3390/f14030502>

Academic Editor: Fred O. Asiegbu

Received: 27 January 2023

Revised: 23 February 2023

Accepted: 25 February 2023

Published: 3 March 2023



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1. Introduction

Forests in many parts of the world are experiencing an increase in the rate of abiotic and biotic disturbances that are fostered by warmer and dryer climate patterns [1]. For instance, the western United States has recently experienced its largest wildfires, and the largest bark beetle outbreak in recorded history affected the region in the last two decades [2]. Trees that survive these disturbances preserve important information recorded in their tree rings that inform us about their anatomical response to colonization by pathogens and insects or to damage by fire, drought, or mechanical injury. The records contained in tree rings can be interpreted by dendrologists and tree physiologists to help reconstruct past disturbance events and inform about how response traits in a tree influence its survival. Such knowledge can help land managers identify conditions that promote healthy trees and forests [3,4].

In conifers, wood produced during and after tree damage present anatomical features associated with the source of damage. Examples include the characteristic resin ducts formed in the xylem as part of the tree's defense. North American conifers in the genera *Pinus*, *Picea*, *Pseudotsuga*, and *Larix* have xylem resin ducts that are constitutively present. Meanwhile, *Abies* and *Tsuga*, in addition those constitutive, form induced resin ducts in

response to abiotic damage or biotic colonization [5,6]. While constitutive resin ducts are scattered in the xylem, those that are induced become supernumerary, often forming a contiguous line of tangentially arranged ducts that are equally distanced from the cambium. These are called traumatic resin ducts (TRDs) and have shapes that vary between different conifer genera. For instance, in *Pinus*, *Larix*, *Picea*, and *Pseudotsuga*, they are elongated (channel-like), whereas in *Abies* and *Tsuga*, they occur normally as isolated cyst-like clusters. If examined tangentially, traumatic resin ducts do not differ anatomically; they are all spaces or channels in the center of a ring made by secretory epithelial parenchyma cells. However, morphological changes in the diameter of resin ducts can be observed within different years in the same tree, which is directly associated with the tree's water availability [7]. Whether biotic agents can drive changes in resin duct diameter size is unknown.

Traumatic resin duct formation is hormone-mediated, where methyl jasmonate initiates their formation by stimulating an increase in ethylene at and away from the area suffering damage [8]. Indeed, we could study TRDs at distances of over a meter away, above, or below, from the point of damage, initially shown in Douglas-fir (*Pseudotsuga menziesii* (Mirbel) Franco and Giant Sequoia (*Sequoiadendron giganteum* (Lindley) J. Buchholz). Because of the above, its characteristic elongated TRDs (easier to find in a cut), and its distribution across a large and contrasting geographic and ecologic landscape, Douglas-fir is a good candidate for studying TRD response to damage. Mature Douglas-fir is known to form TRDs in response to several abiotic and biotic damaging or wood colonizing agents [9–11]. However, whether it forms TRDs in response to one of its primary stem colonizers, the Douglas-fir beetle (*Dendroctonus pseudotsugae* Hopkins), and its suite of associated fungi, has not been explored (Table 1). Gaining this knowledge may help identify past beetle irruptions while reconstructing past disturbance in this tree species.

In the southern Rocky Mountains, *Abies*, *Picea*, and *Pinus* species reach higher elevations than *Pseudotsuga*. Examining TRDs in these other genera can help fill information gaps when studying TRDs over landscapes of varied elevational gradients beyond the range of *Pseudotsugae*. In this mountain range, lodgepole pine (*Pinus contorta* Douglas) occurs over a large portion of Douglas-fir habitat, thus, combined, the two conifers can provide useful information to reconstruct landscape-level disturbance events. However, only two studies suggest TRDs form in lodgepole pine (Table 1, [12,13]). It is unknown if TRDs in lodgepole pine can be as informative as those in Douglas-fir, but studying trees occurring in sympatry with Douglas-fir may provide information about bark beetle resistance traits. Resistance traits in Douglas-fir may explain why, in general, bark beetle irruptions last less time and kill fewer trees in this species than in lodgepole pine.

There is limited general knowledge about TRD formation in conifers, especially regarding how these form in *Pinus* species. Several studies describe anatomical xylem changes in response to a variety of damage in *Pinus* spp., e.g., water deficit/beetle attack in *P. banksiana* Lamb. [14], bark beetle colonization in *P. ponderosa* Douglas ex C. Lawson [15], and seedling fungal inoculation in *P. strobus* L. [14,16]. Although some of these studies have also looked at the response in mature lodgepole pine, only one study has described TRD formation in mature lodgepole pine in response to bark beetle fungal associate colonization (Table 1; [12]). Therefore, a better understanding of TRD formation in this species is essential.

Xylem TRD formations were compared in Douglas-fir and lodgepole pine mature trees in response to sterile agar or with agar inoculated with fungal associates of their respective *Dendroctonus* bark beetles. This study sought to answer three questions with associated hypotheses: (1) Do both species form xylem's TRDs in response to stem fungal inoculations? It was hypothesized that they do. (2) Is there intraspecific variation in the TRD response by Douglas-fir when the inoculation is from phytopathogenic fungi (Aggressive) versus fungi not known to be phytopathogenic (Weak), and in lodgepole pine when inoculated with Aggressive fungal strains versus the Control? It was hypothesized that both tree species would be more sensitive to inoculations with Aggressive fungi, followed by Weak fungi (in Douglas-fir), and lastly by the Control. (3) Is there interspecific variation in the TRD response to the Aggressive inoculation between the two conifers? It was hypothesized that

Douglas-fir would produce a stronger TRD response than lodgepole pine to their respective Aggressive treatment.

Table 1. A summary of studies documenting traumatic resin duct formation in response to abiotic or biotic agents or a combination (comb.) affecting mature Douglas-fir and mature lodgepole pine in North America. * Repeated until Y + 6; dpt = days post treatment.

Disturbance Agent					
Douglas-fir	Type	Y + 0	Y + 1	Y + 2	Author/s
Avalanche	Abiotic	Single	Single	Single *	[17]
Late Summer Fire	Abiotic	none	Single	Single	[9]
Summer Root wounding	Abiotic	Single	Single	n/a	[10]
Late Fall Root pathogen inoculation	Biotic	Double	Sngl/DbI	n/a	[10]
Lodgepole pine	Type	Y + 0	Y + 1	Y + 2	Author/s
Mechanical stem wound (21–99 dpt)	Abiotic	Single	n/a	n/a	[12]
Stem fungal inoculation (21–99 dpt)	Biotic	Single	n/a	n/a	[12]
Stem Bark beetle + fungi (21–99 dpt)	Biotic	Single	n/a	n/a	[12]
Root-collar weevil	Biotic	none	Double	Double	[13]

2. Materials and Methods

The study was performed in the Roosevelt National Forest, between the towns of Red Feather Lakes and Rustic in northern Colorado, at an elevation of 2600 m. The mean annual precipitation in the area is 38 cm, most of which occurs as snowfall between mid-October and April [18]. Vegetation was mixed conifer, composed of co-dominant Douglas-fir, lodgepole pine and ponderosa pine, and scattered aspen (*Populus tremuloides* Michx.). Thirty Douglas-fir and 30 lodgepole pine individuals were treated, and five untreated trees of each species were added one year after to compare structures formed in untreated trees. Selected trees had an approximate diameter at breast height (dbh) of 20 cm (± 2.3 cm) and showed no evidence of beetle activity. All trees were on a north-northeast aspect of a similar slope. Douglas-fir trees were protected from the potential attack of Douglas-fir beetles with the anti-aggregation semiochemical 3-methylcyclohex-2-en-1-one (MCH).

Groups of 10 Douglas-firs and 10 lodgepole pines were treated with Aggressive, Weak, and Control inoculation treatments, except for no Weak treatment, which was included in lodgepole pine as there is no evidence that these occur. Douglas-fir Aggressive fungal suite was collected by the first author two years prior to this study from a site where Douglas-fir beetles killed 100% of the trees. This fungal suite consisted of *Ophiostoma pseudotsugae* (Rumbold) Arx, *Leptographium terebrantis* Barras & Perry (both identified from culture), and the yeasts *Yamadazyma scolity* (Phaff & Yoney.) Billon-Grand and *Nakazawaea holstii* Yamada, K. Maeda & Mikata. The first author collected the mountain pine beetle (*Dendroctonus ponderosae* Hopk.) Aggressive fungal suite from beetles in irruptive state attacking lodgepole pine in 2011–2013 in the Roosevelt National Forest. These were *Grosmannia clavigera* (Rob.-Jeffer. & R.W. Davidson) Zipfel, Z.W. de Beer & M.J. Wingf.; *Leptographium longiclavatum* S.W. Lee, J.J. Kim & C. Breuil; and *Ophiostoma montium* (Rumbold) Ar. (identified morphologically). The Douglas-fir Weak fungal suite was obtained from Douglas-fir beetles collected at a study site where only 50% of trees were killed the year before this study. Basically, this suite lacked the typical Douglas-fir beetle-vectored phytopathogenic fungi affecting Douglas-fir and consisted of *Ceratocystiopsis minuta* (Siemaszko) H.P. Upadhyay & W.B. Kendr., *Sydowia polyspora*, (Bref. & Tavel) E. Müll., and the two yeasts used in the Aggressive treatment. Fungal associates were identified morphologically with the aid of references [19] and based on the first author's personal experience working with this group. The fungi *C. minuta* (CO-JM-115) and *S. polyspora* (CO-JM-147) and the yeasts *N.*

holstii (CO-JM-123) and *Y. scolyti* (CO-JM-149) were also determined molecularly based on the internal transcribed spacer (ITS) region. Each fungal suite was grown in 2% malt extract agar (MEA; Difco, Fisher Scientific) for 14–21 days until homogeneous growth was achieved, and the Control treatment received a sterile MEA plug.

Douglas-fir was inoculated on 8–9 July 2019 and lodgepole pine on 16–17 July 2019 at a density of 400 inoculations/m² (≈ 50 /tree) around a 20 cm wide band (dbh). Basically, a bark plug was removed with a custom made 12 mm diameter punch, a 5 mm diameter agar plug was inserted at the cambium using a syringe with the bottom cut open (BD, Franklin Lakes, NJ, USA), and the hole was covered with the bark plug. To account for the effects caused by mechanical wounding alone, a sterile MEA plug was placed in Control trees. To determine if trees were responding similarly to available soil moisture, or if inoculation treatments affected trees, approximately bi-weekly measurements were taken of predawn leaf water potentials from mid-canopy branches using a PMS 600 pressure chamber (PMS Instrument Company, Albany, OR, USA). For this, random samples of three trees were selected per treatment group for each measurement period. Moreover, soil moisture was measured at three representative sites at a depth of 20 cm with a Hydrosense II soil-water sensor (Campbell Scientific, Logan, UT, USA).

To examine TRD formation, xylem samples were collected after the end of the growing season (22 October 2019) using a 10 mm bark punch (Dogotuls, Germany) at 5 cm above the top of the inoculation band. These samples were collected randomly (from 1 to 360 degrees) and were located around the tree using a compass. Samples included the phloem and several growth rings of sapwood. Samples were placed directly in 50% ethanol (*v/v*) and were transported on ice to the laboratory, where they were trimmed to expose a transverse cross-sectional area of approximately 25 mm² and were dehydrated in a graded ethanol series, starting from 50% and ending with 96% [20]. Sections were infiltrated with a graded ethanol/LR White Acrylic Resin series and were polymerized at 60 °C with LR White Acrylic Resin (SPI Supplies, Westchester, PA, USA) [21]. Sections were sliced to approximately 1 mm in thickness using a rotary tool, sanded with 800 grit sandpaper, and then polished using a rotary tool (Dremel, Racine, WI, USA).

With the aid of a microscope (100 $\times$; CX31, Olympus, Tokyo, Japan), the relative (%) position of TRDs in the 2019 growth rings was calculated by dividing the width of each TRD's trailing edge (closest to cambium) to the cambium and dividing it by the annual ring's width (μm), and this was used as a proxy for the time of TRD formation. Based on a previous study at the same site [22], the growing season in the plot was from approximately 7 May to 7 September. Thus, about 50% of the growing season had passed by the time of inoculation (8–9 July). Therefore, any TRD formed in the initial 50% ring growth was not included in the analyses. In addition, TRD density (TRDs/mm⁻¹) in the three most recent growth rings (2017 to 2019) was estimated. The cross-sectional area (A , μm^2) was calculated by measuring the major (a) and minor (b) radii of each resin duct and using these values as input in the equation for the area of an ellipse:

$$A = \pi ab, \quad (1)$$

One year after treatment, a sterile 4.3 mm increment borer (Haglöf, Sweden) was used to determine whether inocula were present in the xylem. Xylem cores were cultured on MEA and examined every 2–4 days for growth and the depth at which fungi penetrated. Isolated strains were compared to samples of inoculum to determine if recovered species were those inoculated and not contamination from other species.

Microscopy measurements were used to calculate TRD density, cross-sectional area, and ring width. To determine if fungal inoculation (and growth) had different effects on the resin duct position between fungal treatments, a beta regression with the fixed parameters of species and treatment was used. Gamma regressions with the fixed effects of treatment, species, and their interaction were used to determine differences in TRD density and area, and Spearman rank correlations were used to compare TRD density, area, and tree ring width. A Gaussian generalized linear mixed model (GLMM) with a log link mean function

that included the fixed effects of date, treatment, and their interaction was used to test whether weekly water potential differed between treatments across the course of the study period ($n = 4$ weeks; 19 July 2019–25 September 2019). To fit an appropriate statistical model that was bounded above by zero water potential, we multiplied by -1 and fitted the model on the transformed data; the results are presented as back-transformed. A random effect for individual trees was included to account for repeated measures. For all models, all pairwise comparisons between treatment means and adjusted p -values were tested, and p -values were adjusted to account for multiple comparisons using the Tukey method. The depth at which fungi in Weak and Aggressive treatments penetrated the xylem was compared using a t -test on the log-transformed depth. All analyses were performed using R version 4.1.1 (R Core Team, 2021) with the packages lme4 [23], ggplot2 [24], and emmeans [25].

3. Results

3.1. Condition of the Experimental Trees

Experimental trees were healthy at the time of selection. Analyses of the annual growth ring widths in 2019 showed a significant variation between the two tree species (p -value < 0.001), but not among individuals of each species. At $947\ \mu\text{m}$ ($s = 403.4$), the mean tree ring width of Douglas-fir was twice as wide as that of lodgepole pine at $452\ \mu\text{m}$ ($s = 396.3$). Leaf water potential varied significantly through the growing season (p -value < 0.01) after the 2019 treatments and correlated strongly with average soil moisture across the field site (Figure 1a, b). However, leaf water potential did not differ significantly across treatments or tree species (p -value = 0.71) (Figure 1b). Predawn water potential at the end of three months indicated that treatment effects were negligible at that time. Together, tree ring width and water potential indicated that the two tree species had similar vigor and experienced similar water availability levels during the experiment.

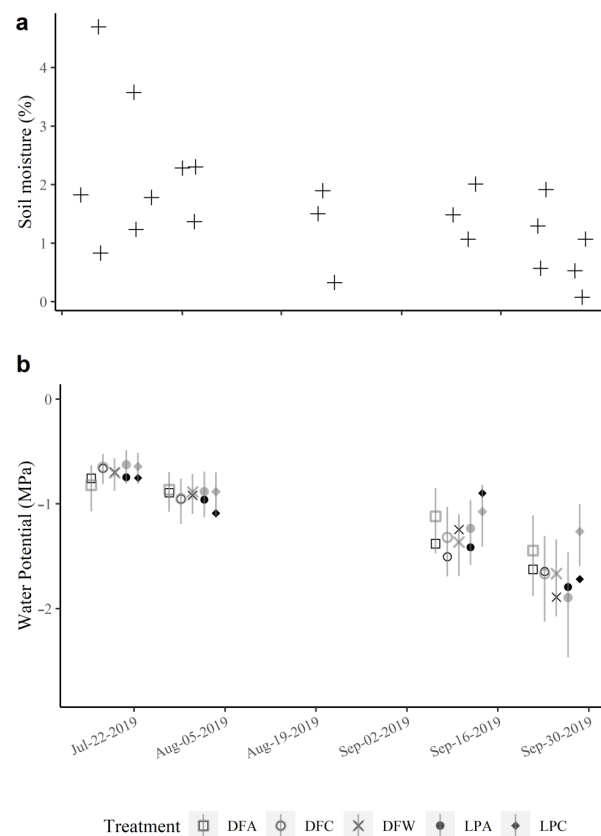


Figure 1. Soil moisture (a) and its direct relationship with leaf water potential (b) to treatments during the first three months post-treatment in 2019 near Red Feather Lakes, CO, USA. DF = Douglas-fir, LP = lodgepole pine, A = Aggressive, C = Control, and W = Weak.

3.2. Characteristics of Traumatic Resin Ducts

3.2.1. Traumatic Resin Duct Formation and Their Relative Position

All sampled trees presented resin ducts scattered throughout the early and latewood of several annual rings in the xylem. However, TRDs (as strictly defined) in the latewood and in reaction to the treatments applied in 2019 formed only on the Douglas-fir Control, Aggressive, and Weak treatments. Resin ducts in lodgepole pine were dispersed and thus were not true TRDs (Figure 2); therefore, it is difficult to determine if these were constitutive resin ducts or induced. For simplicity, all resin ducts formed within the latest 50% formed portion of the annual ring will be treated as induced and are hereafter called TRDs.

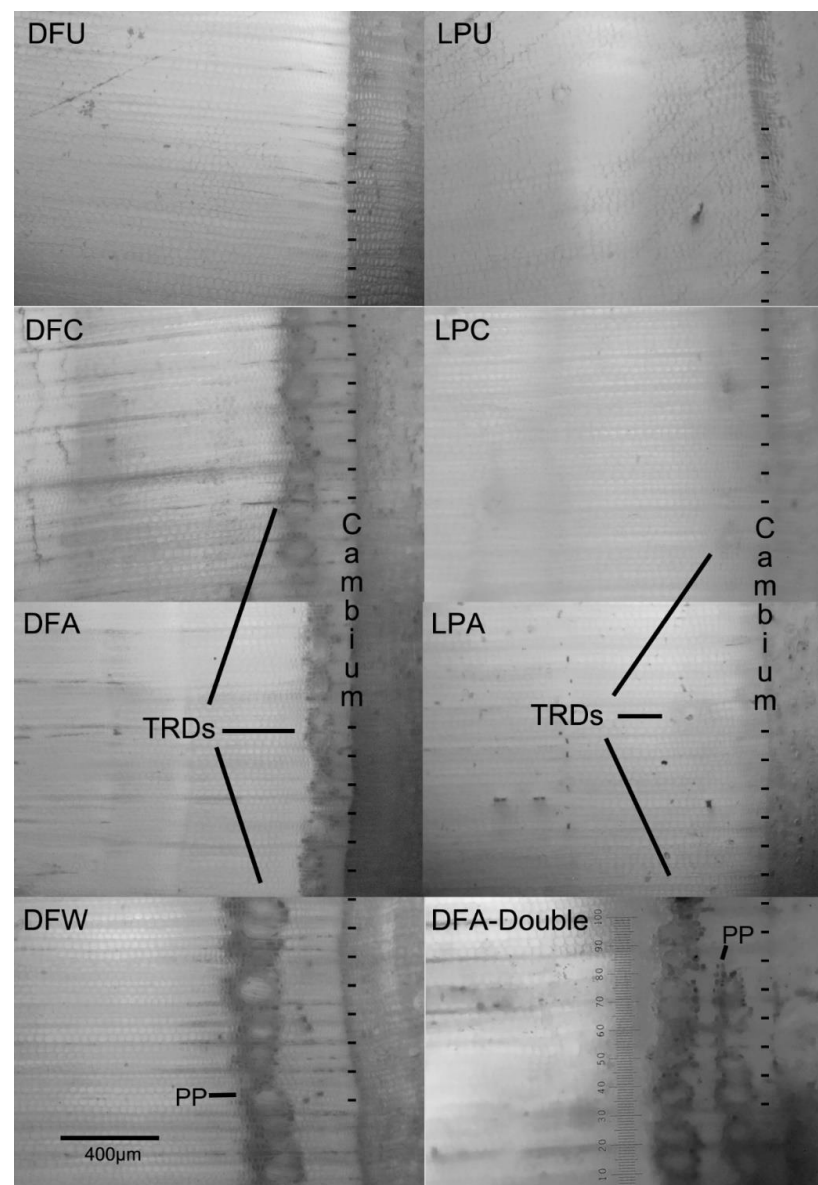


Figure 2. Cross sections of Douglas-fir (DF, left) and lodgepole pine (LP, right) inoculation zones. Phloem aligned to the right. U = Untreated, C = Control, A = Aggressive, and W = Weak, PP = polyphenolic parenchyma. Cambium is indicated by the word Cambium written vertically and dashes. Lower right panel shows a double line of TRDs formed in response to the Aggressive fungal suite. All induced resin ducts are indicated as TRD for convenience.

TRDs in Douglas-fir (including in most Control) were surrounded by 1–5 rows of polyphenolic parenchyma cells, whereas resin ducts in lodgepole pine were surrounded

by a single row. In most cases, a single line of TRDs formed a few sieve cells away from the cambium within the late wood in Douglas-fir; however, a second parallel line of TRDs was observed in two Douglas-fir Aggressive treatment trees (Figure 2). These second parallel lines of TRDs formed closer to the cambium, meaning they were produced more recently. The second TRD line was separated by less than the width of a TRD's diameter and appeared to be forming in one additional Aggressive tree. The relative position of TRDs (Figure 3) in Douglas-fir and lodgepole pine did not differ significantly (p -value = 0.245), but a significant difference was found between the Aggressive and untreated lodgepole pine (p -value = 0.011).

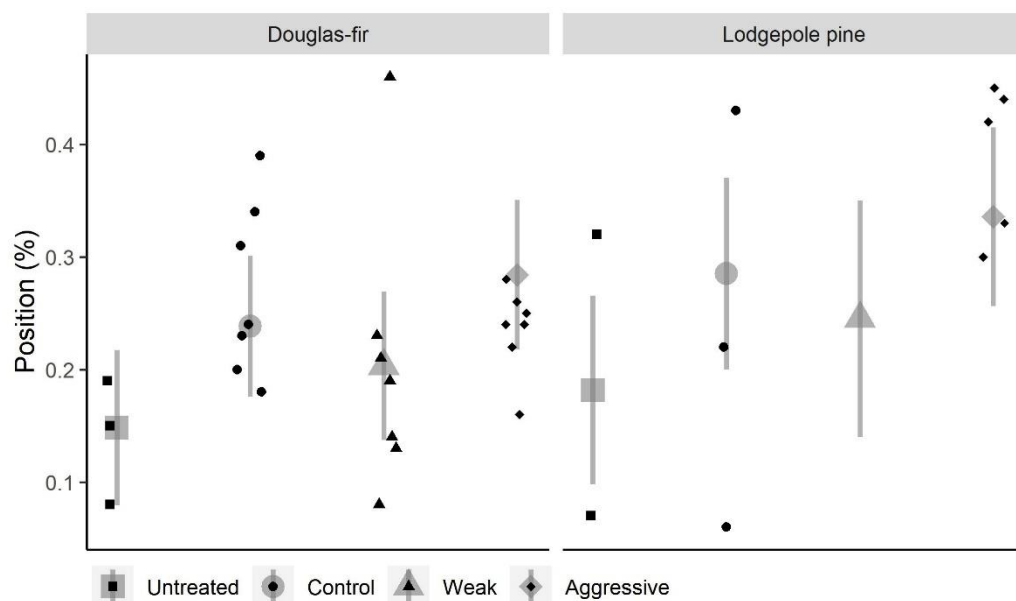


Figure 3. TRD (Douglas-fir) and resin ducts (lodgepole pine) relative position (%) within annual rings in 2019. Observations are shown in black and estimated means are shown in gray with corresponding 95% confidence intervals. No significant differences between species or of treatments within species were found, except between lodgepole pine Aggressive and untreated trees. TRDs that formed in earlier wood (i.e., position (%) > 0.5) were not included in the analysis.

3.2.2. Traumatic Resin Duct Density and Cross-Sectional Area

In Douglas-fir, Aggressive, Weak, and Control treatments produced more TRDs than the untreated trees (p -value < 0.001; Figure 4a). Moreover, a significant difference was found between the untreated and the Control lodgepole pines (p -value = 0.048). Douglas-fir produced over seven times more TRDs than lodgepole pine, at 4.23 mm^{-1} ($s = 2.19$) versus 0.77 mm^{-1} ($s = 0.43$), respectively (Figure 4a). A multiple-year analysis showed no differences between lodgepole pine resin duct densities in 2019 and 2017–18. However, 2019's TRD densities in Douglas-fir were significantly higher than in the two previous years. This reinforces observations that lodgepole pine trees do not respond as strongly as Douglas-fir to wounding or inoculation.

There were no within-species differences in the cross-sectional area of the TRDs between treatments (Figure 4b). Between species, TRDs in Douglas-fir had a cross-sectional area averaging $2637.6 \mu\text{m}^2$ ($s = 1438.4$), which was larger than those in lodgepole pine ($1627.9 \mu\text{m}^2$, $s = 1124.3$). Moreover, Douglas-fir produced larger TRDs than those in lodgepole pine within the Control treatment group (p -value = 0.002). Both Douglas-fir and lodgepole pine exhibited slightly positive correlations between TRD area and density (p -value = 0.344), TRD area and tree ring width (p -value = 0.429), and TRD density and tree ring width (p -value = 0.370).

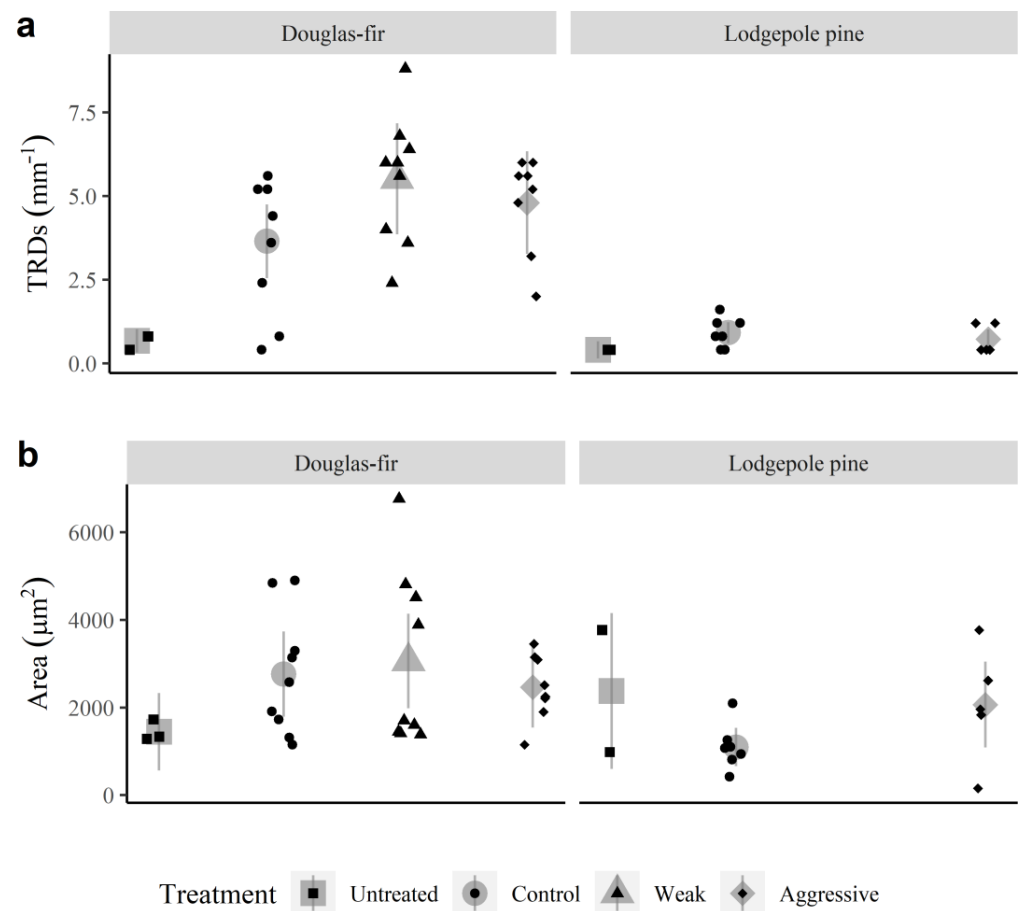


Figure 4. TRD density (a) and cross-sectional area (b) between species and their treatments. Observed points are shown in black and estimated means are shown in gray with corresponding 95% confidence intervals. Within Douglas-fir, TRD density was lower in the untreated trees relative to all treatments, whereas within lodgepole pine, only the Control treatment group was significantly higher than the untreated trees. No significant differences in TRD area were found within species, but within the Control treatment, Douglas-fir trees had significantly larger TRDs than lodgepole pine resin ducts.

3.3. Fungal Colonization and Relationship to TRDs

Fungal colonization was verified one year after treatment and the results showed that all fungi inoculated into Douglas-fir had successfully penetrated the outer ring in the xylem, with at least one of the inoculated fungal taxa plus yeasts present on all Aggressive and Weak inoculated Douglas-fir. In contrast, the Aggressive fungal inoculum was recovered in 40% of lodgepole pines. No other fungal taxa were recovered from the samples of either tree species or their treatment. The depth at which the Aggressive fungi were found in both tree species was similar, with a mean of 3.1 mm ($s = 3.48$) in Douglas-fir and 3.2 mm ($s = 5.25$) in lodgepole pine (p -value = 0.992). Two Douglas-fir trees succumbed to the fungal colonization of their sapwood, a year after treatment.

In Douglas-fir, there were no significant differences in TRD density (p -value = 0.471) or area (p -value = 0.519) among any of the treatments, suggesting that mechanical wounding by the bark punch alone caused the formation of TRDs in these trees. However, the polyphenolic parenchyma (dark cells around TRDs in Figure 2) occurred more densely in the Weak and Aggressive fungal inoculated Douglas-fir, making them easy to differentiate from mechanical damage. Further, the double band of TRD occurred only in some Aggressive treatment trees. Polyphenolic parenchyma was not visible in any of the lodgepole pine treated or untreated trees.

4. Discussion

Anatomical responses such as the formation of TRDs are key traits in conifer defense mechanisms. Traumatic resin ducts help transport a quantitative or qualitative response of oleoresin defensive compounds to areas affected by trauma or experiencing colonization by insects or fungi [26]. This study revealed that the TRD formation response in mature Douglas-fir was stronger than that in mature lodgepole pine trees living in sympatry when inoculated with their correspondent bark-beetle-associated fungi. Elucidating this difference allows us to better explain variations in outbreak duration and tree mortality severity, which is caused by each of their principal bark beetle colonizing species in relation to tree defense.

Different from what was expected, lodgepole pine only produced scattered resin ducts in response to treatments. The results from this study differed from those in Reid (1967), who found lodgepole pine produced TRDs in response to inoculation with *O. montium* and *G. clavigera* 99 days after inoculation (the findings here were after 97 days). The method used for associating the appearance of TRDs was consistent between all Douglas-fir treatments. This allowed to determine that TRDs in Douglas-fir were primarily produced in response to the trauma caused by mechanical wounding during inoculations [10,17]. However, anatomically, the proliferation of polyphenolic parenchyma in response to the Aggressive and Weak fungal inoculation treatments, and largely absent in Controls, indicates that, while both responses are similar, they may vary qualitatively or quantitatively. Morphologically (cross-sectional area), TRDs produced solely by mechanical injury in the Control treatments were larger than when treatments included fungi. It is probable that this difference occurred because the TRDs' lumens were filled by more polyphenolic parenchyma.

Douglas-fir's capacity for producing more and larger TRDs (induced response) may be associated with a defensive strategy primarily directed against bark beetle fungal associates or the colonization by other fungi, which takes several days. This is important to understand when studying resistance traits in trees, as it has been found that bark beetle fungal associates are the primary agents of mortality in pines [27]. The initial defense in *Pinus* spp. resin ducts is directed against mass colonization and synchronous attacking bark beetles (constitutive response) [28]. This is important for lodgepole pine as beetle attacks usually occur massively over a short period of time [29]. Similarly, drastic weather changes such as strong wind and temperatures occur faster at higher elevations where these pine trees live. In combination with thin bark, an effective defense in lodgepole pine must occur immediately to be effective. Conversely, attacks by the Douglas-fir beetle usually occur over a longer period of time [29], and drastic weather changes are less sudden at moderate growing elevations. Additionally, Douglas-fir's thicker bark allows it to have an induced system of TRDs as an effective defense system.

The study of TRDs as a tool to date tree damage or to differentiate resistance between these and other species is relatively recent [10,17,30]. Its potential use as a tool that would allow dendrological reconstructions to elucidate specific types of disturbance has not yet been explored, but the findings regarding Douglas-fir may serve as the basis for future research. It has been shown that Douglas-fir produces a single line of TRDs in response to mechanical damage in the year in which damage occurs, if damage happens before the growing season, or the following year or for more years if the growing season has passed (Table 1). However, biotic disturbances such as the colonization of bark beetles and their fungal associates represented in this study by fungal inoculation, as well as in another study [10], can result in multiple parallel rows of TRDs being produced, as above, during or after the year of the damage. A potential explanation for this may be that, while mechanical injury happens over a discrete period, live phytopathogenic fungi may escape the initial barrier posed by the first row of TRDs and accompanying polyphenolic parenchyma, requiring the tree to allocate more defense resources (if available) to form additional TRD rows. This merits additional research to further develop the fundamental understanding of this phenomenon as well as to explore its potential utility, as expressed here. Preliminarily, the morphological (multiple TRD rows) and anatomical differences

(denser layer of polyphenolic parenchyma) may indicate that TRDs in Douglas-fir are characteristics of a response to a biotic invasion and not just to biotic damage.

Although we applied previously lethal densities of phytopathogenic fungi to both species, tree defensive mechanisms were successful in preventing the fungi from complete penetration and blockage of the xylem, except in two Douglas-fir individuals. This is counterintuitive to the conclusions that Douglas-fir has stronger defenses, but may have resulted from the lower moisture affecting those individuals. The penetration of Weak fungal associates in Douglas-fir and of Aggressive in Douglas-fir also occurring in some Aggressive lodgepole pine matches a resistant tree reaction described as a penetration not exceeding 5 mm [12]. In summary, the study of TRD formation in conifers could be important to understand how different trees respond to their biotic enemies and may explain differing mortality rates. Such anatomical differences may also present opportunities for developing novel information useful in dendrological reconstructions. Therefore, we recommend its use.

5. Conclusions

This study provides novel information about TRD formation in response to stem bark-beetle-associated fungi in Douglas-fir, thus providing a more complete picture of how these structures form in this host. To better understand the formation of tree structures that may take longer to form and the factors that affect response duration, this study should be replicated at a larger scale and with a longer duration. Nevertheless, this study provides information that supports the use of TRDs' characteristics as indicators of specific past disturbances. Interestingly, lodgepole pine did not produce a similar response to that of Douglas-fir. It is possible that the different attack strategies used by each bark beetle may induce xylem defenses in lodgepole that evolved in relation to the rapid mass attack by mountain pine beetle. On the contrary, in Douglas-fir, xylem defenses may respond to the slower attack by Douglas-fir beetle and the slower speed at which its fungi penetrate the different tissues of that tree. An intriguing question remains to be answered: In terms of an evolutionary arms race between host and pathogen, which complex is more well established and which is more recent?

Author Contributions: The research was conceptualized by J.E.M. and R.T.W.; methods were developed by R.T.W., S.K.G. and J.E.M., and were validated by the team; formal analysis was performed by R.T.W. and S.L.K.; B.O.-S. obtained the DNA of fungi to confirm identifications. The investigation was performed by R.T.W., equipment, materials, and organisms were provided by J.E.M. The data were curated by S.F. and J.E.M.; the original draft was prepared by J.E.M. and R.T.W.; the team provided review and editing; visualization was performed by R.T.W.; J.E.M., S.L.K. and S.F. provided supervision; the project funding and administration was provided by S.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the USDA Forest Service, Rocky Mountain Research Station. The findings and conclusions in this publication are those of the authors and should not be construed to represent any official USDA or U.S. Government determination or policy.

Data Availability Statement: Data is publicly available at <https://doi.org/10.2737/RDS-2023-0016>.

Conflicts of Interest: The authors declare no conflict of interest.

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