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Spatial and temporal components of induced plant responses in the context of herbivore life history and impact on host

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Abstract

1. Plants defend against herbivores and pathogens through integrated constitutive and induced defences. Induced responses may be expressed locally or tissue/plant-wide, i.e. systemically, and may also be primed for subsequent attack. Although the elicitation and efficacy of induced responses are increasingly well-characterised, we have little understanding of how timing and within-plant spatial patterns of induced defences relate to different herbivore behaviours and selective pressures.
2. We used interactions between pines and their major mortality agents, native bark beetle-fungal complexes, to explore this dimension. We analysed concentrations of multiple terpenoid and phenolic classes, and lesion formation, to provide a comprehensive profile of specialised metabolites and histological responses in red pine (*Pinus resinosa*) phloem. We examined these profiles in constitutive tissue and following simulated attack, sampling both at the point of challenge and away from the attack site, and following a second simulated attack.
3. Terpenoid concentrations increased by >100-fold at the site of simulated attack. In contrast, systemic induction of terpenoids was absent or weak, with most exhibiting no change and others increasing only 1.5–2 fold. Previous elicitation did not influence terpenoid concentrations, either locally or tissue-wide, when trees were challenged a second time. Phenolics had mixed responses in localised tissues, with some compounds increasing and others decreasing. Like terpenoids, phenolics did not show substantial systemic, tissue-wide changes, and likewise showed no evidence of priming. Collectively, these results indicate that red pine employs a strategy of maximising its response at each point of attack by bark beetles.
4. Pines have been shown to express systemic induced resistance against several canker fungi, so the absence of these responses suggests agent-specific reactions, rather than inherent incapability. Rapid local induction seems to be a better strategy in this instance because if a bark beetle can succeed in entering and producing pheromones from a host, the resulting mass attack usually exceeds defense capacity and kills the tree.
5. These results highlight how plant defence syndromes can modulate the spatial and temporal dynamics of induced responses, in addition to the chemical and morphological traits deployed.

KEYWORDS
bark beetle, conifer, induction, local, *Ophiostoma*, phenolics, plant defence syndrome, systemic, terpenes

1 | INTRODUCTION

When insects locate potential host plants, they encounter defences that are often multifaceted, integrated, and complementary (Agrawal & Fishbein, 2006). These defences may be present prior to herbivore arrival (constitutive), or intensify in response to their actions (induced). Induced defences may include quantitative, proportional and qualitative changes to constitutive conditions (Mithöfer & Boland, 2012; Schuman & Baldwin, 2016). The spatial scale of herbivore-induced changes can range from localised at the site of attack (Balbyshev & Lorenzen, 1997; Fernandes, 1998), to systemic throughout the entire plant or tissue type (Bezemer & Van Dam, 2005; Jones et al., 1993; Orians, Pomerleau, & Ricco, 2000; Park, Kaimoyo, Kumar, Mosher, & Klessig, 2007; Pieterse et al., 2014). The process of elicitation includes reactions to a wide array of stimuli indicative of attack, ranging from relatively general to specific to a particular herbivore (Ali & Agrawal, 2012; Dicke, 2008; Karban & Baldwin, 2007). The temporal scale can also vary: responses to herbivory may be rapid or long term, and in some cases even trans-generational (Agrawal, Laforsch, & Tollrian, 1999; Holeski, 2007). Plants can also undergo "priming," a process by which they modulate their inducibility (Conrath et al., 2006; Mauch-Mani, Baccelli, Luna, & Flors, 2017) to become more responsive to subsequent attack following an initial stimulus (Heil & Bueno, 2007; Helms, De Moraes, Tooker, & Mescher, 2013; Kim, Tooker, Luthe, de Moraes, & Felton, 2012).

Although our knowledge of how induced defences are elicited and contribute to plant resistance has greatly increased, we have less understanding of how different herbivore behaviours and the selective pressures they impose may result in varying spatiotemporal scales of induction within a host plant (van Dam & Heil, 2011). Further, the longevity and architecture of various plants may pose limitations or advantages to various types of response or signalling (Heil & Bueno, 2007; Orians et al., 2000). Our understanding is further limited by logistical constraints that often limit analysis to a single phytochemical group, making inferences about a plant's overall defences difficult.

Conifers include some of the most widely distributed, longest-lived, and largest plants on earth. They often dominate vast terrestrial biomes, and provide a wide range of ecological and socioeconomic services. Like all plants, conifers are confronted with a diverse array of herbivores and pathogens, and induced responses have been demonstrated in phloem (Franceschi, Krokene, Christiansen, & Krekling, 2005; Wallis et al., 2008), roots (Huber, Philippe, Madilao, Rona, & Bohlmann, 2005), and foliage (Mumm & Hilker, 2006; Ralph et al., 2006). Induced responses by conifers can vary among inciting agents (Raffa & Smalley, 1995), and can be systemically expressed or primed against some agents (Bonello, Gordon, Herms, Wood, & Erbilgin, 2006; Eyles, Bonello, & Ganley, 2010).

Bark beetles are the most important herbivores affecting mature conifers (Paine, Raffa, & Harrington, 1997). These insects are particularly threatening because they and their microbial associates develop within the subcortical tissues of the main stem, ultimately disrupting nutrient and water transport and causing tree death (Lieutier, Yart, & Salle, 2009). Conifers have sophisticated physiochemical defences that protect against bark beetle-microbial complexes and play crucial roles in constraining beetle populations below outbreak levels (Raffa, Aukema, Bentz, Carroll, & Hicke, 2008). Physical components include outer bark (Ferrenberg & Mitton, 2014), exudation of oleoresin from wounds (Phillips & Croteau, 1999; Trapp & Croteau, 2001), and induced anatomical responses (Franceschi et al., 2005). Chemical defences include several groups, such as terpenes, which are metabolically costly compounds (Gershenzon, 1994) that can increase by orders of magnitude at the site of bark beetle attack (Keeling & Bohlmann, 2006) and inhibit both the beetles and their associated micro-organisms (Klepzig, Kruger, Smalley, & Raffa, 1995; Kopper, Illman, Kersten, Klepzig, & Raffa, 2005; Mason et al., 2015). Conifers also contain diverse groups of phenolic compounds, which can differ between constitutive and induced states (Schiebe et al., 2012; Sherwood & Bonello, 2013; Villari, Faccoli, Battisti, Bonello, & Marini, 2014; Villari et al., 2012). The various phenolics likewise have differing bioactivities, with some stilbenes, flavonoids, lignans, and lignin exhibiting antifungal properties (Evensen, Solheim, Hoiland, & Stenersen, 2000; Hammerbacher et al., 2013; Hart, 1981; Sherwood & Bonello, 2013). These defences can be elicited by beetle-associated fungi, or exogenous application of the generic defence-eliciting agent methyl jasmonate (Erbilgin, Krokene, Christiansen, Zeneli, & Gershenzon, 2006; Zhao et al., 2010). Accumulation of monoterpenes in response to simulated bark beetle attack using combined mechanical wounding with inoculation of the appropriate fungal symbionts can mimic responses to natural attack (Raffa & Berryman, 1982), and is a good predictor of tree survival under natural conditions in several conifer-beetle systems (Boone, Aukema, Bohlmann, Carroll, & Raffa, 2011; Schiebe et al., 2012).

Outcomes of interactions between conifers and bark beetles typically result in either attack failure or tree death. Beetles engage in pheromone-mediated, cooperative mass attacks, by which many individuals rapidly and jointly exhaust tree defences (Raffa & Berryman, 1983). They exploit host monoterpenes as synergists and precursors of their aggregation pheromones (Blomquist et al., 2010), however, high terpene concentrations can prevent the first beetles that enter from eliciting arrival by conspecifics (Erbilgin et al., 2006). When beetles encounter rapidly mobilised defences, they either abandon the attack, or are killed (Raffa & Berryman, 1983).

Our overall goal was to investigate the spatial and temporal responses of conifer terpenoid and phenolic defences to stimuli signalling bark beetle attack. Specifically, we sought to determine: (1) a more complete profile of terpenoid and phenolic-based induced responses than is currently available for mature trees of the study system species;

(2) whether trees induce tissue-wide responses to simulated bark beetle attack; and (3) whether simulated attacks influence subsequent defence reactions.

2 | MATERIALS AND METHODS

2.1 | Study system

Red pine (*Pinus resinosa* Rudolf) is broadly distributed across north-eastern North America. *Ips pini* (Say), with its principal fungal symbiont *Ophiostoma ips* (Sydow and Sydow) (Klepzig, Raffa, & Smalley, 1991), is the major tree-killing insect affecting mature red pines. Males initiate attacks and produce pheromones that elicit colonisation around the full circumference of the trunk from the base of the crown to slightly above the ground (Thomas, 1961; Wood, 1982). Each male is joined by an average of three females. Following mating, each female constructs an oviposition gallery that extends approximately 10–15 cm from the entry site, and larval feeding galleries radiate 2–5 cm from each oviposition gallery (Wood, 1982). Adults are 3.3–4.3 mm long. In our study area, *I. pini* typically has two or three generations per year, develop in ~30–35 days, and are most abundant from early June to August (Erbilgin et al., 2002).

2.2 | Site description

Experiments were conducted in a red pine plantation planted in 1964 in Dane County, WI (43°12′51.5″N, 89°47′35.5″W). Trees were identical in age, uniformly spaced, ~15 m tall, and ~24 cm in diameter at breast height (Table S1, Supporting Information). Trees exhibited no symptoms of environmental or biotic stress. The experiment was conducted from 5 June 2014 to 23 July 2014.

2.3 | Experimental design

We followed the commonly used definitions of "localised" induction to refer to the point of injury, and "systemic" induction referring to distant from the point of injury with changes throughout a particular tissue type (e.g. foliage, stems, roots) (Karban & Baldwin, 2007; Park et al. 2007, Pieterse & Dicke, 2007, Pieterse et al. 2014). Because *I. pini* attack the entire circumference of the stem, we defined phloem tissue on the opposite side of a tree as the scale for which a systemic response would need to be ecologically operative.

An overview of the experimental design, including nomenclature for the various treatments, is in Figure 1. Each treatment is labelled C, LI, or SI (for control, local induction, and systemic induction respectively) followed by a number indicating the time at which each tissue was sampled. Samples assayed for local induction consisted of the reaction tissue that formed in response to treatment, while samples assayed for systemic induction consisted of phloem on the opposite side of the trunk. At the beginning of the experiment, we selected 60 healthy trees, and randomly assigned them to three categories: control trees from which intact phloem tissue was collected throughout the experiment, trees treated at t_0, and trees treated at t_1.

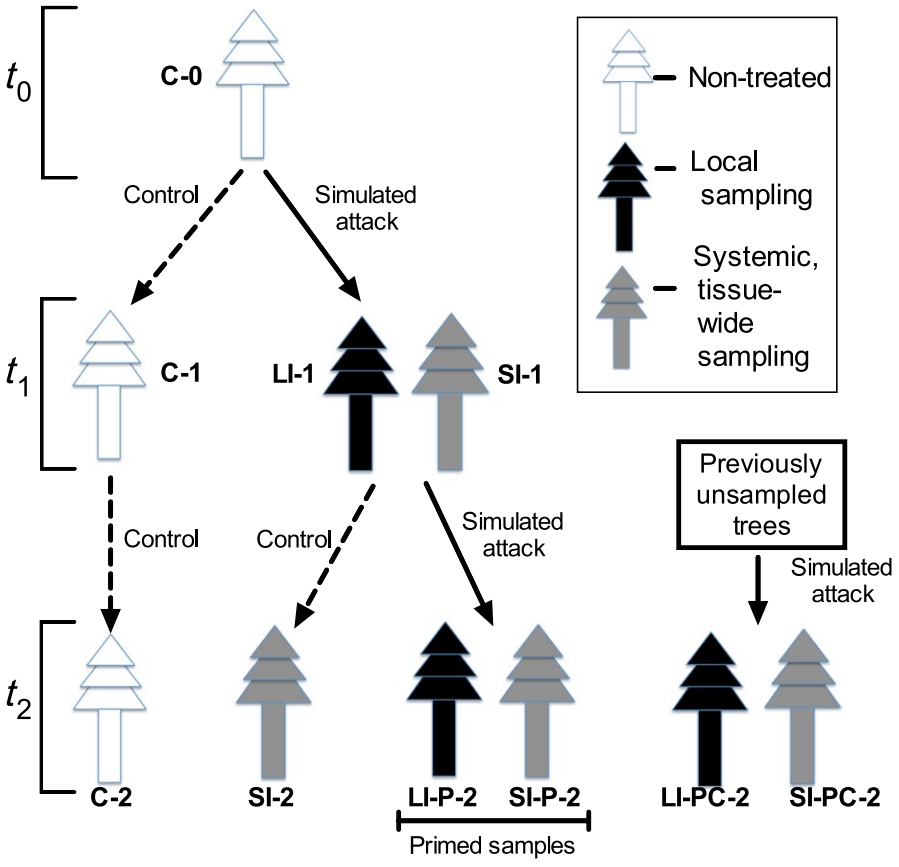

FIGURE 1 Overview of experimental design and treatment designations. Trees were initially separated into three groups: untreated through the duration of the experiment, simulated bark beetle attack at t_0, and simulated bark beetle attack at t_1. Trees that were subjected to simulated attack at t_0 were separated into two groups, repeat challenged or control. Dashed arrows indicate no treatment and solid arrows indicate simulated attack. White symbols represent trees sampled for untreated tissue, black represents trees sampled for potential localised responses, and gray represents trees sampled for potential systemic responses. Sample names consist of an abbreviated treatment (C: control; I: induced; L: local; S: systemic; P: primed) followed by its time point (0 = start of experiment; 1 = 22 days after first treatment, 2 = 22 days after second treatment). This naming scheme is used throughout the text

An initial sample of phloem was obtained from 45 trees (C-0 in Figure 1), and 30 were administered treatments. After 22 days, phloem samples were collected from the untreated trees to control for time (C-1), and to measure local (LI-1) and rapid systemic (SI-1) induction in the treated trees.

Three days after the tissue collection at time t_1 (Figures 1 and S1), and 25 days after the first treatment at t_0, 15 previously challenged trees were administered treatments again (local and systemic priming) and 15 were left alone (long-term systemic, SI-2). These treatments were designed to test for "primed" defences. At this time we treated an additional 15 trees that had not been sampled for either treated or control tissues. Again after 22 days, we collected the following tissues: control for time (C-2), long-term systemic induction (SI-2), locally primed defences (LI-P-2), systemically primed defences (SI-P-2), control for local priming (LI-PC-2), and control for systemic priming (SI-PC-2; Figure 1).

All treatments were standardised at the first time point, with controls on the south and treatments on the north side of each tree. Then when the first treated sample was collected, the systemic sample was randomly collected on either the east or west side of the tree. The second treatment occurred on the opposing side of the initial challenge. When the second induction was collected, the systemic tissue was sampled from the opposite side of the second treatment.

2.4 | Induction procedure

We simulated bark beetle attacks by administering a combination of mechanical injury and application of *O. ips* to the cambium. This method induces chemical and histological responses similar to those against natural attack, responds to environmental stresses and pathogen virulence in similar patterns to natural attack, predicts the likelihood of natural attack, and has been used extensively to investigate the elicitation and expression of conifer resistance defence (Table S2). This method has been used to standardise treatments across a wide range of conifer-bark beetle systems (Boone et al., 2011; Villari et al., 2012; Zhao et al., 2011).

Subcultures of *O. ips* (isolate #C1980, United States Department of Agriculture Forest Products Laboratory, Madison, WI) were grown on malt extract agar (15 g malt extract, 20 g agar /L) at 25°C in darkness for 10 days before treatments. Trees were treated as described previously (Boone et al., 2011). Briefly, a plug of bark and phloem was removed using a cork borer, a 5.0 mm plug of actively growing *O. ips* culture was applied to the exposed xylem, and the bark-phloem plug was replaced. Control samples were unwounded, untreated phloem. Treatments and tissue collections were conducted at ~1.5 m height and consisted of a single inoculation point. After 22 days, we removed bark and phloem from the inoculation site and we photographed and excavated the lesions formed. Samples were transported to the laboratory on ice, flash frozen in liquid nitrogen, and stored at −20°C until processing.

2.5 | Sample processing and phytochemical extractions

Phloem was processed as described previously (Keefover-Ring, Trowbridge, Mason, & Raffa, 2016). Tissues were removed from the freezer, cut into ~1.0 mm pieces, and then divided into two portions. Monoterpenes and sesquiterpenes were extracted by submerging diced phloem in 1 ml of 95% n-hexane containing 0.2 μl/ml toluene in glass vials with PTFE screw caps. Phenolics and diterpenes were extracted by submerging tissues in 1 ml of 200 proof ethanol in microcentrifuge tubes. Extractions were sonicated (10 min) and shake-incubated overnight at 25°C. Supernatants were decanted in fresh vials and diluted in respective solvent when needed. Post-extracted phloem was dried and then weighed.

Phenolics were analysed from the ethanol portion of the extraction. Diterpene acids were reduced in the extraction by precipitation with water containing 0.5 mg/ml resorcinol (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard. Diterpene acids formed a white precipitate, which was separated from the supernatant via centrifugation. The aqueous extracts were then evaporated to dryness and re-dissolved in methanol.

2.6 | Phytochemical analysis

We summarise the chemical analysis methods here, and provide complete details in the Supporting Information. Monoterpenes and sesquiterpenes were analysed using gas chromatography (GC) following Keefover-Ring et al. (2016). We used a Hewlett Packard 5890 GC equipped with a Cyclodex−B enantioselective capillary column (Agilent) and with a flame ionisation detector (FID). Diterpene acids were converted to their methyl esters and analysed by GC-FID (Keefover-Ring & Linhart, 2010; Robert et al., 2010). A 75 μl sample was mixed with 50 μl of 2.0 mol/l (trimethylsilyl) diazomethane (TMS-DAM) in diethyl ether (Sigma-Aldrich). Samples were then vacuum centrifuged to dryness, and re-suspended in 75 μl methanol containing 0.8 μl/ml carvacrol. Diterpene acids were analysed using the same GC-FID equipped with a DB-Wax capillary column (Agilent). Phloem dry weights (dw) were used to calculate compound concentrations (mg compound/g dw) with standard curves ($R^2 > 0.99$) of authentic standards, when available.

Phenolics were identified at the Ohio State University's Targeted Metabolomics Lab by ultra-high pressure liquid chromatography-photo diode array-mass spectrometry (UHPLC-PDA-MS), using an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Santa Clara, CA, USA). Samples (0.5 μl) were injected and separation was performed on an Acquity BEH C18 2.1 × 100 mm column, 1.7 μm particle diameter (Waters, Milford, MA, USA). Metabolites were detected with an Agilent 1260 DAD in line with a hybrid Triple Quadrupole/Ion trap mass spectrometer, QTRAP 5500 (AB Sciex Framingham, MA, USA), run in negative ionisation mode. UV spectral data were recorded from 210 to 400 nm, and compounds were detected at 280 nm. Phenolics were quantified on a Waters Acquity H-class 1200 series UHPLC and detected by PDA, using the same column and conditions described previously. Five-point standard curves ($R^2 > 0.99$) of identified phenolics, or their closest available equivalents, were produced using authentic standards. Phloem dry weights (dw) were used to calculate concentrations of identified compounds (mg compound/g dw). Unknown phenolic compounds were quantified as internal standard-equivalent peak area g/dw.

2.7 | Lesion length

Photographs of lesions alongside a ruler were obtained at the time of tissue collection. We quantified lengths of reaction lesions using ImageJ v.1.38 (Schneider, Rasband, & Eliceiri, 2012).

2.8 | Statistical analyses

Because our objectives required that some but not all trees be repeatedly sampled to distinguish between modalities of induction and control for time (Figure 1), the data do not meet the assumptions of independence required for ANOVA. Therefore, we identified specific questions prior to analysis, formally stated the specific comparison between each treatment, and implemented the most appropriate statistical test for each comparison. The questions, explicit comparisons, and accompanying statistical tests are designated in Table 1. After sample curation and experimental quality control measures, sample sizes for the statistical tests ranged from 13 to 15 (Table 1).

Univariate analyses were conducted in R v. 3.2.1 (R Core Team 2013). Tree chemical was the response variable, and treatment of the phloem sample was the factor of interest. Analyses were conducted on the totals of each group of compounds, and on the individual compounds identified. Transformations could not satisfy normality assumptions for many of the compounds, so nonparametric paired or two-sample tests were used using the function "wilcox.test" in the base R package. We then used false discovery rate (FDR) to account for experimental-wide error, and adjust rejection criteria to control potential false-positives (Benjamini & Hochberg, 1995). Lesion length data satisfied assumptions of normality. Lesion lengths were analysed using a paired *t* test to compare LI-1 vs. LI-P-2, and a two-sample *t* test to compare LI-PC-2 with LI-1 and LI-P-2 with the function "t.test" in the base R package.

We conducted non-metric multidimensional scaling (NMDS) analyses on the compound classes to evaluate whether phloem chemistry following various treatments was characterised by different relative abundances of compounds within a chemical class. Analyses were conducted in PRIMER-E v. 7.0. Samples were standardised within a chemical class (terpenoids or phenolics) by their totals. For unidentified phenolics, the ratio of the peak area to internal standard was used for analysis. Euclidean dissimilarities were generated from the standardised datasets, and used to conduct NMDS.

3 | RESULTS

3.1 | Chemical composition of red pine phloem

The terpenoid fraction included 13 different monoterpenes, one sesquiterpene (longifolene), and six diterpene resin acids (Table 2). Among monoterpenes, the majority was comprised of the (+)- and (−)- enantiomers of α-pinene, and β-pinene. The largest diterpene acid peaks corresponded to co-eluting palustric and levopimaric acid and co-eluting dehydroabietic and abietic acid. Diterpene acids were generally present in higher concentrations than the other terpenoids.

Phenolics were more diverse than terpenoids, and included 19 identifiable (Table 3), and 26 unidentifiable (Table S3) compounds. Here, we focus on the known compounds. Phenols included eight flavonoids, two hydroxycinnamic acids, six lignans, one phenylpropanoid, two stilbenes, and one vanilloid. Of the identified phenolics, lignans and flavonoids had the highest concentrations.

3.2 | Localised responses to simulated bark beetle attack

Localised concentrations of total (Figure 2) and individual (Table 2) terpenes increased dramatically in response to simulated bark beetle attack. Compared to control tissues (C-0), total monoterpenes in the local reaction zone in response to simulated bark beetle attack (LI-1) increased 440 × ($p < .001$), the sesquiterpene longifolene 368 × ($p < .001$), and diterpene acids increased 366 × ($p < .001$) (Figure 2). Individual terpenoids increased to varying degrees in locally induced phloem tissue (Tables 2 and 4). The monoterpenes (+)-α-pinene, (+)-β-pinene, myrcene, and -3-carene increased by >300 × ($p < .001$).

TABLE 1 Summary of the specific questions being addressed, the comparison of sample types being made, and the statistical test being implemented. Nomenclature for comparisons follows naming scheme described in Figure 1. Questions 1 and 2: $n = 13$; Question 3: $n = 14$; Question 4: $n = 15$. False discovery rate was used to adjust *p*-values rejection criteria to account for experimental-wide error and control potential false-positives

Question	Comparison	Statistical test
1. Does red pine undergo local induction in response to simulated bark beetle attack?	C-0 vs. LI-1	Paired nonparametric
2. Does red pine undergo systemic induction in response to simulated bark beetle attack?	Short term: C-0 vs. SI-1 Long term: C-0 vs. SI-2	Paired nonparametric Paired nonparametric
3. Does red pine undergo priming in response to simulated bark beetle attack?	Local primed: LI-1 vs. LI-P-2 Systemic primed: SI-1 vs. SI-P-2 Local time: LI-P-2 vs. LI-PC-2 Systemic time: SI-P-2 vs. SI-PC-2	Paired nonparametric Paired nonparametric Two sample nonparametric Two sample nonparametric
4. Where red pine undergoes putative induction, can it be attributed to simple phenological changes of constitutive tissue through time?	Short-term: C-0 vs. C-1 Long term: C-0 vs. C-2	Paired nonparametric Paired nonparametric

TABLE 2 Concentrations (mg/g) of red pine terpenoids metabolites in phloem in response to induction. Numbers in parentheses represent standard errors. Labelling is consistent with Figure 1

	Control tissues			Systemic tissues				Local tissues		
	C-0	C-1	C-2	SI-1	SI-2	SI-P-2	SI-PC-2	LI-1	LI-P-2	LI-PC-2
Monoterpenes										
(−)-α-pinene	0.18 (0.02)	0.14 (0.06)	0.20 (0.02)	0.19 (0.05)	0.206 (0.018)	0.22 (0.03)	0.26 (0.03)	76.16 (6.97)	67.03 (10.83)	81.46 (9.84)
(+)-α-pinene	0.24 (0.01)	0.19 (0.08)	0.29 (0.03)	0.24 (0.04)	0.26 (0.02)	0.31 (0.04)	0.36 (0.05)	106.81 (8.72)	94.94 (13.91)	107.63 (11.87)
UNKNOWN1	0.01 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.38 (0.04)	0.31 (0.06)	0.35 (0.06)
Camphene	0.01 (0)	0.01 (0.01)	0.01 (0)	0.01 (0.01)	0.01 (0)	0.01 (0)	0.01 (0)	1.17 (0.10)	1.03 (0.16)	1.24 (0.13)
Myrcene	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	2.73 (0.22)	2.44 (0.40)	2.74 (0.25)
(+)-β-pinene	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	2.14 (0.16)	1.93 (0.29)	2.27 (0.19)
(−)-β-pinene	0.16 (0.01)	0.14 (0.07)	0.25 (0.03)	0.17 (0.03)	0.20 (0.03)	0.30 (0.06)	0.25 (0.03)	62.60 (6.34)	52.71 (9.76)	62.51 (6.06)
δ-3-carene	0.02 (0)	0.01 (0.01)	0.02 (0.01)	0.02 (0)	0.02 (0.01)	0.03 (0.01)	0.02 (0)	9.13 (1.62)	7.73 (3.23)	7.96 (1.97)
(−)-limonene	0.001 (0)	0.001 (0.01)	0.003 (0)	0.001 (0)	0.01 (0)	0.003 (0)	0.003 (0)	0.75 (0.06)	0.67 (0.10)	0.82 (0.12)
(+)-limonene	0.002 (0)	0.001 (0)	0.003 (0)	0.001 (0)	0.003 (0)	0.01 (0)	0.01 (0)	1.57 (0.11)	1.43 (0.21)	1.65 (0.22)
p-cymene	0.01 (0)	0.01 (0.01)	0.01 (0.001)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0)	2.83 (0.26)	2.39 (0.43)	3.10 (0.45)
γ-terpinolene	0.001 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.10 (0.14)	0.90 (0.26)	0.91 (0.15)
UNKNOWN2	0.01 (0.001)	0.01 (0.01)	0.01 (0)	0.01 (0)	0.01 (0)	0.01 (0.010)	0.01 (0)	0.11 (0.02)	0.08 (0.03)	0.13 (0.03)
Sesquiterpenes										
Longifolene	0.01 (0.001)	0.01 (0.012)	0.01 (0.002)	0.01 (0)	0.01 (0)	0.01 (0.01)	0.01 (0)	2.578 (0.25)	2.51 (0.43)	2.76 (0.39)
Diterpeneoids										
Sandaracopimaric	0.09 (0.02)	0.09 (0.04)	0.14 (0.03)	0.12 (0.01)	0.11 (0.02)	0.08 (0.01)	0.08 (0.01)	30.77 (1.60)	30.84 (3.03)	28.95 (3.36)
Pimaric	0.14 (0.02)	0.32 (0.24)	0.32 (0.05)	0.42 (0.07)	0.31 (0.06)	0.38 (0.04)	0.40 (0.07)	3.04 (0.46)	4.17 (0.74)	2.59 (0.37)
Palustric/ levopimaric	0.41 (0.02)	0.55 (0.22)	0.70 (0.10)	0.65 (0.05)	0.66 (0.06)	0.48 (0.04)	0.58 (0.03)	244.55 (12.63)	251.01 (28.31)	226.12 (26.24)
Isopamaric	0.10 (0.02)	0.08 (0.05)	0.14 (0.04)	0.12 (0.01)	0.11 (0.01)	0.10 (0.02)	0.15 (0.01)	37.21 (2.21)	38.59 (3.52)	40.80 (4.65)
Dehydroabietic/ abietic	0.37 (0.09)	0.27 (0.11)	0.43 (0.12)	0.36 (0.04)	0.43 (0.06)	0.34 (0.04)	0.35 (0.022)	106.14 (4.46)	116.53 (9.77)	104.26 (12.57)
Neoabietic	0.26 (0.02)	0.32 (0.11)	0.39 (0.04)	0.35 (0.02)	0.34 (0.04)	0.29 (0.02)	0.36 (0.023)	83.64 (4.43)	85.61 (8.92)	92.78 (7.38)

TABLE 3 Mean concentrations (mg/g) of red pine secondary metabolites in phloem in response to induction. Numbers in parentheses represent standard errors. Labelling is consistent with Figure 1

	Control tissues			Systemic tissues				Local tissues		
	C-0	C-1	C-2	SI-1	SI-2	SI-P-2	SI-PC-2	LI-1	LI-P-2	LI-PC-2
Flavonoids										
Epicatechin	1.19 (0.05)	1.31 (0.26)	1.40 (0.09)	1.36 (0.08)	1.40 (0.12)	1.38 (0.16)	1.57 (0.09)	0.05 (0.05)	0.12 (0.12)	0.24 (0.17)
Isorhamnetin derivative	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.22 (0.08)	0.25 (0.11)	0.14 (0.08)
Pinocembrin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.56 (0.07)	0.61 (0.12)	1.52 (0.42)
Procyanidin dimer	0.46 (0.04)	0.41 (0.32)	0.46 (0.08)	0.28 (0.06)	0.39 (0.10)	0.32 (0.10)	0.64 (0.023)	0 (0)	0 (0)	0 (0)
Procyanidin trimer	3.83 (0.10)	2.57 (1.09)	3.74 (0.26)	2.58 (0.25)	3.54 (0.40)	3.82 (0.30)	4.11 (0.17)	2.07 (0.50)	3.35 (0.93)	5.05 (0.51)
Quercetagetin dimethyl ether	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3.78 (0.62)	6.60 (0.96)	6.37 (0.74)
Taxifolin	0.11 (0.03)	0.09 (0.17)	0.21 (0.05)	0.03 (0.02)	0.11 (0.05)	0.15 (0.05)	0.18 (0.05)	0.04 (0.04)	0.14 (0.09)	0.12 (0.08)
Taxifolin hexoside	1.02 (0.05)	1.07 (0.34)	1.31 (0.09)	1.02 (0.08)	1.09 (0.09)	1.17 (0.11)	1.01 (0.07)	1.13 (0.12)	1.35 (0.17)	1.36 (0.18)
Hydroxycinnamic acids										
Ferulic acid hexoside	4.95 (0.16)	5.22 (1.60)	6.17 (0.33)	5.16 (0.28)	5.32 (0.34)	5.51 (0.40)	5.99 (0.39)	2.31 (0.29)	3.05 (0.42)	2.06 (0.40)
Caffeic acid hexoside	0.97 (0.05)	0.65 (0.65)	0.62 (0.13)	0.44 (0.09)	0.72 (0.13)	0.61 (0.13)	0.77 (0.09)	0 (0)	0.15 (0.15)	0 (0)
Lignans										
Lignan coumaroyl Glucoside derivative	0.53 (0.013)	0.55 (0.08)	0.59 (0.04)	0.52 (0.04)	0.65 (0.04)	0.63 (0.03)	0.59 (0.03)	0 (0)	0.07 (0.07)	0 (0)
Lignan deoxyhexoside	2.49 (0.07)	2.73 (0.46)	2.88 (0.12)	2.82 (0.10)	2.99 (0.17)	2.86 (0.12)	2.86 (0.14)	1.47 (0.14)	1.52 (0.19)	1.23 (0.23)
Lignan hexoside	2.32 (0.09)	2.39 (0.63)	2.46 (0.18)	2.82 (0.13)	2.70 (0.24)	2.88 (0.16)	2.87 (0.18)	1.20 (0.19)	1.58 (0.21)	1.14 (0.23)
Lignan xyloside 1	0.97 (0.02)	1.03 (0.18)	1.12 (0.04)	1.03 (0.04)	1.15 (0.07)	1.13 (0.03)	1.17 (0.05)	0 (0)	0.17 (0.12)	0.09 (0.09)
Lignan xyloside 2	3.30 (0.11)	3.51 (0.67)	3.98 (0.20)	3.66 (0.13)	4.13 (0.17)	3.81 (0.19)	3.88 (0.26)	1.26 (0.22)	1.56 (0.37)	1.18 (0.28)
Pinoresinol	0.02 (0.02)	0.02 (0.07)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.54 (0.17)	0.70 (0.25)	2.71 (0.89)
Phenylpropanoids										
Dihydroconiferin	3.25 (0.10)	3.68 (0.61)	3.62 (0.10)	3.56 (0.13)	3.71 (0.26)	3.52 (0.15)	3.58 (0.37)	1.89 (0.24)	2.27 (0.25)	1.83 (0.28)
Stilbenes										
Pinosylvin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.86 (0.11)	1.00 (0.16)	1.43 (0.16)
Pinosylvin monomethyl ether	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4.31 (0.43)	4.45 (0.41)	5.69 (0.47)
Vanilloids										
Hydroxypropiovanillone hexoside	2.38 (0.06)	2.51 (0.32)	2.73 (0.10)	2.50 (0.09)	2.74 (0.13)	2.70 (0.08)	2.50 (0.18)	1.52 (0.13)	1.67 (0.15)	1.39 (0.17)

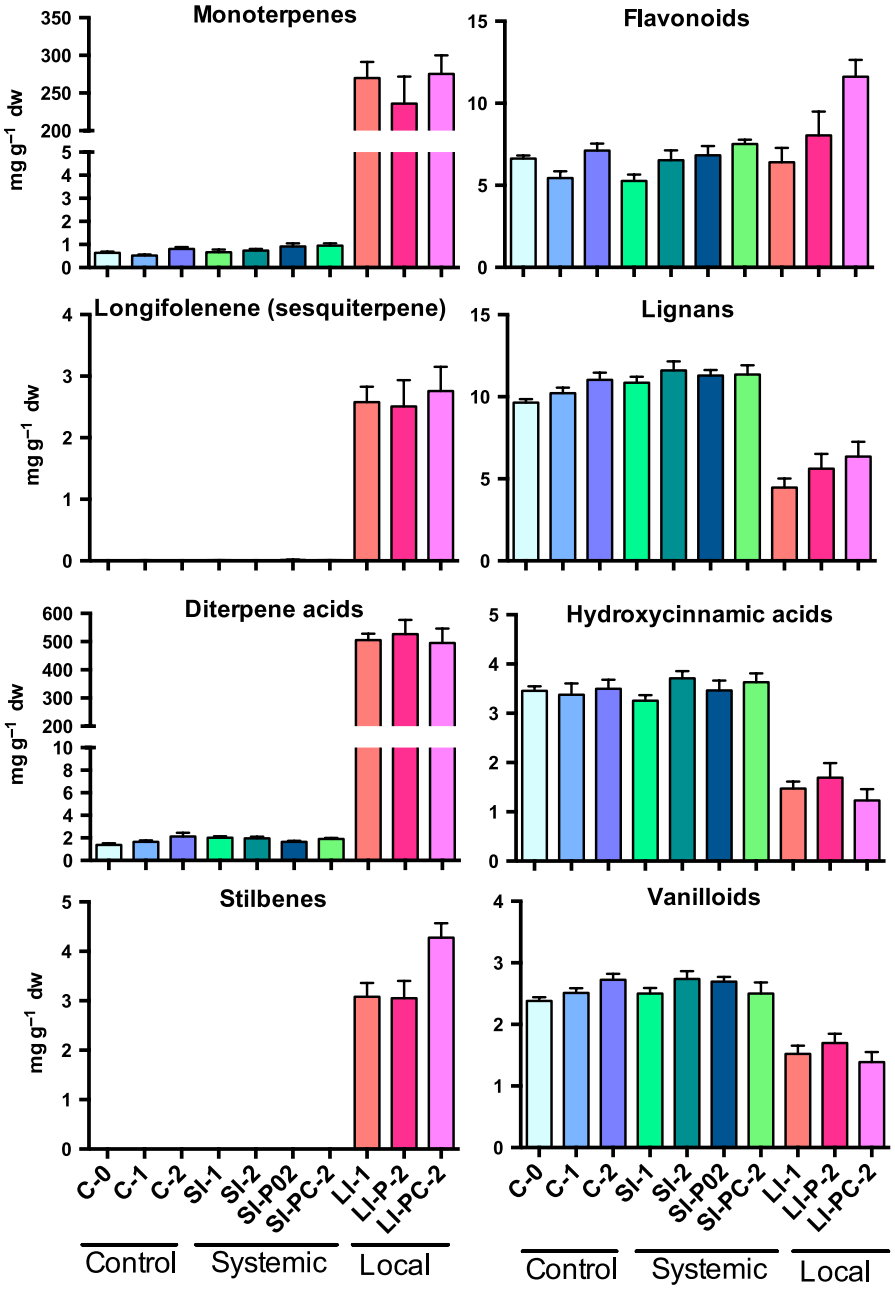

FIGURE 2 Total concentrations (mg compound/g dw; mean ± *SE*) of three terpene and five phenolic classes in red pine phloem. Tissues were measured in control, local, and systemic tissues following labeling scheme in Figure 1

Individual diterpene acids increased by orders of magnitude ($p < .001$). Among all secondary chemicals, terpenoids had the lowest concentrations in constitutive tissue, but the highest concentrations in locally induced tissue.

There were also pronounced changes in phenolics between control (C-0) and locally treated (LI-1) tissues (Tables 3 and 4). The two stilbenes, pinosylvin and pinosylvin monomethyl ether, were not detected in control tissue, yet accumulated in induced tissues ($p < .001$). The flavonoids pinocembrin and quercetagetin dimethyl ether had similar phytoalexin-like responses ($p < .001$). In contrast, there were reduced concentrations of the flavonoids epicatechin (22×) and procyanidin trimer (1.83×), ferulic (2.1×) and caffeic acid (undetectable), dihydroconiferen (1.7×), and hydroxypropiovanillone hexoside (1.6×) in locally treated tissues. All lignans decreased in locally induced tissue, with the exception of pinoresinol, which did not change. Likewise, isorhamnetin, taxifolin, and taxifolin hexoside did not differ between local inoculations and controls.

3.3 | Systemic responses to simulated bark beetle attack

Systemic responses of terpenoids to simulated bark beetle attack were either absent or substantially less pronounced than in locally wounded tissues (Figure 2). Neither mono- nor sesquiterpenes differed between short-term systemic (SI-1) and control (C-0) tissue. However, the diterpene acids levopimaric and neoabietic acid exhibited minor increases in the short-term systemic tissues. Long-term systemic tissues (SI-2) exhibited increases in two individual monoterpenes,

TABLE 4 Wilcox and pairwise-Wilcox *p*-values of terpenoid and phenolic concentrations between the different tissues. Sample naming conventions and specific comparisons are described in Figure 1 and Table 1. "nt" indicates that the test was not conducted. The # symbol identifies *p*-values below that of the false discovery rate (FDR) calculated α. FDR modifies the rejection criteria to help control for false positives in the analysis. FDR calculated rejection criteria and the accompanying true *p*-values are provided in Table S4

	Q1:Local induction?	Q2: Systemic induction?		Q3: Effects of previous attack?				Q4: Effects of time?	
	C-0: LI-1	C-0: SI-1	C-0:SI-2	LI-1: LI-P2	LI-P-2: LI-PC-2	SI-1: SI-PC-2	SI-P-2: SI-PC-2	C-0: C-1	C-0: C-2
Monoterpenes									
(−)-α-pinene	<0.001#	0.685	0.027	0.622	0.432	0.003#	0.227	0.804	0.048
(+)-α-pinene	<0.001#	0.588	0.233	0.970	0.595	0.003#	0.487	0.208	0.015#
UNKNOWN1	<0.001#	nt	nt	0.970	0.536	nt	nt	nt	nt
Camphene	<0.001#	0.893	0.301	0.622	0.462	0.042	0.487	0.934	0.107
Myrcene	<0.001#	0.168	0.007#	0.569	0.494	0.01#	0.817	0.035	<0.001#
(+)-β-pinene	<0.001#	0.040	0.009#	0.733	0.347	0.005	0.425	0.151	0.055
(−)-β-pinene	<0.001#	0.497	0.092	0.569	0.252	0.002	0.980	1.000	<0.001#
δ-3-carene	<0.001#	0.76	0.407	0.722	0.657	0.022	0.620	0.024	0.477
(−)-limonene	<0.001#	0.234	0.151	0.470	0.560	0.022	0.394	0.100	0.005
(+)-limonene	<0.001#	0.236	1	0.569	0.560	0.006#	0.554	0.726	0.197
p-cymene	<0.001#	0.094	0.042	0.791	0.212	0.005#	0.817	1	0.048
γ-terpinolene	<0.001#	nt	nt	0.791	0.631	0.014	0.192	nt	0.584
UNKNOWN2	0.008#	0.266	0.636	1.000	0.256	0.722	0.935	0.802	0.124
Sesquiterpenes									
longifolene	0.002#	0.196	0.894	0.204	0.487	0.148	0.117	0.706	0.89
Diterpene acids									
Sandaracopimaric	<0.001#	0.147	0.092	0.733	0.980	0.007#	0.589	0.018	<0.001#
Pimaric	<0.001#	0.002	0.002	0.176	0.067	0.465	0.589	0.121	0.229
Palustric/levopimaric	<0.001#	0.004#	0.003#	0.850	0.374	0.083	0.042	0.001#	<0.001#
Isopamaric	<0.001#	0.127	0.151	0.791	0.462	0.123	0.003	0.003#	<0.001#
Dehydroabietic/abietic	<0.001#	0.305	0.176	0.151	0.560	0.638	0.520	0.015	<0.001#
Neoabietic	<0.002#	0.002#	0.002#	1.000	0.742	0.24	0.060	0.121	0.007#
Flavonoids									
Epicatechin	<0.001#	0.244	0.129	1.000	0.610	0.966	0.462	0.489	0.639
Isorhamnetin derivative	0.1	nt	nt	0.834	0.489	nt	nt	nt	nt
Pinocembrin	0.002#	1	1	0.850	0.003	nt	nt	nt	nt
Procyanidin dimer	0.006#	0.083	0.689	nt	nt	0.813	0.028	0.188	0.754
Procyanidin trimer	<0.001#	0.068	0.85	0.919	0.142	0.067	0.403	<0.001#	0.33

(Continues)

TABLE 4 (Continued)

	Q1:Local induction?	Q2: Systemic induction?		Q3: Effects of previous attack?				Q4: Effects of time?	
	C-0: LI-1	C-0: SI-1	C-0:SI-2	LI-1: LI-P2	LI-P-2: LI-PC-2	SI-1: SI-PC-2	SI-P-2: SI-PC-2	C-0: C-1	C-0: C-2
Quercetagetin dimethyl ether	0.003#	nt	nt	0.027	0.820	nt	nt	nt	nt
Taxifolin	0.855	0.201	1	0.371	0.902	0.402	0.737	0.906	0.073
Taxifolin hexoside	0.787	0.455	0.016	0.850	0.900	0.365	0.403	0.489	0.403
Hydroxycinnamic acids									
Ferulic acid hexoside	<0.001#	0.017	0.012	0.791	0.142	0.7	0.595	0.804	0.095
Caffeic acid hexoside	0.003#	0.055	0.119	1.000	0.316	0.415	0.552	0.055	0.008#
Lignans									
Lignan coumaroyl glucoside derivative	<0.001#	0.273	0.042	1.000	0.316	0.024	0.494	0.89	0.121
Lignan deoxyhexoside	<0.001#	0.094	0.027	1.000	0.180	0.32	0.742	0.229	0.022
Lignan hexoside	<0.001#	0.080	0.012#	1.000	0.156	1	0.860	0.978	0.107
Lignan xyloside 1	<0.001#	0.376	0.007#	0.371	0.547	0.054	0.820	0.762	0.015#
Lignan xyloside 2	<0.001#	0.147	0.021	0.689	0.358	0.32	0.980	0.041	0.004#
Pinoresinol	0.1	1	1		0.005	nt	nt	1	1
Phenylpropanoids									
Dihydroconiferin	<0.001#	0.191	0.063	0.970	0.396	0.831	0.705	0.135	0.303
Stilbenes									
Pinosylvin	<0.001#	nt	nt	0.677	0.053	nt	nt	nt	nt
Pinosylvin monomethyl ether	<0.001#	nt	nt	0.470	0.003	nt	nt	nt	nt
Vanilloids									
Hydroxypropiovanillone hexoside	0.002#	0.167	0.005#	0.850	0.193	0.278	0.432	0.600	0.064

myrcene and (+)-β-pinene, compared to control. Of the diterpenes, only levopimaric (p = .003) and neoabietic acid (p = .002) increased in long-term systemic induction.

No phenolics exhibited differences between controls and short-term systemic tissues. Compared to the control, long-term systemic responses (SI-2) exhibiting elevated concentrations (~1.2×) only occurred in hydroxypropiovanillone hexoside (p = .005), lignan hexoside (p = .012), and lignan xyloside 1 (p = .007).

3.4 | Primed responses to simulated bark beetle attack

Terpene compositions of reaction zones in trees receiving a second simulated attack ("primed": LI-P-2) were not substantially different from the initial localised responses on the same trees (LI-1) (Table 4). This absence of priming was observed among both total and most individual terpenoids. Previously challenged (LI-P-2) trees did not differ in terpenoid concentrations from trees that had not previously been challenged with simulated bark beetle attack (LI-PC-2).

Where systemic priming of terpenoids appeared (SI-P-2), it was mostly limited to monoterpenes, and in small quantities. With the exception of the two unidentified peaks, monoterpene concentrations were 1.2–1.4× higher in systemically primed (SI-P-2) than systemic tissue at the first sampling (SI-1). Total diterpene acids did not differ between the SI-1 and SI-P-2 treatments, but sandaracopimaric acid concentration was lower in SI-P-2 (p = .007). Concentrations of monoterpenes and longifolene did not differ between the "primed" systemic samples (SI-P-2) and those receiving a single simulated attack (SI-PC-2). The diterpene acids levopimaric and isopimaric acid were elevated in SI-PC-2 by 1.2× and 1.5× respectively.

Differences in localised concentrations of individual phenolics were absent or minor between trees receiving second inoculations (i.e. test for priming: LI-P-2) compared to those only receiving one inoculation (LI-1). Likewise, no increases in phenolics were observed between the localised reactions of previously unchallenged (LI-PC-2) and previously challenged (LI-P-2) trees. No compounds exhibited substantial decreases between the LI-P-2 and LI-PC-2.

Few differences were observed between the systemic tissues across the different sampling times. No phenolics were elevated between the systemic tissues of trees receiving a second attack (SI-P-2) compared to short-term systemic responses (SI-1). Likewise, no phenolics differed between the previously challenged (SI-P-2) and previously unchallenged (SI-PC-2) trees.

3.5 | Constitutive changes over time in red pine phloem chemistry

Constitutive monoterpenes did not change between the first (C-0) and second (C-1) sampling times. Diterpene acids exhibited only minor changes: levopimaric acid increased 1.3 × (p = .001) and isopimaric acid decreased 0.9 × (p = .003). No other terpenes differed between these two time points. Changes in monoterpenes from C-0 to C-2 were likewise relatively minor. Total monoterpenes increased by 1.3 × (p = .007). The diterpene acids sandaracopimaric (1.5×), levopimaric (1.7×), isopimaric (1.4×), dehydroabietic acid (1.2×), and neoabietic (1.5×) had higher concentrations at C-2 than C-0 (p < .001).

Similar to the terpenoids, phenolics exhibited only minor changes in constitutive phloem tissue over time. Total flavonoids and procyanidin trimer were 1.4× greater in C-0 than in C-1, but not in C-2. Lignan deoxyhexoside and lignan xyloside 1 and 2 were 1.2× greater in the second control for time (C-2) compared to the initial time point, but exhibited no difference between the initial and first control for time (C-1). Caffeic acid was lower in C-2 compared to the tissues sampled at C-0 (p = .008).

3.6 | Chemical profiles reflect differences between constitutive and locally induced phloem

NMDS of the relative abundances of various compounds indicated that localised host responses had different compositions than either control tissues or tissues distant from inoculation (Figure 3). Within the terpenoids, tissues sampled from the localised reaction zones (LI-1, LI-P-2, LI-PC-2) were clustered together (Figure 3a). These localised responses were not altered by prior treatment, as would be explained by priming. There was no separation between control tissue and samples that were distant from fungal challenges (i.e. systemic, tissue-wide induction). Phenolics showed similar patterns, but exhibited even greater separation between local induction vs. controls and phloem distant from treatments (Figure 3b). Localised induction

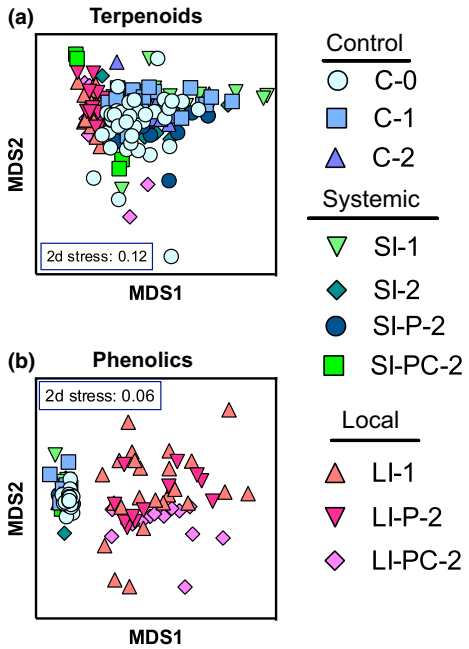

FIGURE 3 Non-metric multidimensional scaling (NMDS) plots of relative abundances of the terpenoids (a) and phenolics (b) present in red pine phloem samples. NMDS plots were generated from Euclidean distances of the relative abundances of dry weight concentrations of terpenoids, and using normalised absorbance units with phenolics. Naming scheme follows that of Figure 1

samples showed more variability than the other treatments yet did not exhibit overlap with control or systemically tissues.

3.7 | Lesion formation in response to simulated bark beetle attack

As in previous studies, trees formed lesions in response to inoculation, and no lesions were formed distant from the point of treatment. The timing of simulated bark beetle attack influenced lesion length (Figure 4). Overall, lesions from treatments on July 1 (t_1) were longer than those administered 25 days later. The lesions from the t_2 simulated attack (LI-P-2) were 1.3× longer than the first simulated attacks (LI-1) on the same trees (t = 3.66; df = 11, p = .004). Likewise, lesions on separate trees that had not been previously challenged (LI-PC-2) were also 1.3× greater than LI-1 (t = 3.221; df = 22.351, p = .004). Lesion length did not differ between previously inoculated and uninoculated trees in July (t = 0.385; df = 22.72, p = .704).

4 | DISCUSSION

The ability to deploy effective defences against tree-killing bark beetle-microbial complexes is critical for conifer success. Mature red pines responded to simulated attack by undergoing pronounced, multifaceted induced reactions, which included quantitative and proportional changes of terpenoids and phenolics and the expression of histological changes. These responses were localised and rapid, and there was no evidence of tissue-wide systemic induction, or priming, i.e. heightened responsiveness to subsequent treatment (Figure 5).

Conifer induced responses suggest a defence syndrome that targets multiple components of an enemy complex. The local concentrations of monoterpenes produced during induction exceed the lethal dose for *I. pini* adults (Raffa & Smalley, 1995), and concentrations of α-pinene and β-pinene (which are stimulatory at low doses)

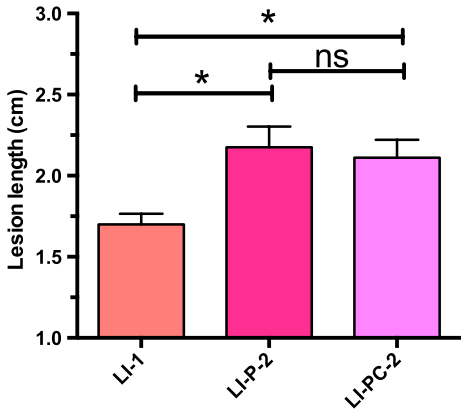

FIGURE 4 Lesion length (cm) of reaction zones formed in response to simulated bark beetle attack. Asterisks indicate significant differences (p < .05) between the samples. The first induction (LI-1) resulted in significantly different lesion lengths from later simulated attacks. Prior induction did not influence length of lesion in response to subsequent treatment

exceed those needed to inhibit *I. pini* tunneling (Wallin & Raffa, 2000). Components of this response also inhibit the beetles' mutualists. For example, -3-carene strongly inhibits most beetle-associated bacteria (Adams, Boone, Bohlmann, & Raffa, 2011), and diterpene acids and stilbenes suppress mycelial growth and conidial germination of beetle-associated fungi (Hammerbacher et al., 2013; Klepzig, Smalley, & Raffa, 1996; Kopper et al., 2005). Because most monoterpenes do not strongly inhibit beetle-associated fungi (Klepzig et al., 1996), diterpenes have minimal activity against beetles (Kopper et al., 2005), and stilbenes only weakly affect beetle behaviour, the composition and inducibility of conifer phloem suggests defensive complementarity among various phytochemical groups.

Different groups of compounds underwent variable changes in concentration, reflecting distinct and perhaps interacting biosynthetic pathways. Increases were much higher among terpenes, products of mevalonic acid and 1-deoxy-D-xylulose-5-phosphate pathways, than phenolics, products of shikimic acid metabolism. Among phenolics, however, stilbenes underwent a phytoalexin-like response, being undetectable in constitutive tissue but abundant in locally induced reaction tissue, as observed elsewhere (Villari et al., 2012; Wallis et al., 2008). Three other classes of phenolics, lignans, hydroxycinnamic acids, and vanilloids, decreased after treatment. Flavonoids showed no quantitative change, but underwent substantial changes in composition that may reflect biodegradation (Wadke et al., 2016). Future studies should partition to what extent phenolic decreases in this system reflect allocation trade-offs, cross-talk with terpene induction, fungal metabolism, or other mechanisms (Hammerbacher et al., 2013; Thaler, Humphrey, & Whiteman, 2012; Wadke et al., 2016; Wallis et al., 2011).

Our results illustrate how the spatiotemporal scale of induced responses to specific enemies is an important dimension of plant defence syndromes. In this system, induced responses were almost entirely localised. Yet pines have shown the ability to express both local and systemic induced resistance responses against fungal necrotrophic canker pathogens (Bonello & Blodgett, 2003; Bonello, Gordon, & Storer, 2001; Bonello et al., 2006; Eyles et al., 2010; Sherwood & Bonello, 2013; Wallis et al., 2008). There are two major differences between those systems and bark beetle-microbial complexes. First, the prior systems involved saplings, which are hosts to a different assemblage of biotic agents than are mature pines. For example, saplings are not at risk to *I. pini*, because only mature trees are large enough to support brood. Second, canker pathogens such as *Diplodea pinea* and *Fusarium circinatum* cause disease symptoms in their hosts, whereas Ophiostomatoid fungi associated with bark beetles are typically not pathogenic by themselves in coevolved systems (Six & Wingfield, 2011), but rather assist beetles as cofactors during mass attack (DiGuistini et al., 2011; Lieutier et al., 2009; Six & Klepzig, 2004). Additionally, terpenoid defenses are energetically demanding (Gershenzon, 1994; Goodsman, Lusebrink, Landhäusser, Erbilgin, & Lieffers, 2013) and bark beetle life history and behaviour may favour a strategy in which trees commit their resources directly and immediately at the attack site. Trunk-wide induction could conceivably provide some advantage against mass attack, but response to the first entering

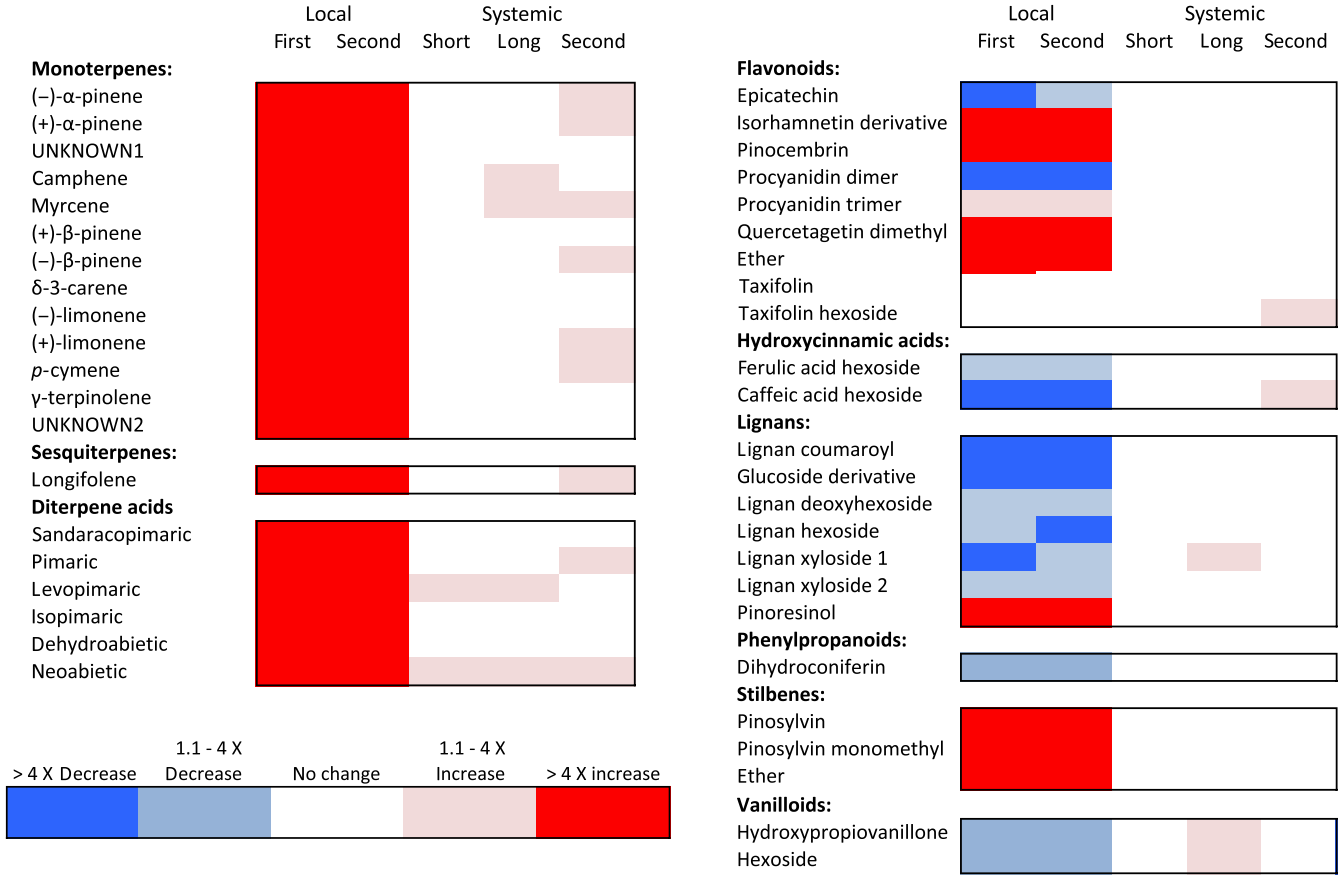

FIGURE 5 Overview of local and systemic chemical profiles in response to simulated bark beetle attack relative to control (C-0) tissue. Increases by 1.1–4 and >4× are denoted by pink and red shading, and decreases by 1.1–4 and >4× are indicated by pale blue and blue shading. Compounds exhibiting no difference have no colouring. Local responses are to the first inoculation (LI-1) and the second, primed inoculation (LI-P-2). Systemic responses include short term (SI-1), long term (SI-2), and primed responses (SI-P-2)

beetle is particularly critical. If the initial beetle succeeds in eliciting mass attack, tree survival is unlikely, but if the beetle encounters high concentrations of monoterpenes, it may fail to attract other beetles and hence be confined within defensive reactions (Erbilgin, Powell, & Raffa, 2003; Erbilgin et al., 2006).

These results also add to our understanding of how plant architecture and the attacking agent interact to influence the expression of induced responses. Chemical induction in conifers can extend beyond anatomical responses, as previous studies have detected changes well beyond lesions, sometimes in needles of trees induced on the stem (Arango-Velez et al., 2016; Bonello & Blodgett, 2003; Krokene, Solheim, & Krekling, 2003; Sherwood & Bonello, 2016; Villari et al., 2012). The extent to which the distances involved reflect local chain reactions due to cell injury, hormonal elicitors, or diffusion is unknown. Pineaceae have relatively short tracheids (typically <5 cm) compared to angiosperm vessels (often several meters long) (Kramer, 1983; Kramer & Kozlowski, 1960), and axial resin ducts and rays that can increase and enlarge during induction (Franceschi et al., 2005). Increased concentrations of secondary compounds over short distances are consistent with either diffusion or short-distance signal eliciting further biosynthesis.

We did not find evidence of prior induction influencing subsequent inducibility. The multivoltinism of *I. pini* might suggest some advantage of priming, but the lethal consequence of beetle success may instead favour a strategy of immediate and total commitment to local defence. Priming might likewise provide an advantage in subsequent years, but bark beetle populations are highly variable in time and space, and *I. pini* numbers 1 year do not closely predict abundance at the same location during the next year (Erbilgin et al., 2002). Similarly mass-inoculating Norway spruce (*Picea abies* (Beck)) with *O. polonica* can reduce injury to inoculations two (Christiansen et al., 1999) and 3 weeks later, but this protection is localised (Krokene, Christiansen, Solheim, Franceschi, & Berryman, 1999). The scale of treatment may also affect response. Spraying Norway spruce stems with methyl jasmonate, a plant defence elicitor, raised terpene levels and resistance to *Ips typographus* (L.) (Erbilgin et al., 2006; Zhao et al., 2011), and painting stems with methyl jasmonate decreased lesions produced in response to *O. polonica* 4 weeks later (Zeneli, Krokene, Christiansen, Krekling, & Gershenzon, 2006), although methyl jasmonate and beetle-vectored fungi elicit qualitatively different chemical responses (Zhao et al., 2010).

A longer period between treatment and subsequent challenge might reveal some modality of heightened defence. However, lesion lengths in loblolly (*P. taeda* L.) and shortleaf pine (*P. echinata* [Mill]) in response to *O. minus*, and in jack (*P. banksiana* [Lamb]) and red pine

in response to *O. ips*, were not affected by inoculation 1 year earlier (Cook & Hain, 1987; Raffa & Smalley, 1988). Similarly, constitutive and induced monoterpene levels in jack pine were not influenced by inoculation 2 months or 1 year earlier (Wallin & Raffa, 1999). A potential mechanism for long-term induction could be formation of traumatic resin ducts (Christiansen et al., 1999), a general response to a variety of abiotic and biotic stresses (Bräuning et al., 2016; Hood, Sala, Heyerdahl, & Boutin, 2015; Slack, Zeibig-Kichas, Kane, & Varner, 2016). Several studies have shown that trees that were alive following bark beetle outbreaks had more and larger resin ducts than trees that had been killed (Ferrenberg & Mitton, 2014; Kane & Kolb, 2010). Christiansen et al. (1999) observed increased resin duct density after administering 325 *O. polonica* inoculations plus another 325 mechanical wounds per square metre. However, that density greatly exceeds the number of *I. typographus* entries typically associated with failed attacks.

Our results illustrate that herbivore behaviour and the type of damage they impose can influence the spatial and temporal components of plant defence responses. Rapid local induction may be particularly adaptive in systems where insects develop within host tissues whose loss can be lethal or not easily tolerated. For example, plants often exert localised defences against gall forming midges and wasps that can suddenly and irreparably co-opt host physiology (Harris et al., 2003; Tooker & Helms, 2014). In contrast, tissue-wide or systemic responses may be more adaptive against herbivores that impose more chronic injury, continually increase populations on individual plants, and can move among plant parts. For example, plants commonly respond systemically to folivores (Karban, Baldwin, Baxter, Laue, & Felton, 2000; Kim et al., 2012). Priming may be more adaptive in systems where injury or herbivore cues are highly predictive of future attack, which have been demonstrated against both gall forming and leaf feeding insects (Frost et al., 2008; Helms et al., 2013). Further sources of diverse strategies likely arise from plant architecture, the physical properties of bioactive compounds, and the phylogenetic lineage of different plant-herbivore associations.

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AUTHORS' CONTRIBUTIONS

C.J.M. and K.F.R. conceptualised the study. C.J.M., K.F.R., and S.J. conducted the experiments and collected plant tissues and measurements. C.J.M., K.K.R., C.V., and P.B. performed chemical analyses. C.J.M., J.Z., and K.F.R. analysed and interpreted the data. C.J.M. and K.F.R. wrote the first draft of the manuscript, and all authors contributed to subsequent revisions. All authors approved the submitted manuscript.

DATA ACCESSIBILITY

Data used in this paper are available at Dryad Digital Repository under the accession https://doi.org/10.5061/dryad.401kf (Mason et al., 2017).

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SUPPORTING INFORMATION

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