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Publisher's version / Version de l'éditeur:
https://doi.org/10.1094/MPMI-06-23-0077-R
Molecular Plant-Microbe Interactions, 37, 5, pp. 445-458, 2024-05-22

MPMI Vol. 37, No. 5, 2024, pp. 445–458, https://doi.org/10.1094/MPMI-06-23-0077-R

Evidence that Ophiostomatoid Fungal Symbionts of Mountain Pine Beetle Do Not Play a Role in Overcoming Lodgepole Pine Defenses During Mass Attack

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Accepted for publication 8 January 2024.

Mountain pine beetle (MPB; *Dendroctonus ponderosae* Hopkins) is a devastating forest insect pest that has killed millions of hectares of pines in western North America over the past two decades. Like other bark beetles, MPB vectors ophiostomatoid fungal species, some of which are pathogenic to host pine species. The phytopathogenicity of these fungal symbionts has sparked considerable debate regarding their role in facilitating MPB attack success. We tested the hypothesis that MPB ophiostomatoid fungal associates like *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield contribute to overwhelming host defenses during MPB mass attack. We compared responses of mature lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) trees growing in natural stands that were mass attacked by MPB with those inoculated with *G. clavigera* by examining host defense hormones, secondary metabolites, and gene expression profiles. The jasmonate and ethylene signatures of necrotrophic pathogen-triggered response were identified in *G. clavigera*-inoculated trees, but only the jasmonate signature of a herbivore-triggered response was measured in MPB-attacked trees. Several *G. clavigera*-induced changes in pine phenolic metabolite profiles and phenolic biosynthesis gene expression patterns were absent in MPB-attacked pines. These findings indicate that ophiostomatoid fungi like *G. clavigera* are not a major factor in overwhelming host defenses during MPB mass attack. Instead, fungal pathogenicity likely is more important in aiding MPB colonization and development within the host tree. Phenolics appear to play a larger role in the host response to *G. clavigera* than to MPB, although phenolics may also influence MPB feeding and behavior.

Keywords: bark beetle, *Dendroctonus ponderosae*, ethylene, *Grosmannia clavigera*, jasmonic acid, phenolics, *Pinus contorta*, plant defense

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Author contributions: C.E.F., A.E.M., and J.E.K.C. designed the research project; C.E.F., A.E.M., and L.I.Z. performed the research and data analysis; results were interpreted by C.E.F. and J.E.K.C.; primary writing of the manuscript was carried out by C.E.F. and J.E.K.C.; all authors contributed to editing and approval of the final version.

Data availability: The scripts and data that support the findings of this study are openly available in Borealis, the Canadian Dataverse Repository, at https://doi.org/10.5683/SP3/QNUVEZ.

Funding: This research was supported by grants to J. E. K. Cooke and M. L. Evenden from the Natural Sciences and Engineering Research Council of Canada (NSERC) to the TRIA Network (Strategic Network grant no. NET GP 434810-12) with contributions from Alberta Agriculture and Forestry, fRI Research, Manitoba Conservation and Water Stewardship, Natural Resources Canada–Canadian Forest Service, Northwest Territories Environment and Natural Resources, Ontario Ministry of Natural Resources and Forestry, Saskatchewan Ministry of Environment, West Fraser, and Weyerhaeuser; a grant to J. E. K. Cooke from NSERC (Discovery Grant no. RGPIN-2016-04672); a grant to J. E. K. Cooke and M. L. Evenden from Genome Alberta (grant no. L20TF); a grant to J. E. K. Cooke and M. L. Evenden from Genome Canada (grant no. LSARP 18202); and graduate student scholarships to C. E. Fortier from NSERC and Alberta Innovates. C. E. Fortier and A. E. Musso were supported by graduate teaching assistantships from the University of Alberta during this work.

e-Xtra: Supplementary material is available online.

The author(s) declare no conflict of interest.

Mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins; Coleoptera: Curculionidae: Scolytinae) is an aggressive bark beetle native to western North America that attacks and kills lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.). MPB utilizes a mass attack strategy to overwhelm host tree defenses (Six and Klepzig 2021). An aggregation pheromone that initiates mass attack is produced soon after a pioneer female lands and determines that a host is suitable (Safranyik and Carroll 2006). Ensuing attacks generally peak 1 to 2 days following initial attack and typically do not persist for more than 7 days (Raffa and Berryman 1983; Safranyik and Carroll 2006). During this time, host constitutive defenses are exhausted by attacking MPB, and synthesis of induced defenses may not be sufficient to prevent successful MPB colonization (Krokene 2015).

Trees form distinct lesions containing defense compounds following MPB attack, including terpene-rich oleoresin (Kolosova and Bohlmann 2012; Safranyik and Carroll 2006; Shrimpton 1973). Terpenes fulfill an important role in Pinaceae defense against pests and pathogens, including in *Pinus* spp. responses to MPB. MPB exploits host (−)-α-pinene as a precursor to the aggregation pheromone (−)-*trans*-verbenol (Chiu and Bohlmann 2022; Chiu et al. 2019), while other monoterpenes such as limonene are toxic to MPB (Reid et al. 2017). In addition to resinous compounds, lesions formed in MPB-attacked trees contain phenolics (Raffa et al. 2008), which are synthesized and accumulated in an attempt to slow or contain pathogen growth

(Wallis and Galarneau 2020; Witzell and Martín 2008). The effects of phenolics on MPB are not as well understood as those of terpenoids, although polar extracts of lodgepole pine, presumably containing some phenolics, encourage MPB feeding (Raffa and Berryman 1982). Oxidation of phenolics can trigger production of reactive oxygen species (Barbehenn and Constabel 2011), and foliar phenolics are believed to play a role in resistance to insect herbivores (Moctezuma et al. 2014; Qazi et al. 2018).

MPB vectors sapwood-colonizing ophiostomatoid fungi in specialized structures called mycangia (Bleiker et al. 2009). Fungal assemblages found in the mycangia typically comprise multiple ophiostomatoid species (Roe et al. 2011a; Six and Bentz 2007), such as *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield, *Ophiostoma montium* (Rumbold) von Arx., and *Leptographium longiclavatum* Lee, Kim and Breuil (Bleiker et al. 2009; Roe et al. 2010, 2011b; Six 2020a). MPB-vectored fungi spread into xylem via ray parenchyma cells (Ballard 1982; Ballard et al. 1984; Safranyik and Carroll 2006; Six 2020a). Perception of the fungi by the host pine triggers formation of tyloses, blocking movement of materials through tracheids in an effort to restrict fungal access (Arango-Velez et al. 2016; Parmeter et al. 1992; Solheim 1995). Continued fungal colonization of sapwood results in blockage of water transport and is a major contributor to tree mortality following MPB attack (Arango-Velez et al. 2016; Hubbard et al. 2013).

Several studies have demonstrated that the three main ophiostomatoid fungal associates—*G. clavigera*, *O. montium*, and *L. longiclavatum*—are an important nutritional resource for MPB, effectively translocating nutrients from sapwood to the galleries and pupal chambers (Six 2020a). This concentration of resources by ophiostomatoid symbionts provides nitrogen (Bleiker and Six 2007; Cook et al. 2010; Goodsman et al. 2012), sterols (Bentz and Six 2006), and other nutrients to developing MPB (Ayres et al. 2000; Klepzig and Six 2004). MPB larvae and teneral adults show preferential feeding of phloem containing hyphae and fungal spores, respectively, over uncolonized phloem (Bleiker and Six 2007), and beetles reared on inoculated phloem develop faster with less gallery construction (Myrholm and Langor 2016). MPB fitness also depends on these fungi, as MPB lacking fungal associates produce no brood (Six and Paine 1998).

The role that ophiostomatoid fungal associates play in MPB's ability to successfully attack host trees is much less clear. One prevailing paradigm states that ophiostomatoid fungal associates help to exhaust host defenses (Lieutier et al. 2009; Six and Wingfield 2011). This paradigm is based in large part on the observation that *G. clavigera* and other ophiostomatoid symbionts of MPB are pathogenic to pines: that is, fungal benefits derived from host colonization cause harm to the tree host (Arango-Velez et al. 2016; Parker and Gilbert 2004). Trees inoculated with *G. clavigera* exhibit defense responses similar to those invoked by MPB attack, including lesion development (Arango-Velez et al. 2014, 2016), suggesting that *G. clavigera* and other ophiostomatoid associates trigger these responses during MPB attack.

In their thought-provoking review, Six and Wingfield (2011) challenge the long-held view that ophiostomatoid fungal associates of bark beetles such as MPB contribute to the beetle's capacity to overcome defenses of healthy trees during mass attack. They outline several studies that provide indirect evidence that these fungi do not significantly contribute to the ability of mass-attacking bark beetles to overwhelm tree constitutive and induced defenses. The authors propose an alternative model in which the pathogenicity of ophiostomatoid fungal associates provides an advantage in competing with other species of the MPB microbiome, enabling these fungi to colonize the host more efficiently for resource capture (Six and Wingfield 2011). However, to date, no rigorous test of their hypothesis has been carried out.

In this study, we use the distinctive hormone signatures of plant defense against herbivorous insect pests and fungal pathogens to assess the contribution of MPB-vectored ophiostomatoid fungal symbionts to the ability of MPB to overcome lodgepole pine defenses during mass attack. In conifers, as in angiosperms, jasmonic acid (JA) plays a central role in activating defense responses against both insect herbivores and necrotrophic fungal pathogens (Arango-Velez et al. 2016; Lombardero et al. 2013; Miller et al. 2005; Pieterse et al. 2012). In contrast, extensive studies with angiosperms show that salicylic acid (SA) is synthesized in response to biotrophic fungal pathogens (Glazebrook 2005; Ullah et al. 2019). We have previously demonstrated that lodgepole pine synthesizes JA and its active form, JA-isoleucine (JA-Ile), in response to *G. clavigera* challenge (Arango-Velez et al. 2016), providing evidence that pines perceive *G. clavigera* as a necrotrophic pathogen. JA has also been implicated in conifer responses to other necrotrophs, including Norway spruce (*Picea abies* (L.) Karst) response to *Heterobasidion annosum* (Fr.) Bref. s.l. (Arnerup et al. 2013; Lundén et al. 2015). In angiosperms, ethylene (ET) acts synergistically with JA to regulate plant defense responses against necrotrophic pathogens (Broekgaarden et al. 2015) but is not involved in defense signaling triggered by herbivorous insects (Garcia et al. 2021). The role of ET in conifer defense has received considerably less attention than that of JA, although there is indirect evidence implicating ET in conifer defense against necrotrophs from transcriptomic studies of the Norway spruce–*H. annosum* pathosystem (Arnerup et al. 2011).

We hypothesized that if ophiostomatoid fungi contribute to MPB's capacity to overwhelm host defenses during the critical mass attack phase, then lodgepole pine should perceive attack by both MPB and its ophiostomatoid symbionts, triggering synthesis of both JA and ET. Alternatively, if ophiostomatoid fungi do not contribute meaningfully to MPB's capacity to overwhelm host defenses during the attack phase, then lodgepole pine should perceive MPB but not its necrotrophic fungal symbionts, and we should detect increased JA but not ET. To test this hypothesis, we needed to establish whether ET is invoked by lodgepole pine in response to MPB ophiostomatoid fungal associates. Accordingly, we quantified the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) together with JA, JA-Ile, and SA in lodgepole pines undergoing MPB mass attack and in *G. clavigera*-inoculated lodgepole pines. We focused on the attack phase, during which host defenses are being challenged and overcome, rather than the subsequent colonization phase that follows successful attack. Finally, we compared chemical defense responses of MPB-attacked and *G. clavigera*-inoculated lodgepole pines to determine whether lodgepole pine's perception of an herbivorous insect versus a necrotrophic fungal pathogen triggers markedly different suites of chemical defenses. Given that lesion development is a hallmark of pine responses to both MPB attack and inoculation with ophiostomatoid MPB fungal associates, but that lesion development takes an extended period, we profiled phenolic compounds that are characteristic of these lesions. This experimental approach allowed for the most rigorous test to date of the role that ophiostomatoid fungi play in MPB's ability to overcome host defenses during mass attack.

Results

In this study, we relied on natural MPB mass attack of mature lodgepole pine trees in a natural unmanaged stand baited with a semiochemical mixture designed for *D. ponderosae* containing *exo*-brevicomin and *trans*-verbenol (https://semiochemical.com/bark-beetles; Borden et al. 2008). Unlike conventional

infestation experiments in which herbivorous insects are introduced to the host on a single day at the outset of the experiment, natural MPB mass attack of the baited trees resulted from multiple MPB attacks that occurred over several days. Samples collected at 1 day postwound (dpw) corresponded to 1 to 7 days after initial MPB attack, representing the active phase of MPB mass attack. Based on the observation that an MPB mass attack is typically completed within 7 days of initial attack (Safranyik and Carroll 2006), we expected to see evidence of mass attack by 7 dpw, which corresponded to 7 to 13 days after initial MPB attack for all trees. Indeed, by 7 dpw, we observed that all MPB-attacked trees had reached the threshold of more than 40 attacks, generally used as a benchmark of mass attack by MPB (Raffa and Berryman 1983; Safranyik and Carroll 2006).

JA signaling represents an early step in activation of host specialized defenses in response to insect herbivory (Aerts et al. 2021), while the hallmark of a plant's response to necrotrophic fungal pathogen attack is synthesis of both JA and ET. To determine whether both JA and ET signatures triggered by necrotrophic fungal pathogen attack could be detected in MPB-attacked lodgepole pine, we first needed to establish whether *G. clavigera* challenge induced both JA and ET biosynthesis in lodgepole pine. To assess the impact of *G. clavigera* inoculation on JA and ET levels in host lodgepole pine, we designed our experiment to sample trees at 7 and 14 days postinoculation (dpi). Unlike the MPB attacks, which occurred over a period of 1 week, *G. clavigera* was applied to inoculated trees in a single day. Because of these differences in the duration over which these biotic challenges were applied, careful consideration of sampling time points was required for the *G. clavigera* experiment to best coincide with the MPB mass attack sampling time points. Because MPB attacks began 7 days before the first sampling time point at 1 dpw, we chose to designate the first sampling time point for *G. clavigera*-inoculated trees as 7 dpi. The 7 dpi time point for the *G. clavigera* inoculation experiment also coincided with the key time point for which we had previously measured significant increases in JA of *G. clavigera*-inoculated lodgepole pine seedlings (Arango-Velez et al. 2016). We then chose 14 dpi as the second sampling time point to approximate the 7 dpw MPB mass attack time point.

In addition to measuring JA, we measured levels of the active form of JA, JA-Ile. Because ET is a gas and as such must be collected from the tree headspace, we quantified the stable precursor of ET, ACC, as an indicator of ET levels (Bulens et al. 2011; Chauvaux et al. 1997). As predicted, levels of JA, JA-Ile, and ACC all increased in phloem for *G. clavigera*-inoculated trees relative to mock-inoculated controls at both 7 and 14 dpi (Fig. 1A to C ; Supplementary Table S2). JA and JA-Ile levels also increased significantly in xylem for *G. clavigera*-inoculated trees relative to mock-inoculated controls by 14 dpi (Fig. 1K and L; Supplementary Table S2). SA and conjugated forms of SA did not differ significantly in xylem or phloem between *G. clavigera*- and mock-inoculated trees at 7 or 14 dpi (Fig. 1D, E, N, and O; Supplementary Table S2).

Having determined that lodgepole pine synthesizes both JA and ET in response to *G. clavigera*, we next looked at defense hormone levels in MPB-attacked versus mock-attacked lodgepole pine. Consistent with a response to infestation by an herbivorous insect, JA and JA-Ile levels were significantly higher in secondary phloem of MPB-attacked trees relative to mock-attacked trees at 7 dpw (Fig. 1F and G; Supplementary Table S2). JA and JA-Ile levels were significantly higher in secondary xylem of mock-attacked trees at 1dpw relative to MPB-attacked trees. (Fig. 1P and Q; Supplementary Table S2). JA levels in secondary xylem then increased significantly at 7 dpw for both mock-attacked and MPB-attacked trees, at which point the two treatments had similar JA levels (Fig. 1P; Supplementary Table S2). JA-Ile levels also increased in MPB-attacked trees at 7 dpw relative to 1 dpw but were significantly higher than mock-attacked trees at 7 dpw (Fig. 1Q; Supplementary Table S2). There were no significant differences in phloem ACC levels between MPB-attacked and mock-attacked trees at 1 or 7 dpw, although ACC measured in xylem at 7 dpw was significantly higher for mock-attacked trees than MPB-attacked trees at 7 dpw and mock-attacked trees at 1 dpw (Fig. 1R; Supplementary Table S2). No significant differences were observed for SA or conjugated SA in phloem or xylem of MPB-attacked trees relative to mock-attacked trees at either time point, except for a decrease in SA levels in xylem of MPB-attacked trees at 7 dpw versus 1 dpw (Fig. 1I, J, S, and T; Supplementary Table S2).

To determine whether the differences in defense hormone profiles invoked by MPB attack versus *G. clavigera* challenge were related to differences in chemical defense responses, we examined phenolic metabolite profiles. Ordination plots of nonmetric multidimensional scaling (NMDS) values, paired with analysis of similarities (ANOSIM) results, revealed that phloem phenolic profiles varied significantly across treatments but not time points (Fig. 2A). The greatest resolution between treatments was between the three control treatments and the two challenge treatments. There was little resolution, however, in the NMDS analysis between the phloem phenolic profiles of MPB- attacked and *G. clavigera*-inoculated trees. Closer inspection of total phenolic levels and of individual phenolic compounds identified as significant contributors to variation between samples in the NMDS model supported this (Fig. 2B to O; Supplementary Table S3). For example, pinocembrin and pinosylvin increased dramatically in *G. clavigera*-inoculated phloem at 14 dpi and were low or undetected in all other treatments (Fig. 2G and H; Supplementary Table S3). Increased pinoresinol levels were also observed in *G. clavigera*-inoculated trees at 14 dpi (Fig. 2D; Supplementary Table S3) as well as in both MPB-attacked and mock-attacked trees at 1 dpw (Fig. 2K; Supplementary Table S3). Ferulic acid, pinosylvin monomethyl ether (PMME), and catechin hydrate levels did not show any treatment-specific responses in phloem (Fig. 2C, E, F, J, L, and M).

Relative to the results obtained with phloem-derived phenolic metabolite levels (Fig. 2), xylem phenolic metabolite profiles showed greater resolution across treatments (Fig. 3). ANOSIM results indicated that both treatment and time point were significant factors contributing to differences in xylem metabolite profiles (Fig. 3A). Two-dimensional visualization of NMDS values illustrated clear separation of MPB-attacked phenolic profiles from *G. clavigera*-inoculated phenolic profiles along NMDS1 (Fig. 3A). *G. clavigera*-inoculated samples were further differentiated from the mock controls along NMDS2 (Fig. 3A). Significant increases in total phenolics were observed in mock-attacked and mock-inoculated xylem samples relative to unwounded controls (Fig. 3G; Supplementary Table S4) that were not observed in phloem (Fig. 2I). Most notably, xylem total phenolics increased significantly in *G. clavigera*-inoculated trees at 14 dpi relative to mock-inoculated trees (Fig. 3B; Supplementary Table S4). Increases in pinostrobin, PMME, and pinosylvin were observed in *G. clavigera*-inoculated xylem, principally at 14 dpi (Fig. 3C, D, and F). Interestingly, while pinosylvin levels remained negligible in MPB-attacked and mock-attacked trees, pinostrobin and PMME levels increased in response to mock treatments and MPB attack (Fig. 3H and I). We found that *p*-coumaryl alcohol levels increased substantially in xylem of MPB-attacked trees at 1 dpw relative to the low or negligible levels quantified in other samples (Fig. 3J).

To complement these biochemical metabolite analyses, we also looked at changes in the expression of genes encoding enzymes at key branch points of phenolic biosynthesis: *stilbene synthase* (*STS*), *O-methyltransferases 1* and *2* (*OMT1*

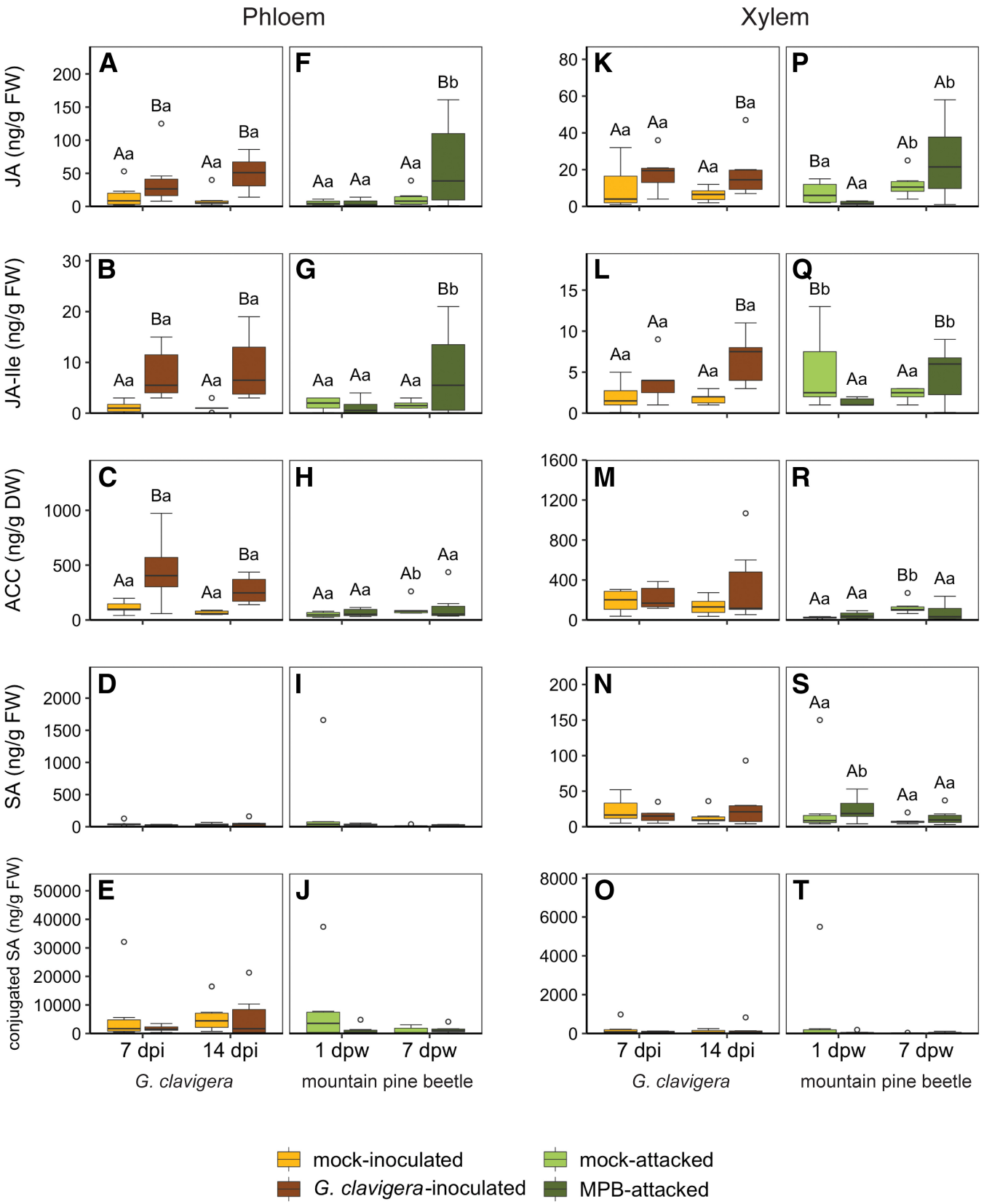

Fig. 1. Defense hormone profiles in *Grosmannia clavigera*-inoculated or mountain pine beetle (MPB)-attacked lodgepole pines, and the corresponding mock-treated control trees. **A to E,** Secondary phloem of mock- or *G. clavigera*-inoculated trees (Experiment 1 from "Materials and Methods"). **F to J,** Secondary phloem of mock- or MPB-attacked trees (Experiment 2 from "Materials and Methods"). **K to O,** Secondary xylem of mock- or *G. clavigera*-inoculated trees (Experiment 1). **P to T,** Secondary xylem of mock- or MPB-attacked trees (Experiment 2). Boxes for each box plot indicate the median value bounded by the 75th (upper) and 25th (lower) quantiles. Box plot whiskers represent boundaries of 1.5 times the interquartile range added to each quantile. Uppercase letters indicate significant differences between treatments within a time point within Experiment 1 or 2, while lowercase letters indicate significant differences between time points within treatments within Experiment 1 or 2 (Tukey-adjusted $P < 0.05$, $n = 6$). JA, jasmonic acid; JA-Ile, JA-isoleucine; ACC, 1-aminocyclopropane-1-carboxylic acid; SA, salicylic acid; FW, fresh weight; DW, dry weight.

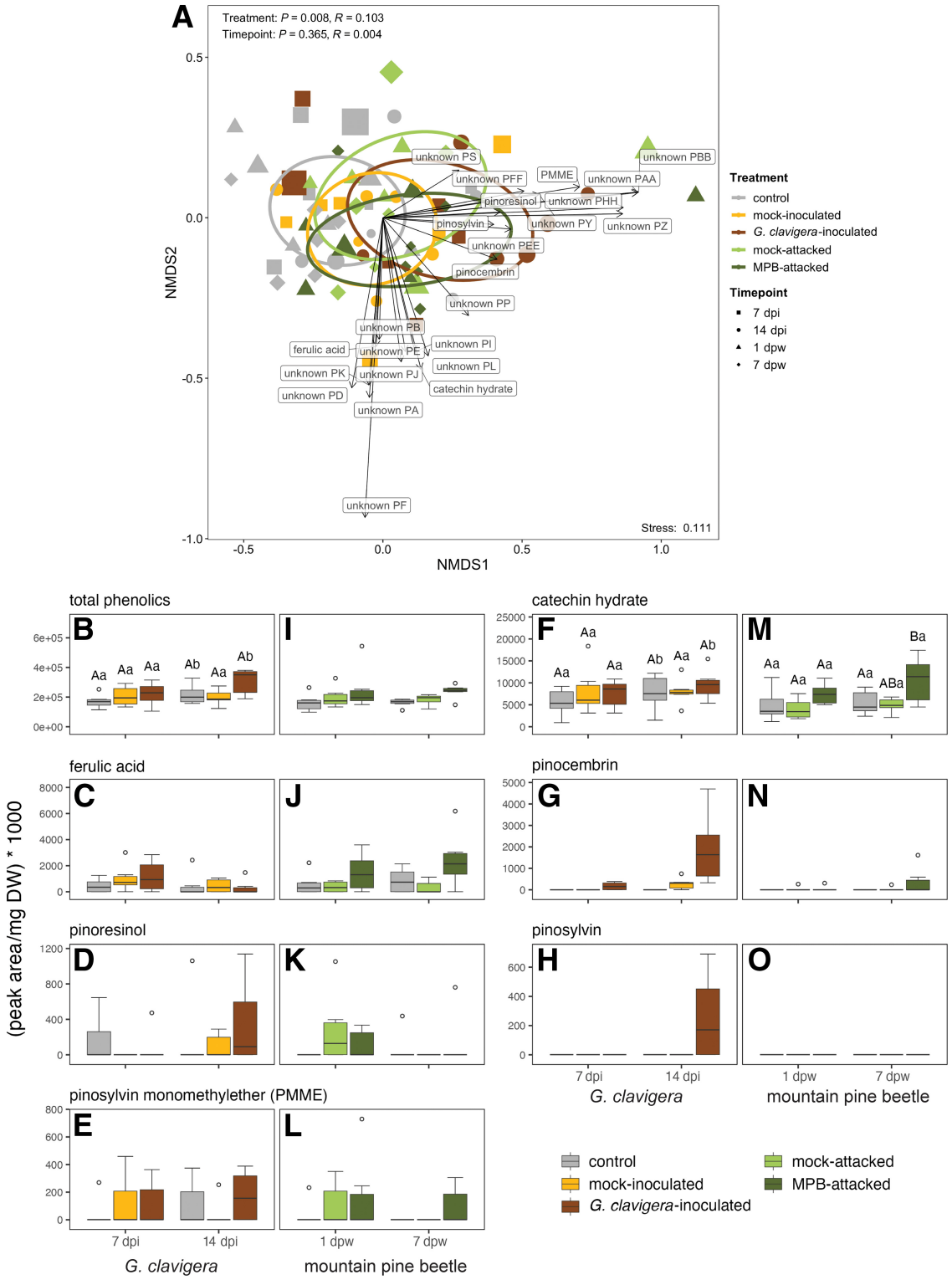

Fig. 2. Phenolic profiles of secondary phloem of mountain pine beetle (MPB)-attacked or *Grosmannia clavigera*-inoculated lodgepole pines and their corresponding control trees. **A,** Two-dimensional nonmetric multidimensional scaling (NMDS) of phenolic compounds in secondary phloem. Point color indicates uninoculated, mock-inoculated, and *G. clavigera*-inoculated treatments (Experiment 1 from "Materials and Methods"), and unattacked, mock-attacked, and MPB-attacked treatments (Experiment 2 from "Materials and Methods"). Point shape indicates time point, while point size is proportional to the goodness of fit of the ordination model for each sample. Ellipses represent covariance across each treatment. Arrows represent the strength and direction of significant ($P < 0.05$) phenolic predictors. Compounds that did not match the retention time of standards were alphabetically labeled with the prefix "Unknown P-" to indicate they were found in phloem. The stress value is representative of the fit of the ordination model to the sample data, with values less than 0.2 considered a good fit. Analysis of similarities (ANOSIM) *P* values represent the significance of each factor, while *R* values represent the similarity of profiles within a treatment (*R* values of 1 indicate that samples are most similar). **B to O,** Phenolic metabolites identified as significantly contributing to differences across phenolic profiles in secondary phloem by NMDS. Box plot shading is used to denote uninoculated, mock-inoculated, and *G. clavigera*-inoculated treatments for Experiment 1 (B to H), and unattacked, mock-attacked, and MPB-attacked treatments for Experiment 2 (I to O). Boxes for each box plot indicate the median value bounded by the 75th (upper) and 25th (lower) quantiles. Box plot whiskers represent boundaries of 1.5 times the interquartile range added to each quantile. Uppercase letters indicate significant differences between treatments within a time point within Experiment 1 or 2, while lowercase letters indicate significant differences between time points within treatments within Experiment 1 or 2 (Tukey-adjusted $P < 0.05$, $n = 6$). DW, dry weight.

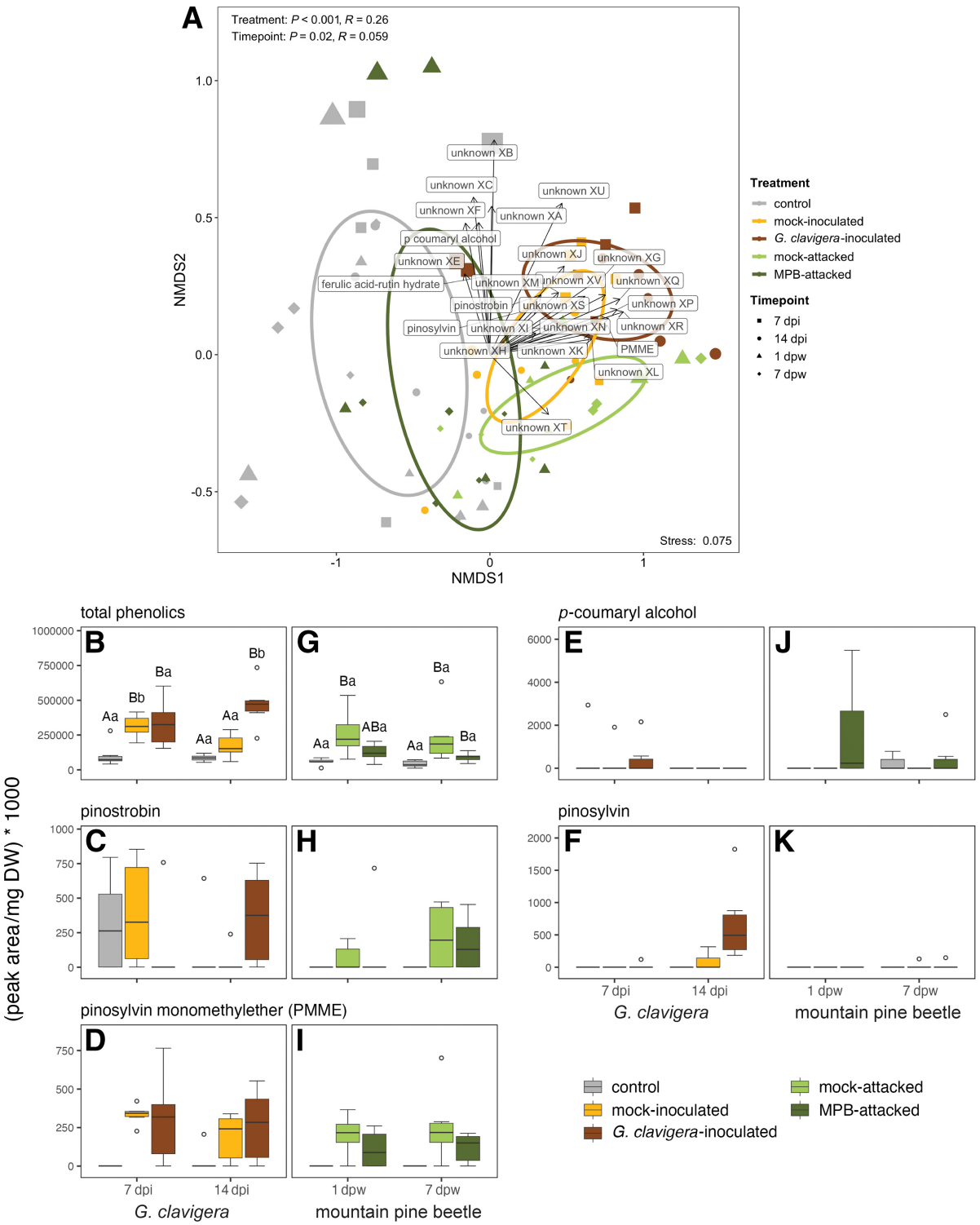

Fig. 3. Phenolic profiles of secondary xylem of mountain pine beetle (MPB)-attacked or *Grosmannia clavigera*-inoculated lodgepole pines and their corresponding control trees. **A,** Two-dimensional non-metric multidimensional scaling (NMDS) of phenolic compounds in secondary xylem. Point shape indicates time point and point size is proportional to the goodness of fit of the ordination model for each sample. Point color indicates uninoculated, mock-inoculated, and *G. clavigera*-inoculated treatments (Experiment 1 from "Materials and Methods") and unattacked, mock-attacked, and MPB-attacked treatments (Experiment 2 from "Materials and Methods"). Ellipses indicate the covariance across each treatment. Phenolic compounds identified as significant contributors to sample differences within the ordination model are included ($P < 0.05$); arrows represent the strength and direction of each predictor. Compounds that did not match the retention time of standards were alphabetically labeled with the prefix "Unknown X-" to indicate they were found in xylem. The stress value is representative of the fit of the ordination model to the sample data; values less than 0.2 are considered a good fit. Analysis of similarities (ANOSIM) *P* values represent the significance of each factor, while *R* values represent the similarity of profiles within a treatment (*R* values of 1 indicate that samples are most similar). **B to K,** Phenolic metabolites identified as significantly contributing to differences across phenolic profiles in secondary phloem by NMDS. Box plot shading is used to denote uninoculated, mock-inoculated, and *G. clavigera*-inoculated treatments for Experiment 1 (B to F), and unattacked, mock-attacked, and MPB-attacked treatments for Experiment 2 (G to K). Boxes for each box plot indicate the median value bounded by the 75th (upper) and 25th (lower) quantiles. Box plot whiskers represent boundaries of 1.5 times the interquartile range added to each quantile. Uppercase letters indicate significant differences between treatments within a time point within Experiment 1 or 2, while lowercase letters indicate significant differences between time points within treatments within Experiment 1 or 2 (Tukey-adjusted $P < 0.05$, $n = 6$). DW, dry weight.

and *OMT2*), *dihydroflavanol reductase 1* and *2* (*DFR1* and *DFR2*), and *chalcone synthase* (*CHS*). In this study of mature lodgepole pine, *PcSTS*, *PcOMT1*, and *PcDFR1* showed significant increases in transcript abundance in phloem from *G. clavigera*-inoculated trees relative to mock-inoculated trees for at least one time point (Fig. 4A, B, and D; Supplementary Table S5). *PcSTS*, *PcOMT1*, *PcDFR1*, *PcDFR2*, and *PcCHS* showed significant increases in transcript abundance in phloem from *G. clavigera*-inoculated trees relative to uninoculated controls for at least one time point (Fig. 4A, B, D, and F), while *PcSTS*, *PcOMT1*, *PcDFR1*, and *PcCHS* also showed significant increases in phloem transcript abundance in mock-inoculated trees relative to uninoculated controls for at least one time point (Fig. 4A, B, D, and F), suggesting that expression of these phenolic biosynthesis genes was also induced by wounding (Supplementary Table S5). Expression profiles for these five genes in xylem of *G. clavigera*-inoculated trees relative to mock-inoculated and uninoculated control trees were similar to those in phloem, but fewer contrasts were statistically significant. *PcSTS* showed a significant increase in transcript abundance in xylem from *G. clavigera*-inoculated trees versus mock-inoculated trees, and only at 7 dpi (Fig. 4M). *PcSTS*, *PcDFR1*, *PcDRF2*, and *PcCHS* showed significant increases at 7 and 14 dpi in xylem of *G. clavigera*-inoculated trees relative to uninoculated control trees and in mock-inoculated trees relative to uninoculated controls (Fig. 4M, P, and R; Supplementary Table S5).

Two of these phenolic biosynthesis genes, *PcOMT1* and *PcDFR2*, exhibited significantly greater transcript abundance in phloem sampled from MPB-attacked trees versus mock-attacked trees (Fig. 4H and K; Supplementary Table S5). These two genes also showed a significant increase in transcript abundance in phloem of mock-attacked trees relative to control trees. *PcSTS* and *PcDFR1* showed increased expression in MPB-attacked and mock-attacked trees relative to control trees (Fig. 4G and J). In xylem, *PcOMT1* exhibited significantly greater expression in MPB-attacked trees than in mock-attacked trees (Fig. 4T), while *PcSTS*, *PcDFR1*, *PcDFR2*, and *PcCHS* showed significantly greater expression in xylem of MPB-attacked trees relative to controls but not relative to mock-attacked trees (Fig. 4S and V to X; Supplementary Table S5). *PcSTS* and *PcCHS* exhibited significantly greater transcript abundance in xylem from mock-attacked versus control untreated trees (Fig. 4S and X; Supplementary Table S5).

Discussion

In this study, we used lodgepole pine molecular responses to test the extent to which MPB ophiostomatoid fungal symbionts contribute to MPB's capacity to overcome host defenses during mass attack. We first established that lodgepole pine synthesizes both JA and ET—but not SA—in response to *G. clavigera* challenge. We chose to use *G. clavigera* because it is (i) one of the most common MPB fungal associates (Lee et al. 2006; Roe et al. 2011a), (ii) sufficiently virulent to kill pines in the absence of MPB (Owen et al. 1987; Yamaoka et al. 1995), and (iii) the earliest and fastest colonizer of the MPB ophiostomatoid fungal associates (Solheim 1995; Solheim and Krokene 1998). That JA rather than SA is invoked in lodgepole pine's response to *G. clavigera* challenge agrees with our previous findings that *G. clavigera* is perceived by lodgepole and jack pine (*Pinus banksiana* Lamb.) seedlings as a necrotrophic pathogen, as it colonizes xylem primarily via ray parenchyma (Arango-Velez et al. 2016), and extends this evidence to include mature trees. We also confirmed our earlier finding that lodgepole pine synthesizes JA-Ile (Arango-Velez et al. 2016), the active form of JA in angiosperms (Ruan et al. 2019). We presume that JA-Ile is also an active form of JA in conifers, although this remains to be tested rigorously. Compared with JA, ET and its role in conifer defense against necrotrophic pathogens has received little attention. This study provides the first direct evidence that, like angiosperms (Pieterse et al. 2012; van Loon et al. 2006), lodgepole pine responds to necrotrophic fungal invasion by synthesizing both ET and JA. These in planta measures of ET support the few studies in other conifer pathosystems carried out to date examining expression of genes involved in ET biosynthesis and signaling (Arnerup et al. 2011, 2013; Barnes et al. 2008; Hudgins and Franceschi 2004; Hudgins et al. 2006).

We used a pheromone bait strategy to ensure that MPB-attacked trees were subject to at least 40 MPB attacks during the experiment period, a threshold commonly considered sufficient for MPB to successfully attack and colonize the host (Raffa and Berryman 1983; Safranyik and Carroll 2006). The lack of ACC accumulation in MPB-attacked pines during the attack phase provides experimental evidence that lodgepole pine does not perceive challenge by MPB-vectored *G. clavigera* in either the phloem or xylem during this critical window when the mass attack strategy is sufficient for MPB to overcome host defenses and proceed to host colonization. Consequently, our findings do not support the oft-cited but contested theory that MPB-vectored ophiostomatoid fungi are critical for MPB success by acting in concert to overwhelm host tree defenses during the period of mass attack and initial beetle establishment (Lieutier et al. 2009; Six and Wingfield 2011).

If these MPB-vectored ophiostomatoid fungi do not play a role in overwhelming host defenses during the attack phase of MPB, then it is likely that these fungi play alternative roles that provide benefit to MPB following establishment in the host and that the pathogenic nature of these fungi facilitates some of these roles. There is compelling evidence, for example, that MPB-vectored ophiostomatoid fungi contribute to nutrient provisioning for MPB at larval and adult stages (Adams and Six 2007; Six 2020b). Relative to the nutritional needs of developing bark beetles such as MPB, phloem is considered to be limiting, particularly for nitrogen and phosphorous (Six 2020a; Six and Elser 2019). Numerous studies of the MPB–ophiostomatoid fungi nutrient-based mutualistic relationship have demonstrated that ophiostomatoid symbionts aid MPB development by supplementing sterols and nitrogen (Bentz and Six 2006; Bleiker and Six 2007; Goodsman et al. 2012; Paine et al. 1997), improving MPB fitness. For example, association with *G. clavigera* resulted in MPB producing more adult progeny that emerged earlier, while *G. clavigera*-free MPB were unable to produce a brood (Six and Paine 1998). Finally, the ability of ophiostomatoid fungi to extract and supplement nutrition for bark beetles helps reduce the constraints of intraspecific competition resulting from mass attack and likely has contributed to the close relationship many aggressive bark beetles have with ophiostomatoid fungal species (Six 2020b).

To acquire and assimilate these essential nutrients, MPB ophiostomatoid fungal associates must colonize the host phloem and sapwood (Six and Klepzig 2021) and thus must either evade detection by the host or tolerate and exhaust host defenses to effectively colonize these tissues. For example, *G. clavigera* has the capacity to detoxify terpene defenses (DiGuistini et al. 2011). The strong induction of JA, JA-Ile, and ET synthesis in both xylem and phloem of lodgepole pine within a few days of *G. clavigera* challenge, together with increases in phenolic defenses, suggests that its pathogenic lifestyle enables *G. clavigera* to overcome host defenses to colonize the host without the aid of MBP mutualism. MPB's primary fungal symbionts—*G. clavigera*, *L. longiclavatum*, and *O. montium*—exhibit different degrees of pathogenicity (Rice and Langor 2008; Rice et al. 2007), growth rates (Addison et al. 2015; Moore and Six 2015), and tem-

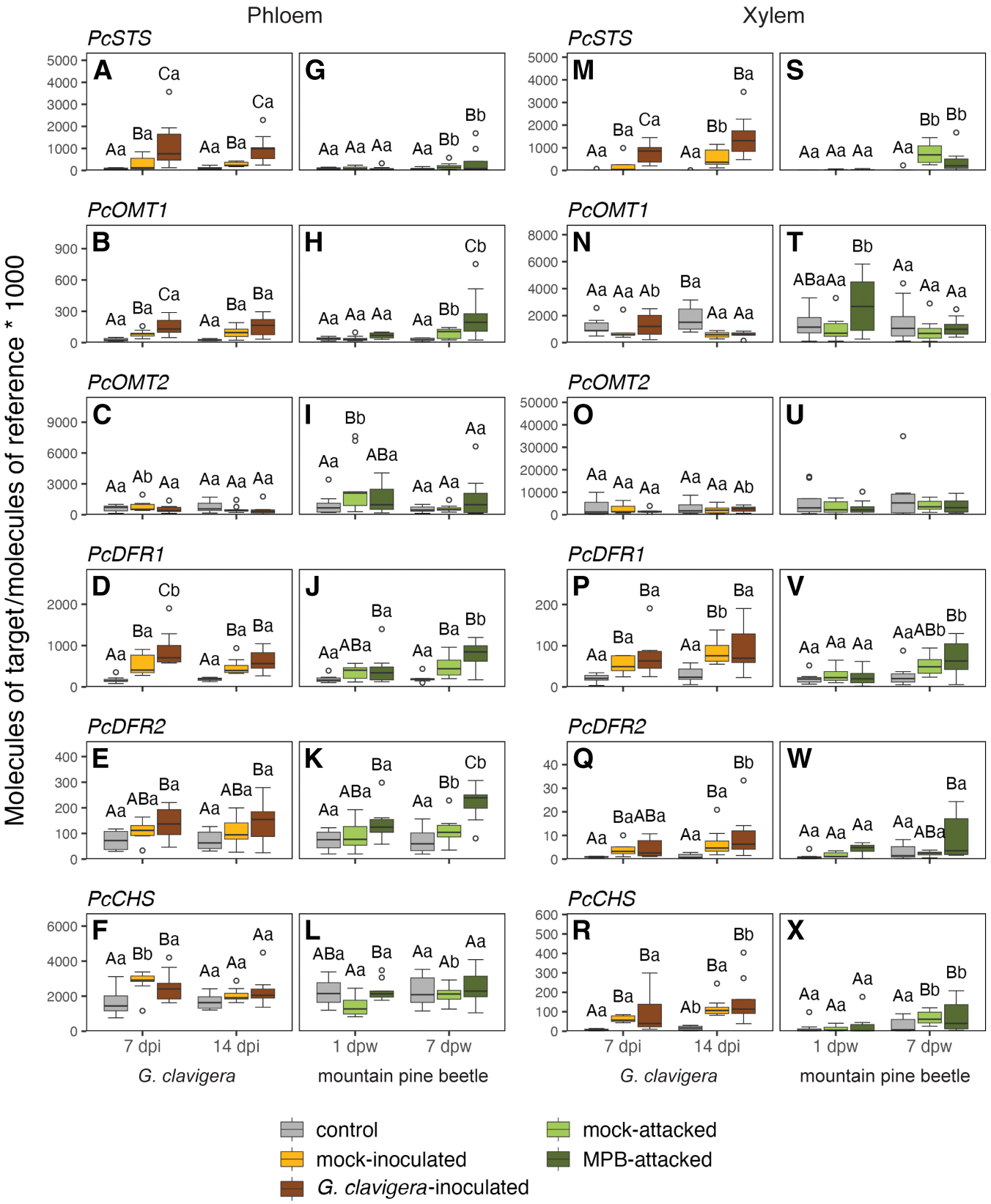

Fig. 4. Expression profiling for genes encoding key enzymes of stilbene and flavonoid biosynthesis in mountain pine beetle (MPB)-attacked and *Grosmannia clavigera*-inoculated lodgepole pines and their corresponding control trees. **A to F,** Secondary phloem of uninoculated or mock- or *G. clavigera*-inoculated trees (Experiment 1 from "Materials and Methods"). **G to L,** Secondary phloem of unattacked or mock- or MPB-attacked trees (Experiment 2 from "Materials and Methods"). **M to R,** Secondary xylem of uninoculated or mock- or *G. clavigera*-inoculated trees (Experiment 1). **S to X,** Secondary xylem of unattacked or mock- or MPB-attacked trees (Experiment 2). Box plot shading is used to denote uninoculated, mock-inoculated, and *G. clavigera*-inoculated treatments for Experiment 1, and unattacked, mock-attacked, and MPB-attacked treatments for Experiment 2. Boxes for each box plot indicate the median value bounded by the 75th (upper) and 25th (lower) quantiles. Box plot whiskers represent boundaries of 1.5 times the interquartile range added to each quantile. Uppercase letters indicate significant differences between treatments within a time point within Experiment 1 or 2, while lowercase letters indicate significant differences between time points within treatments within Experiment 1 or 2 (Tukey-adjusted $P < 0.05$, $n = 8$ to 10 for phloem; $n = 5$ to 8 for xylem).

perature tolerance (Rice et al. 2007). It has been suggested that the phenotypic differences exhibited by these ophiostomatoid species allow for niche partitioning and coexistence of these fungi in this multipartite relationship (Ojeda Alayon et al. 2017). Of the MPB ophiostomatoid fungal symbionts, *G. clavigera* generally is the quickest to colonize its pine host, an indicator of its success as an early colonizer (Addison et al. 2015; Six and Wingfield 2011). This characteristic may allow for denser growth early in the host colonization phase when host defenses are stronger, enabling *G. clavigera* to occupy this niche within this complex microbial community.

Polyphenolic parenchyma cells in secondary phloem fill with phenolic compounds in response to fungal inoculation, contributing to the appearance of lesions (Arango-Velez et al. 2016; Franceschi et al. 2005). Within the time frame of this study, we did not measure significant changes to phloem total phenolic levels in response to mock treatments, *G. clavigera* inoculation, or MPB attack, suggesting that phenolic compounds are not a key defense in bark during mass attack or during the initial phases of *G. clavigera* host colonization. Phenolic compounds also accumulate in cells of the sapwood that constitute lesions (Brignolas et al. 1995; Franceschi et al. 2005). We observed significant increases in xylem total phenolics in response to *G. clavigera* inoculation, MPB attack, and mock treatments, with *G. clavigera* inoculation inducing the greatest accumulation of phenolics. This suggests that phenolic compounds are a more important component of the lodgepole pine induced defense arsenal in sapwood than in bark. Mechanical wounding resulting from mock inoculation and mock attack was sufficient to significantly increase phenolic levels and expression of most phenolic biosynthesis genes profiled in xylem. Together, these results suggest that phenolic defenses constitute a more generalized defense strategy for lodgepole pine in response to attack rather than a defense mechanism specifically induced in response to a particular biotic threat. However, our phenolic profiling and gene expression analyses also demonstrate that *G. clavigera* colonization of sapwood induces greater phenolic defenses than wounding alone. These findings indicate that colonization of the xylem by this necrotrophic pathogen is perceived by host tissues, increasing phenolic biosynthesis. In contrast, MPB attack had a negligible effect on xylem phenolic profiles relative to mock-attacked trees during mass attack, likely reflecting both the limited physical damage that MPB incurred to sapwood and the limited time that MPB-vectored ophiostomatoid fungi have to colonize sapwood during this period.

That lesion development is a key part of conifer response to fungal establishment is further supported by the compounds we identified. The stilbenes pinosylvin and PMME exhibit substantive increases in response to *G. clavigera* inoculation in phloem and xylem, similar to that in Scots pine (*Pinus sylvestris* L.) infected with *H. annosum* (Kovalchuk et al. 2017) and in Eastern white pine (*Pinus strobus* L.) following pine wood nematode (*Bursaphelenchus xylophilus* Nematoda: Aphelenchoididae) infection (Hwang et al. 2021). Stilbenes have been identified as important phytochemicals involved in containment of decay in trees (Hart 1981; Hart and Shrimpton 1979), with many such as pinosylvin exhibiting fungitoxicity (Lee et al. 2005). Another stilbene, resveratrol, is linked to Norway spruce resistance to *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf. (Hammerbacher et al. 2011, 2013). Strains of fungi have been identified that have developed detoxification mechanisms and are even able to use resveratrol as a carbon source (Hammerbacher et al. 2013; Wadke et al. 2016; Zhao et al. 2019). Accumulated phenolics may oxidize, creating oxygen radicals that can trigger host cell death (Appel 1993). This plant-triggered cell death is potentially beneficial to necrotrophic pathogens that rely on release of nutrients from dead host cells (Balint-Kurti 2019). Oxidation of foliar phenolic compounds has been linked with oxidative stress in some herbivores (Barbehenn and Constabel 2011; Barbehenn et al. 2008). Additionally, phenolic oxidation can trigger accumulation of reactive oxygen species, peroxidases, and other host repair mechanisms that alter tissue quality (Almagro et al. 2009). Although it does not appear that phenolics are a significant part of host responses to MPB, changes in phenolic profiles triggered by wounding and fungal colonization may still influence MPB feeding and behavior.

Conclusion

Our objective was to ascertain the contribution of ophiostomatoid fungal symbionts to the capacity of MPB successfully overwhelming host defenses during mass attack. We demonstrated that lodgepole pine synthesizes both JA and ET in response to *G. clavigera*, but not SA, consistent with the classification of *G. clavigera* as a necrotrophic pathogen. This is the first study to show that, like angiosperms, conifers synthesize ET in response to attack by a necrotrophic fungal pathogen. From this finding, we infer that ET is part of the defense signaling pathway invoked by the tree to regulate induced defense responses. Our future studies will explore the interplay between JA and ET in regulating defense gene expression. This finding enabled us to discriminate between the defense signaling pathways triggered by the host tree's perception of herbivore attack versus necrotrophic pathogen attack. We used these hormone signatures to demonstrate that MPB-attacked lodgepole pines do not perceive MPB-vectored ophiostomatoid fungal symbionts like *G. clavigera* during the mass attack phase. Instead, MPB mass attack triggers JA synthesis by lodgepole pine, consistent with host tree perception of attack by an herbivorous insect. These findings suggest that there is insufficient colonization of host tissues by these ophiostomatoid fungi during the mass attack phase to stimulate necrotrophic-triggered defense responses. An alternative explanation is that colonizing fungi vectored by MPB evade detection by the host. Notwithstanding this possibility, this study provides the strongest evidence to date that ophiostomatoid fungi do not contribute to overwhelming host tree defenses during the MPB mass attack phase by stimulating and thus assisting in exhausting the host tree's induced defenses.

Instead, our findings support the model of Six and Wingfield (2011), which posits that MPB has evolved mechanisms for vectoring ophiostomatoid fungi and maintaining these relationships because of the role these fungal symbionts play in supporting MPB colonization, reproduction, and development within the host tree. The pathogenic relationship of *G. clavigera* with the tree host reflects the aggressive colonization of host tissues by this fungus and the mechanisms by which host nutrient resources are acquired by the fungus. *G. clavigera* colonization of lodgepole pine sapwood results in formation of tyloses in water-conducting tracheids, effectively leading to cavitation and reduced hydraulic conductivity (Arango-Velez et al. 2014). These tyloses form before visible hyphal growth of *G. clavigera* in sapwood (Arango-Velez et al. 2014), implying that tylose formation is proactively triggered by the tree to limit fungal colonization. Reduced water conduction resulting from these tyloses contributes to tree mortality, which occurs weeks or months after host defenses have been overwhelmed during MPB mass attack and MPB colonization is established. Collectively, these lines of evidence support the notion that ophiostomatoid fungi do not contribute to overwhelming host defenses during MPB mass attack of lodgepole pine, but ultimately contribute to tree mortality through triggering of the host's myriad defense responses that include strategies to contain fungal growth.

Materials and Methods

Field experiment

The study was conducted in a natural unmanaged stand composed predominantly of even-aged mature lodgepole pine located south of Grande Prairie, AB, Canada (54°27′N, 118°37′W), as outlined in McAllister et al. (2018). Healthy lodgepole pine trees aged approximately 45 to 75 years old with no signs of recent damage or disease were randomly assigned to one of two experiments. Experiment 1 was the *G. clavigera* inoculation experiment: trees inoculated with *G. clavigera*, mock-inoculated, and uninoculated controls. Experiment 2 was the MPB attack experiment: trees designated for exposure to mass attack by MPB, mock-attacked, and uninoculated controls (Supplementary Fig. S1). These experiments are described in more detail in the following sections. Mean diameter at breast height (1.3 m) for all experimental trees was 31 cm (SD = 4 cm). Each tree in this study is a genetically unique individual. Based on our population genetic studies of lodgepole pine in this region (Cullingham et al. 2014; Peery et al. 2022), most individual trees in this study exhibit a low or negligible degree of kinship. We elected to conduct the *G. clavigera* inoculation experiment before the emergence window of MPB to avoid the possibility that *G. clavigera*-inoculated or control trees would be attacked by MPB during the experiment. Accordingly, the *G. clavigera* inoculation experiment was conducted in June 2016, while the MPB attack portion coincided with MPB emergence in July and August 2016.

G. clavigera inoculation experiment

Twenty-nine trees were randomly assigned to one of three treatments: (i) *G. clavigera*-inoculated ($n = 10$), (ii) mock-inoculated ($n = 9$), and (iii) uninoculated control ($n = 10$). Each individual tree was considered to be a biological replicate. For each *G. clavigera*- or mock-inoculated tree, a 1.25-cm round drive punch (Tandy Leather) was used to remove bark plugs to create two horizontal rows of 6 to 8 inoculation holes: one row positioned at 1.9 m above ground level and a second row at 1.3 m. Rows were positioned on opposite sides of each tree and covered half of the tree circumference. For mock-inoculated trees, each hole was filled with a sterile malt extract agar (MEA) plug. For *G. clavigera*-inoculated trees, each hole was filled with a MEA-mycelium plug of *G. clavigera* single-spore isolate M002-12-03-03-UC10G11 SS496 (Roe et al. 2010). This culture, which is available from the UAMH Centre for Global Microfungal Biodiversity (https://www.uamh.ca/index.html), was obtained from a single spore isolated from a *G. clavigera* culture that in turn was derived from a sample of gallery wood collected from a naturally MPB-attacked tree near Sparwood, BC, Canada (49°53′N, 114°54′W). Details of the original *G. clavigera* culture, including taxonomic confirmation by multilocus sequencing and generation of the subsequent single-spore isolate, are described in Roe et al. (2010, 2011b). Bark plugs were replaced and trunks wrapped with stretch wrap (Uline Canada) to cover inoculation sites. Uninoculated control trees were left untouched until sampling.

Secondary xylem and secondary phloem, that is, xylem and phloem tissues generated via the vascular cambium, were sampled from each of the 29 trees at 7 dpi and resampled at 14 dpi. Sampling details and a schematic are included in Supplementary Figure S2. Collected tissues were immediately flash frozen using liquid nitrogen and stored on dry ice until transfer to –80°C for long-term storage.

MPB attack experiment

Twenty-eight trees were assigned to one of three treatments: (i) MPB-attacked ($n = 9$), (ii) mock-attacked ($n = 9$), and (iii) control (untreated; $n = 10$). Each individual tree was considered to be a biological replicate. Bark beetle tree baits designed for *D. ponderosae* (product no. 3122, Synergy Semiochemicals Corporation) were attached to trees designated for the MPB-attack treatment. Baited trees were then monitored twice daily for evidence of MPB attack. To prevent MPB attack of mock-attacked and control trees, trunks were wrapped in 18 × 16 aluminum mesh screen from the base of the tree to 3 m high. Styrofoam was used to fill in gaps at both ends of the wrapped sections between the mesh and trunk. Trees were monitored to ensure that no MPB attacks occurred before sampling.

When all trees designated for the MPB-attacked group had received more than one MPB attack, MPB attacks were simulated on each of the trees in the mock-attacked group using the drive punch procedure described earlier to create the same configuration of two offset horizontal rows of wound sites. The day that treatments were applied to mock-attacked group of trees was designated Day 0, that is, 0 dpw. The first set of secondary phloem and secondary xylem samples was collected the following day from MPB-attacked, mock-attacked, and control untreated trees (Supplementary Fig. S2), and baits were removed from MPB-attacked trees. This first sampling time point is collectively referred to as 1 dpw for all treatments and corresponded to 1 to 7 days following initial detection of MPB attack for all trees in the MPB-attacked treatment group. The second set of samples for the MPB-attack experiment was collected from all three treatment groups at 7 dpw, which corresponded to 7 to 13 days following initial detection of MPB attack. At 7 dpw, all MPB-attacked trees had at least 40 attacks. Each sampling of MPB-attacked trees encompassed 0 to 2 pitch tubes.

Plant hormone analysis

Phytohormones were quantified from six independent biological replicates (trees) at the National Research Council of Canada (Saskatoon, SK, Canada). Quantitative analysis was performed by UPLC-ESI-MS/MS (ACQUITY UPLC, Waters Canada, Mississauga, ON, Canada) using deuterium-labeled internal standards. Analytes used were JA, SA, ACC (Sigma-Aldrich) and JA-Ile (OlChemim Ltd.), while deuterated forms of the hormones used as internal standards included 2,2-d_2-jasmonic acid and 12,12,12-d_3-jasmonic acid isoleucine, synthesized according to Galka et al. (2005). 1-Amino-[2,2,3,3-d_4]ACC (d_4-ACC) and 3,4,5,6-d_4-2-hydroxybenzoic acid standards were purchased from CDN Isotopes. ACC was extracted using a procedure modified from Lulsdorf et al. (2013) and was quantified using a procedure modified from Chauvaux et al. (1997). The procedure for quantification of JA and SA was performed as described in Murmu et al. (2014), while hydrolysis of conjugated salicylic acid (conj. SA) was based on a procedure modified from Malamy et al. (1992).

Phenolic metabolite analysis

Twenty-five microliters of 4 mg/ml gallic acid monohydrate (Sigma-Aldrich) was added to approximately 100 mg of ground frozen tissue before extraction in 1 ml high-performance liquid chromatography (HPLC)-grade methanol by vortexing, then shaking, covered, for 1 h at 4°C. Samples were centrifuged for 15 min at 14,000 rpm, and the supernatant was collected. The extraction was repeated three times. Eight hundred microliters of the pooled supernatant was transferred to a glass vial and evaporated to dryness under a stream of nitrogen gas. Residues were stored at –20°C for no more than 1 month before analysis. Six biological replicates were prepared for each treatment, and three technical replicates were extracted separately from each sample.

Sample residues that had been resuspended in 200 µl HPLC-grade methanol were filtered through an Ultrafree-MC 0.2-µm

polytetrafluoroethylene (PTFE) centrifugal filter (EMD Millipore) and kept at 4°C for no more than 16 h before injection. Ten microliters of filtered extract was injected onto a Luna 5-μm C18(2) 100Å 250 × 4.6 mm column (Phenomenex) maintained at 25°C (± 8°C). Extracts were separated using a gradient modified from Lin and Harnly (2012) on an Agilent 1200 series HPLC. Formic acid (0.2% v/v, in HPLC-grade water; ThermoFisher Scientific) and acetonitrile (ThermoFisher Scientific) represent mobile phases A and B, respectively; mobile phase flow rate was 1 ml/min; gradient profile: 0 to 35 min, 5 to 20% B in A; 35 to 65 min, 20 to 65% B; 65 to 80 min, 79% B; 80 to 90 min, 100% B; 95 to 100 min, 0% B to recover the column. A diode array detector (Agilent G1315C) was used to measure eluting compounds at 270 nm.

Compounds were identified based on similarities in retention time to the following standards: coniferyl alcohol, dihydromyrcetin, taxifolin, pinoresinol, *p*-coumaroyl alcohol, pinosylvin, (±)-dihydrokaemperol, kaempferol 3-glucoside, *trans*-cinnamic acid, *p*-coumaric acid, dihydromyricetin, coniferyl alcohol, matairesinol, quercetin 3-glucoside (all Sigma-Aldrich); and (+)-catechin hydrate (Fisher Scientific). Compounds not matching retention times of standards were labeled alphabetically with the prefix "Unknown P-" or "Unknown X-" to indicate they were identified in phloem or xylem tissues, respectively.

Gene expression analysis

Approximately 150 to 200 mg of frozen ground tissue was used for extraction of total RNA according to Pavy et al. (2008). RNA was quantified with a NanoQuant 200 (Tecan Infinite) and treated with DNase 1 (New England Biolabs). cDNA was synthesized using Invitrogen Superscript III Reverse Transcriptase (ThermoFisher Scientific). Primers used for qRT-PCR (including GenBank accession numbers; Supplementary Table S1) were designed manually or with Geneious Prime 2020.0 (https://www.geneious.com). qRT-PCR reaction conditions and quantification by standard curve are outlined in El Kayal et al. (2011). Six to nine biological replicates and two technical replicates were used for each of the 12 treatments. Target gene data from secondary phloem were normalized to the arithmetic mean of *Eukaryotic translation initiation factor 5A-1* (*PcTIF5A*), *Vacuolar ATP synthase subunit A* (*PcVHA-A*), and *Ubiquitin-activating enzyme 1* (*PcUBA1*). Target gene data from secondary xylem were normalized to the arithmetic mean of *PcTIF5A*, *PcUBA1*, and *Ubiquitin-conjugating enzyme 11* (*PcUBC11*).

Statistical analysis

All statistical analyses were conducted with R v4.2.3 (R Core Team 2023) and RStudio v2021.9.0.351 (RStudio Team 2021), and all plots were generated using ggplot2 v3.4.2 (Wickham 2016) from the tidyverse package v2.0.0 (Wickham et al. 2019), cowplot v1.1.1 (Wilke 2020), and ggpubr v0.6.0 (Kassambara 2023) packages for R. Raw data and R scripts used for analyses are available at https://doi.org/10.5683/SP3/QNUVEZ.

Distinct peaks (not overlapping with other compounds) identified in the HPLC data as consistently present across technical replicates and present in most ($n = 4$) biological replicates within at least one treatment were fit to a NMDS model with Bray-Curtis distances in the vegan package v2.6-4 (Oksanen et al. 2022). Covariance ellipses for each treatment were calculated using the vegan package. Statistical differences between treatments and time points in the NMDS model were determined using the ANOSIM test (Clarke 1993) within the vegan package. Total phenolics were calculated as the sum of compounds included in ordination, based on peak area normalized to the sample dry weight (per mg).

Hormone data (ng hormone/g), reference-normalized gene expression data (normalized transcript abundance), and metabolite data for phenolics identified as significant predictors in the NMDS models (peak area/mg) were fit to generalized linear mixed models (GLMMs) using the lme4 package v1.1-32 (Bates et al. 2015): responding variable ~ treatment * time point + (1 | tree), family = Gamma (link = log). Suitability of reference gene combinations for qRT-PCR was analyzed using Normfinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and GeNorm2 (Vandesompele et al. 2002) using the R package ctrlGene v1.0.1 (Zhong 2019), and by GLMM. GLMMs were determined to meet assumptions of normality and homoscedasticity based on Shapiro-Wilk test (Shapiro and Wilk 1965) and Bartlett's test (Snedecor and Cochran 1989) results, as well as visual assessments of model residual distributions. Post hoc comparisons were made using the emmeans package v1.8.5 (Lenth 2023) to determine significant differences between groups, and letters were assigned using the multcomp package v1.4-15 (Hothorn et al. 2008). Models for some phenolic compounds did not meet assumptions, so post hoc comparisons were not completed. Tables summarizing these comparisons were generated using the gt package v0.9.0 (Iannone et al. 2023).

Acknowledgments

We thank Caroline Whitehouse, Devon Letourneau, and Jeff Atkinson (Government of Alberta) for their assistance in locating a field site; Shucong Nie, Rhiannon Peery, Eden McPeak, Chandra McAllister, Sebastian Lackey, and Natalie Hoffman (University of Alberta) for assistance with fieldwork; and Marion Mayerhofer, Anh Dao, and Kate Stolnikova (University of Alberta) for help with sample preparation.

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