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ARTICLE

Seasonal changes in eastern hemlock (*Tsuga canadensis*) foliar chemistry

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Abstract: Eastern hemlock (*Tsuga canadensis* (L.) Carrière) is an eastern North American conifer threatened by the invasive hemlock woolly adelgid (*Adelges tsugae* Annand). Changes in foliar terpenes and phenolics were evaluated in new (current-year growth) and mature (1-year-old growth) hemlock needles during the growing season and into plant dormancy. From April through September, foliar concentrations of nonvolatile soluble phenolics, condensed tannins, lignin, mono- and sesquiterpenes, α-pinene, camphene, isobornyl acetate, and diterpene resin were quantified. After September, additional analyses of metabolites that continued to differ significantly between new and mature foliage were carried out. Total soluble phenolic and condensed tannin concentrations in new foliage remained low relative to those of mature foliage throughout the growing season and converged in December. Lignin concentration in new foliage converged with that of mature foliage by July. Concentrations of α-pinene, camphene, isobornyl acetate, and diterpene resin in new foliage converged with those of mature foliage within 1 month of budbreak. The convergence of terpene concentrations in new and mature foliage suggests that these metabolites may play a role in herbivore defense during the peak growing season. Conversely, soluble phenolics, including condensed tannins, may defend foliage from herbivory outside of the spring growth period.

Key words: phenology, terpenes, phenolics, conifers, hemlock.

Résumé : La pruche du Canada (*Tsuga canadensis* (L.) Carrière) est un conifère de l'est de l'Amérique du Nord menacé par un insecte invasif, le puceron lanigère de la pruche (*Adelges tsugae* Annand). Les modifications dans les composés phénoliques et les terpènes foliaires ont été évaluées dans les nouvelles aiguilles (pousses de l'année en cours) et les aiguilles matures (pousses âgées d'un an) de la pruche durant la saison de croissance et la période de dormance. La concentration foliaire des composés phénoliques solubles non volatiles, des tannins condensés, des mono- et sesquiterpènes, α-pinène, camphène, acétate d'isobornyle, et des diterpènes résiniques a été quantifiée du mois d'avril au mois de septembre. Après le mois de septembre, d'autres analyses des métabolites qui continuaient à différer significativement dans le nouveau feuillage comparativement au feuillage mature ont été effectuées. La concentration totale de composés phénoliques solubles et de tannins condensés dans le nouveau feuillage demeurait faible comparativement au feuillage mature durant toute la saison de croissance et convergeait en décembre. La concentration de lignine dans le nouveau feuillage convergeait vers celle du feuillage mature vers le mois de juillet. La concentration de α-pinène, de camphène, d'acétate d'isobornyle et de diterpènes résiniques dans le nouveau feuillage convergeait vers celle du feuillage mature à moins d'un mois du débourrement. La convergence de la concentration de terpènes dans le nouveau feuillage et le feuillage mature indique que ces métabolites jouent possiblement un rôle dans la défense contre les herbivores au plus fort de la saison de croissance. À l'inverse, les composés phénoliques solubles, incluant les tannins condensés, pourraient protéger le feuillage contre les herbivores en dehors de la période de croissance printanière. [Traduit par la Rédaction]

Mots-clés : phénologie, terpènes, composés phénoliques, conifères, pruche.

Introduction

Phenology is the study of seasonally varying developmental events driven by environmental cues. Plant phenology is characterized by temporal patterns of growth associated with abiotic factors such as degree-day, amount of transmitted light, and precipitation (Nault 2003; Rathcke and Lacey 1985; Smith 1982). In seasonal climates, leaf growth and development largely occur during a specific time of year (Fenner 1998). In temperate forests of the northern hemisphere, for example, plants leaf out rapidly in the spring to take full advantage of the growing season. Rapid leaf expansion and development are associated with substantial physiological changes during tissue maturation (Koricheva and Barton 2012; Wiggins et al. 2016). In mountain birch (*Betula pubescens* subsp. *czerepanovii* Ehrh.), for example, leaf toughness increases, amino acid levels decrease, and different phenolic compounds (e.g., proanthocyanidins, gallotannins, and flavonoids) show discrete accumulation patterns throughout the growing season (Riipi et al. 2002).

Knowledge of plant leaf phenology is largely derived from deciduous trees (e.g., Fenner 1998; Mauffette and Oechel 1989; Schultz et al. 1982; Wiggins et al. 2016); however, evergreen coni-

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fers make up a significant proportion of global woody plant biomass (Waring and Franklin 1979) and exhibit unique foliar expansion patterns. For instance, loblolly pine (*Pinus taeda* L.) needles have three distinct periods of seasonal development, shifting from winter dormancy to an April–September period of rapid growth and completing needle expansion before the end of the year (Sampson et al. 2003). Conifer foliar chemistry also varies temporally, with shifting amounts of various secondary metabolites that are central to defense against herbivory as needles mature. Expanding jack pine (*Pinus banksiana* Lamb.) needles, for example, have high levels of a *Neodiprion* Rohwer sawfly antifeedant compound that rapidly decreases in concentration as the needles mature (Ikeda et al. 1977). Phenolic compounds and terpenes are the two primary classes of defensive metabolites present in conifer needles (Raffa et al. 2017); their concentrations generally relate to needle age (Mumm and Hilker 2006). Understanding the phenology of secondary metabolites is important, as changes in these compounds can alter plant resistance to abiotic (e.g., drought and excess light) and biotic (e.g., herbivore and pathogen attack) stressors.

Terpenes function in a wide array of ecological processes vital to conifer survival. These include regulating community dynamics through allelopathic inhibition of seed germination, altering rates of soil nutrient cycling and nitrification, and conferring resistance to herbivores and pathogens (Langenheim 1994; Michelozzi 1999; White 1994). For individual terpene compounds in conifer needles, the time–concentration relationship is often nonlinear and can vary throughout the growing season. In newly emerged needles of Scots pine (*Pinus sylvestris* L.), for instance, α-pinene accumulates during the growing season, whereas δ-3-carene levels decrease rapidly after budbreak (Thoss et al. 2007). Emerging Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) needles show similarly dynamic changes in monoterpene levels, with the abundances of α-pinene and β-pinene reversing as young needles expand (Nealis and Nault 2005).

Phenolic compounds in conifers contribute to needle structural development, tissue toughness, and defense against damage by pests and pathogens (Isah 2019). In response to leaf damage, various phenolic compounds are polymerized and covalently bound to cell walls, sealing off sites of infection or injury and strengthening leaf tissue against further damage (Beckman 2000). Phenolics also play a direct role in defense (i.e., toxicity to herbivores and pathogens) and can deter insect herbivore oviposition and affect larval performance (Pasquier-Barre et al. 2000). Phenolics also have well-known roles in abiotic stress resistance, including oxidative stress relief and protection from ultraviolet (UV) radiation (Appel 1993; Isah 2019). These compounds also vary substantially with needle age; Hatcher (1990) found that the immature needles of five conifer species had lower phenolic concentrations than mature needles on the same branch.

Research exploring temporal variation in conifer foliar chemistry has focused on a few species in the pine family (Pinaceae) and excluded other ecologically significant species (see Hatcher 1990; Nault 2003; Nealis and Nault 2005; Nerg et al. 1994; Thoss et al. 2007). Eastern hemlock (*Tsuga canadensis* (L.) Carrière) (hereafter referred to simply as "hemlock") is one such conifer — a long-lived, canopy-dominant species endemic to forests of the eastern United States (U.S.) (Orwig et al. 2008). Hemlock is responsible for important ecological functions, including soil moisture regulation and protecting riparian habitat from lethal temperature extremes (Ellison et al. 2005). It is currently under threat of extirpation by hemlock woolly adelgid (*Adelges tsugae* Annand) (hereafter referred to simply as "adelgid"), an invasive, stylet-feeding insect from Japan that has caused widespread mortality and decline of hemlock (see Dharmadi et al. 2019; McClure 1987) since its introduction nearly 70 years ago (Havill et al. 2006).

Both the sistens and progrediens adelgid generations preferentially feed on mature hemlock foliage (Lagalante et al. 2006), and this is likely driven by chemical differences between new and mature needles. Adelgid resistance in western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and Asian hemlock species, for instance, has been linked to terpene profiles that differ substantially from those of adelgid-susceptible species (Lagalante and Montgomery 2003). Adelgid-resistant eastern hemlock cultivars also have unique foliar terpene profiles (Lagalante et al. 2007). Additionally, resistance to adelgid has been documented in rare individual eastern hemlocks lingering in adelgid-devastated forests (Ingwell and Preisser 2011); chemical analyses found higher terpene concentrations in the needles and twigs of these putatively adelgid-resistant eastern hemlocks compared with adelgid-susceptible controls (McKenzie et al. 2014).

Increasing terpenoid concentrations in expanding hemlock needles have also been correlated with reduced fecundity of elongate hemlock scale (*Fiorinia externa* Ferris), another pest of eastern hemlock introduced from Japan (McClure and Hare 1984). Herbivores of other feeding guilds are also impacted by hemlock's foliar chemical phenology. Hemlock looper (*Lambdina fiscellaria* (Guenée in Boisduval and Guenée, 1858)), an important defoliator of eastern hemlock, feeds preferentially on specific needle age classes, with early-instar larvae feeding on expanding needles and mature larvae shifting to old-growth foliage (Carroll 1999).

Although multiple eastern hemlock studies have explored chemical defense induction in response to adelgid and other herbivores (e.g., Broeckling and Salom 2003; Rigsby et al. 2019), there has been less work addressing constitutive levels of foliar terpenes (but see McKenzie et al. 2014). Only two studies have addressed phenological changes in hemlock terpene emission rates (Lagalante et al. 2006; McClure and Hare 1984). These studies considered only the volatile fraction of hemlock terpenes, and there has been no work addressing nonvolatile terpenes in hemlock. Although volatile terpene emissions are ecologically significant (e.g., antixenosis), as herbivores feed directly on plant tissue, nonvolatile terpenes are more important in direct resistance to herbivore attack (i.e., antibiosis).

At least three studies have identified the foliar terpenes α-pinene, camphene, and isobornyl acetate as the most significant terpenes in hemlock's interactions with adelgid (Lagalante et al. 2006, 2007; Lagalante and Montgomery 2003), which suggests that they play a role in hemlock's defense against adelgid herbivory. We are unaware of any studies examining the role of these terpenes in eastern hemlock's interactions with hemlock looper; however, relationships between these terpenes and other lepidopteran folivores are well documented. Western spruce budworm (*Choristoneura occidentalis* Freeman) resistance in Douglas-fir, for example, is associated with higher foliar concentrations of camphene and isobornyl acetate (Chen et al. 2002). Likewise, hemlock foliar phenolics have also been explored, but only in the context of induced defenses (Rigsby et al. 2019; C.M. Rigsby et al., unpublished data). Increased concentrations of phenolics and lignin bound to cell walls, for example, have been documented in adelgid-infested hemlock foliage without corresponding changes in oxygenated terpenes (Rigsby et al. 2019). Ultimately, within the context of phenology, dynamic changes in nonvolatile phenolic and terpene accumulation in eastern hemlock remain unexplored.

In the present study, changes in nonvolatile phenolics and terpenes were evaluated in both newly produced and mature eastern hemlock foliage through a growing season and into plant dormancy. Temporal changes in the foliar concentration of some major defensive secondary metabolites were outlined, including monoterpene compounds, nonvolatile resin, soluble phenolics, and condensed tannins, in both expanding and mature needles. Foliar concentrations of lignin, a structural and defensive secondary metabolite, were also measured. These data were used to identify when metabolite levels in expanding needles were statistically indistinguishable from those found in mature needles. First, we hypothesized that relatively low levels of lignin in new foliage would be accompanied by relatively greater levels of phenolic and terpene defensive metabolites. Our reasoning was that new foliage, being more attractive to herbivores (Lempa et al. 2001) and having greater fitness value (Heath et al. 2014), would rely on these non-lignin defensive metabolites while actively growing. Second, we hypothesized that concentrations of all metabolites would converge with levels in mature needles by the end of the spring growth period. Studies of the chemical phenology of model conifer species have indicated that terpene and structural metabolite levels in expanding needles become indistinguishable from those of mature needles by the end of the growing season (Hatcher 1990; Nault 2003; Thoss et al. 2007). Specifically for eastern hemlock, volatile terpene concentrations in new foliage have been shown to converge with those of mature foliage by fall leaf-off (Lagalante et al. 2006). In the present study, we provide a first look at eastern hemlock's chemical phenology.

Materials and methods

Common garden

In April 2014, 320 adelgid-free, chemically untreated hemlock saplings (0.5–0.7 m tall; 2 years in age) grown from seed collected in Pennsylvania, U.S., were purchased from Van Pines Nursery (West Olive, Mich., USA). These saplings were planted in five blocks of 64 plants, with ≥1.5 m between each sapling, in the understory of a mixed hardwood stand at the Kingston Wildlife Research Station (South Kingstown, R.I., USA). Plants were protected from both vertebrate and invertebrate herbivory with chicken-wire cages covered in mesh bags (Agribon-15, Johnny's Selected Seeds, Waterville, Maine, USA; 90% light transmission). In early spring 2018, we randomly selected 12 herbivore-free saplings with heights of 1.0–1.2 m from four of the five blocks for the present study. Between two and five plants were sampled from each block. This discrepancy in the number of plants sampled per block existed because we desired to include insect-free trees from each block, but multiple experiments were either occurring or had occurred within this common garden. Several of the trees in this garden also experienced a spruce spider mite (*Oligonychus ununguis* Jacobi) infestation that was avoided by our plant selection. These factors necessitated the selection of our specific plants.

Beginning on 26 April 2018, we removed one 20 cm terminal branch of mature (1-year-old growth) foliage per plant; newly produced foliage was not sampled in April, as budbreak did not occur until mid-May. We returned to each sapling on 31 May 2018 and destructively sampled two 20 cm terminal branches: one of mature foliage and another of expanding (current-year growth) foliage. This protocol meant that we collected one branch per tree during the April sampling and two branches per tree during the May sampling and all subsequent sampling dates. Each terminal branch was excised with pruning shears, wrapped in aluminum foil, placed in a cooler on ice, and brought back to the laboratory, where it was stored at −80 °C until processed. The total sampling time (i.e., from when the first sample was clipped to the time all samples were placed at −80 °C) always took <1 h, samples were immediately immersed in ice as soon as they were sampled, and trees were sampled haphazardly (with regards to the order of sampling) to avoid treatment artifacts due to our sampling procedure. We chose this procedure over clipping individual needles from sampled branches in the field (thus leaving behind a completely defoliated woody stem) based on time. Whereas our chosen method always took <1 h (first branch clip to when all tissue was flash-frozen), in a pilot experiment, we found that carefully removing each individual needle from each sampled branch in the field more than tripled the time between tissue removal and flash-freezing. Branch clippings occurred monthly on the following dates: 26 April, 31 May, 28 June, 26 July, 30 August, and 27 September 2018. After this final date, we continued to assay only secondary metabolites that differed significantly between new and mature foliage. We added two additional sampling dates (19 December 2018 and 28 January 2019) on which we quantified these remaining metabolites. By the end the experiment, our cuttings had removed <5% of the total foliage from each sapling.

Tissue preparation

Needles were removed from each branch sample and ground to a powder in liquid nitrogen using a mortar and pestle. New foliage was processed separately from mature foliage. The powder was partitioned into three tubes: 100 ± 5 mg in a 1.5 mL microtube for the phenolic analyses (total soluble phenolics, condensed tannins, and lignin), 100 ± 5 mg in another 1.5 mL microtube for gas chromatography – flame ionization detector (GC–FID) analysis of major mono- and sesquiterpenes, and 1 ± 0.05 g in a 15 mL Falcon tube (BD Biosciences, Bedford, Mass., USA) for nonvolatile resin analysis. Tubes were stored at −30 °C until analysis, and analyses were conducted within 2 days of tissue grinding.

Total soluble phenolics

Soluble phenolics were extracted in methanol of high-performance liquid chromatography (HPLC) grade, and total soluble phenolic levels were quantified similarly to the method of Rigsby et al. (2019), via Folin assay, with minor enhancements for optimization. The 25 μL extract was first diluted in 75 μL of methanol, and 500 μL of water were then added, followed by 40 μL of Folin–Ciocalteu reagent (Sigma–Aldrich, St. Louis, Mo., USA). Tubes were incubated at room temperature for 10 min, then 40 μL of 1 M sodium bicarbonate ($NaHCO_3$) were added, and the tubes were incubated at room temperature for 1 h. Absorbance was then quantified at a wavelength of 725 nm using a SpectraMax M2 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, Calif., USA) and Greiner UV-Star 96-well plates (Greiner Bio-One, Monroe, N.C., USA). In place of gallic acid, chlorogenic acid (Sigma–Aldrich) was used to generate a standard curve, and phenolic concentration was expressed as chlorogenic acid equivalents (in milligrams per gram of fresh weight (FW)). Condensed tannin concentration was also quantified as per Rigsby et al. (2019), by incubating methanol extracts (250 μL) with 750 μL of a 95:5 butanol:hydrochloric acid (HCl) solution for 3 h at 95 °C. After cooling, absorbance at 550 nm was quantified (expressed as optical density at 550 nm (OD_{550}) per gram FW) using a Turner SP-830 cuvette spectrophotometer (Barnstead Thermolyne, Ramsey, Minn., USA) and plastic cuvettes.

Although Appel et al. (2001) raised concerns regarding the use of Folin assays in quantifying temporal or species variation in foliar phenolic levels in ecological studies, recent work by our research group has revealed that derivatives of chlorogenic acid dominate (≥70%) the profile of soluble phenolics in hemlock foliage (C.M. Rigsby et al., unpublished data) throughout the year. We conducted preliminary experiments showing that total soluble phenolic content estimated with the spectrophotometric procedure previously described, using chlorogenic acid as standard, is highly correlated with phenolics quantified via HPLC–$UV_{280\ nm}$ (coefficient of determination (R^2) ≥ 0.88; C.M. Rigsby et al., unpublished data), regardless of sampling month or tissue age. This background work led us to conclude that this more cost- and time-effective Folin spectrophotometric assay provided reasonable estimations of foliar soluble phenolic concentrations in lieu of phenolics quantified via HPLC. We acknowledge, however, that despite this preliminary work and the fact that we could obtain a close estimation of HPLC-quantified phenolics with Folin-quantified phenolics, the Folin method could, and likely did, miss quantitative variation in the contents of phenolic compounds.

Lignin

Lignin levels were quantified as per Villari et al. (2012). Briefly, the leftover pellet from the soluble phenolic extraction was washed twice with methanol, allowed to air-dry overnight, then resuspended in 400 μL of 1 M sodium hydroxide (NaOH) and incubated for 24 h at 40 °C. The homogenate was acidified with 200 μL of 1.5 M formic acid, and 400 μL of methanol were added. The tubes were centrifuged at 16 000*g* for 5 min, and the supernatant was discarded. Pellets were then washed twice with 1 mL of methanol, and 1 mL of 2 M HCl was added to the tubes, followed by 250 μL of thioglycolic acid. The tubes were incubated for 4 h at 85 °C. Once cooled to room temperature, tubes were centrifuged at 16 000*g* for 5 min, and the supernatant was discarded. Pellets were then washed once with 1 mL of water, and thioglycolic acid – lignin pellets were extracted overnight in 1 mL of 1 M NaOH. The tubes were centrifuged at 16 000*g* for 5 min, and the supernatant was saved; this extraction step was repeated, and supernatants were combined. Extracts were acidified with 300 μL of concentrated HCl and incubated at room temperature for 4 h. Tubes were then centrifuged at 20 000*g* for 5 min, supernatants were discarded, and pellets were allowed to dry overnight at 40 °C. The following day, pellets were resuspended in 1 mL of 1 M NaOH. The absorbance of 20-fold dilutions (with 0.5 M NaOH) at 280 nm was quantified using the SpectraMax microplate reader and Greiner UV-Star 96-well plates against a standard curve of spruce lignin (expressed as milligrams per gram FW).

Mono- and sesquiterpenes

α-Pinene, camphene, and isobornyl acetate (Sigma–Aldrich) were extracted and quantified via GC–FID similarly to a method previously used by our research group (C.M. Rigsby et al., unpublished data), with minor adjustments in the oven program to optimize rapid quantification of these three compounds. These are the three dominant terpene species of hemlock foliage and constitute ≥75% of all terpenes identified in eastern hemlock (Broeckling and Salom 2003; C.M. Rigsby et al., unpublished data). Foliar terpenes were extracted in 700 μL of *n*-hexane containing 1 μL·mL^{-1} *m*-xylene as an internal standard by sonicating homogenates for 10 min in an ice bath. Tubes were then vortexed for 10 s, and the supernatant from the 5 min centrifugation at 20 000*g* and 0 °C was transferred to a 2 mL glass autosampler vial, capped with a screw cap coated in polytetrafluoroethylene (PTFE), and stored at –30 °C until injected into the GC (within 48 h). The instrument, settings, gases, column, injection volume, and external standards previously used by our research group (C.M. Rigsby et al., unpublished data) were used here. Mono- and sesquiterpene identification and quantification took place using a Shimadzu GC-2010 Plus gas chromatograph equipped with an AOC-20i autosampler and a flame ionization detector (GC–FID) (Shimadzu, Kyoto, Japan). Nitrogen was used as the carrier gas at a flow rate of 1.0 mL·min^{-1} in an HP-5ms column (30 m × 0.25 mm internal diameter; 0.25 μm film thickness; Agilent Technologies, Santa Clara, Calif., USA). Terpene extract (2 μL) was injected using a split flow ratio of 30:1, and the injector and detector temperatures were set to 260 °C and 300 °C, respectively. The adjusted oven program was as follows: 40 °C for 5 min, increased by 5 °C·min^{-1} to 225 °C, increased by 25 °C·min^{-1} to 280 °C, and held at 280 °C for 5 min (total run time = 49.2 min). Peaks were matched to external standards based on retention times, and tissue amounts of terpenes (in micrograms per gram FW) were estimated using three-point standard curves of standards (R^2 > 0.99).

Diterpene resin

Nonvolatile diterpene resin concentration was estimated gravimetrically using standard techniques for estimating the nonvolatile resin concentrations of pine foliage, which is highly correlated with diterpene resin acid concentration measured via gas chromatography – mass spectrometry (GC–MS) (Moreira et al. 2016). Briefly, 1 g of tissue powder was extracted in 3 mL of *n*-hexane for 10 min in a sonicator, then centrifuged at 4000*g*, and the supernatant was transferred to a 15 mL Falcon tube. This extraction procedure was repeated twice, and the supernatants were combined in a preweighed 15 mL Falcon tube. Uncapped tubes were then placed in a fume hood, and the solvent was evaporated to dryness (approximately 4 days) before reweighing. The before–after difference in tube mass was considered the mass of nonvolatile resin (expressed as milligrams per gram FW). It is worth noting that hemlock diterpenes have not been characterized; therefore, we were unable to directly relate diterpene content quantified by GC–MS with content quantified by this gravimetric method, as has been shown and used in many species of pine (e.g., Moreira et al. 2016). Our *n*-hexane extracts would certainly have extracted other nonpolar, nonvolatile metabolites aside from diterpenes, and these could have contributed to the change in tube masses. Our results should, therefore, be interpreted cautiously until hemlock diterpenes can be characterized.

Statistical analysis

R software version 3.5.0 was used for all analyses (R Core Team 2018). Secondary metabolite concentrations in new and mature foliage were analyzed via linear mixed-effects models using the lme4 package (Bates et al. 2015). Foliage age, month, and their interactions were treated as fixed effects, and block and tree were treated as random effects. A type III analysis of variance (ANOVA) was used to evaluate each model term for significance. Month-specific differences in metabolite concentrations in new and mature foliage were analyzed for significance using difflsmeans in the lmerTest package (Kuznetsova et al. 2017), and the Holm–Bonferroni procedure for controlling family-wise error rate was used to correct for multiple comparisons.

Results

Phenolics

Phenolic concentration differed significantly between new and mature foliage for all measured compounds (all $F_{[1,1]}$ > 183.17, P < 0.05; Figs. 1A–1C). Phenolic levels also varied by month (all $F_{[1,7]}$ > 77.82, P < 0.001), and there was a significant month × foliage age interaction (all $F_{[1,6]}$ > 7.47, P < 0.001). Specifically, total soluble phenolic and condensed tannin concentrations were lower in new foliage than in mature foliage throughout the growing season (May–September) (means separation test, both P < 0.001; Figs. 1A and 1B). It took until December for concentrations of total soluble phenolics and condensed tannins in new and mature foliage to converge (means separation test, both P > 0.05). At that point, mean total soluble phenolic concentrations in new and mature foliage reached 59.6 mg·g^{-1} FW and 73.5 mg·g^{-1} FW, respectively, and mean concentrations of condensed tannins in new and mature foliage reached 11.7 mg·g^{-1} FW and 14.4 mg·g^{-1} FW, respectively. Conversely, lignin levels in new foliage rapidly increased at the start of the growing season and became indistinguishable from those of mature foliage by July, with mean concentrations reaching 11.6 mg·g^{-1} FW in new foliage and 14.0 mg·g^{-1} FW in mature foliage (means separation test, P = 0.097; Fig. 1C).

Terpenes

In contrast with phenolics, terpene concentrations in new foliage rapidly converged with those of mature foliage. Statistically similar levels of quantified terpenes in new and mature foliage occurred within 1 month of budbreak (means separation test, all P > 0.05; Figs. 2A–2D). By June, for instance, mean α-pinene concentrations in new and mature foliage were 0.71 mg·g^{-1} FW and 0.75 mg·g^{-1} FW, respectively. Terpene levels varied by month (all $F_{[1,5]}$ > 154.38, P < 0.001), and there was a significant month × foliage age interaction (all $F_{[1,4]}$ > 61.04, P < 0.001). Levels of camphene, α-pinene, and isobornyl acetate were higher, and resin concentration was lower, in new foliage than in mature foliage in September (means separation test, all P < 0.05; Figs. 2A–2D). Mean resin concentrations in new and mature foliage in September, for instance, were 50.4 mg·g^{-1} FW and 56.7 mg·g^{-1} FW, respectively. Conversely, mean levels of isobornyl acetate in new and mature

Fig. 1. Mean concentrations of (A) total soluble phenolics, (B) condensed tannin, and (C) lignin in new (current-year growth) and mature (1-year-old growth) eastern hemlock foliage throughout the growing season. New foliage was not produced until after the April sampling date. Values on the *x* axis represent month and year (e.g., 4/18 represents April 2018). Asterisks (*) represent significant differences in metabolite concentrations between new and mature foliage; vertical lines represent ±1 standard error (SE). Additional samples were analyzed in December and January to identify when phenolic and condensed tannin levels in new foliage converged with the levels in mature foliage. FW, fresh weight.

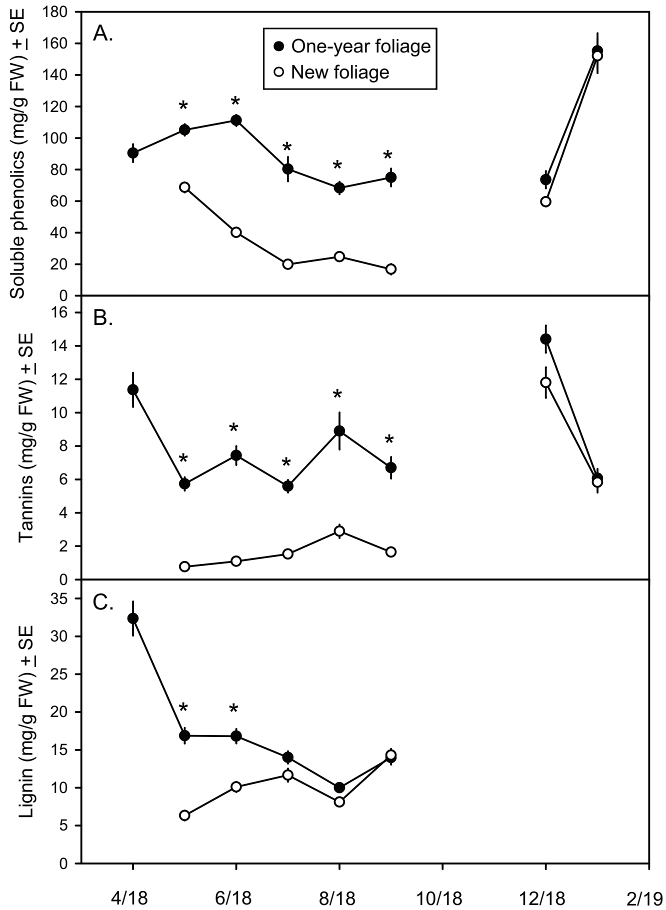
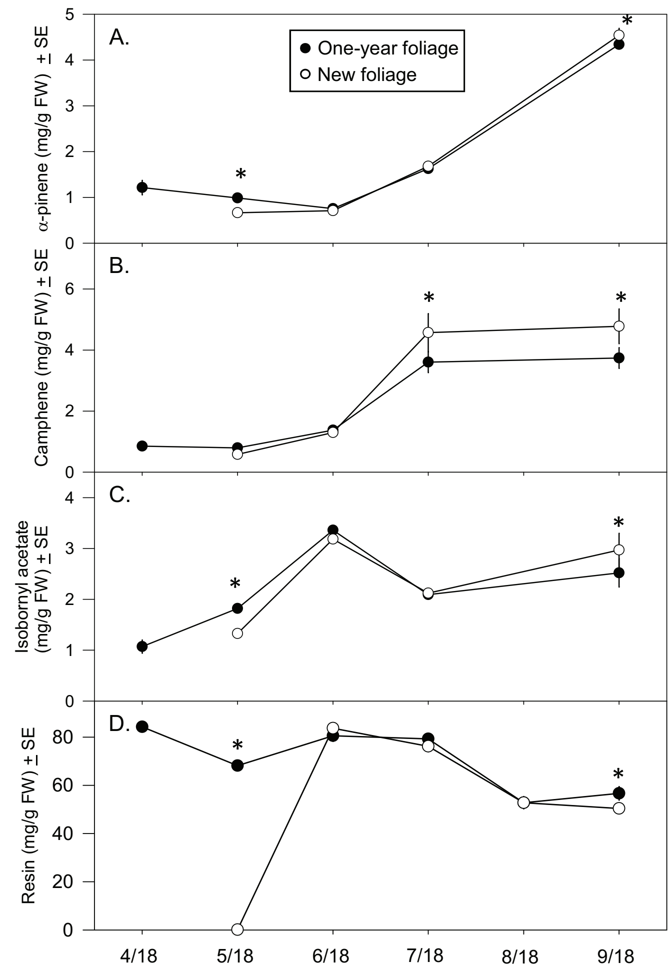


Fig. 2. Mean concentrations of (A) α-pinene, (B) camphene, (C) isobornyl acetate, and (D) resin in new and mature eastern hemlock foliage throughout the growing season. New foliage was not produced until after the April sampling date. Asterisks (*) represent significant differences in metabolite concentrations between new and mature foliage; vertical lines represent ±1 SE.



foliage were 2.9 mg·g^{-1} FW and 2.5 mg·g^{-1} FW, respectively, and mean camphene levels in new and mature foliage reached 4.7 mg·g^{-1} FW and 3.7 mg·g^{-1} FW, respectively.

Discussion

New foliage contained significantly lower concentrations of total soluble phenolics, condensed tannins, and lignin than those of mature hemlock foliage (Figs. 1A–1C). Phenolic concentrations also varied by month, and the monthly change in the concentration of each compound was different between new and mature foliage. Total soluble phenolic and condensed tannin concentrations were lower in new foliage than in mature foliage throughout the growing season (May–September), resulting in the rejection of our hypothesis that concentrations of all metabolites would converge with levels in mature needles by the end of the spring growth period (Figs. 1A and 1B). Moreover, concentrations of both classes of compounds in new foliage did not converge with levels present in mature foliage until December (Figs. 1A and 1B). Concentrations of the structural metabolite lignin in new foliage became indistinguishable from levels in mature foliage as early as July (Fig. 1C). The incidence of low levels of lignin with similarly low levels of both phenolics and terpenes did not support our first hypothesis that relatively low levels of lignin in new foliage would be accompanied by relatively greater levels of phenolic and terpene defensive metabolites. Instead, concentrations of the different metabolite groups measured in new foliage remained low relative to those of mature foliage until the new foliage lignified.

α-Pinene and isobornyl acetate levels in new foliage were significantly lower than those of mature foliage immediately after budbreak in May, increased to convergence by June, and exceeded those of mature foliage in September (Figs. 2A and 2C). Resin concentration in new foliage was also initially low compared with that of mature foliage but became indistinguishable from levels in mature foliage by June (Fig. 2D). Camphene concentrations in new and mature foliage were not significantly different immediately after budbreak, and in September, camphene levels were higher in new foliage than in mature foliage (Fig. 2B).

This broad pattern of early-season convergence of terpene concentrations in new and mature foliage suggests that this class of secondary metabolites may play a significant role in eastern hemlock's defense against herbivores that are active at the beginning of — and during — the peak growing season. Conversely, soluble phenolics, including condensed tannins, may be responsible for defending hemlock foliage from herbivory that occurs outside of the spring growth period. It is important to note, however, that the phenolic content of most plants varies not only in association with tissue age and (or) herbivore activity, but also according to other stressors such as growing conditions (reviewed by Levin 1971). For example, latitudinal variation in temperature, growing season duration, and sun exposure has a strong effect on the total phenolic concentration of Scots pine needles (Nerg et al. 1994). Thus, although the apparent seasonal trade-off between terpene and phenolic concentrations observed in this study may be an element of hemlock's antiherbivore defense complex, it may also be affected by seasonal variation in certain biophysical factors. Future studies should evaluate the extent to which seasonal herbivores, biophysical factors, and their interactions drive these patterns.

Phenolics

Total soluble phenolic and condensed tannin concentrations in new foliage did not converge with levels in mature foliage until December (Figs. 1A and 1B). Total soluble phenolic concentration in new foliage was relatively high immediately after budbreak but declined throughout the growing season (Fig. 1A). Like terpenes, phenolics are a class of secondary metabolites that play a significant role in conifer defense against insect herbivory (Mumm and Hilker 2006). Because hemlock retains its needles throughout the year, it resumes photosynthesis well before new foliage emerges (Hadley 2000). As a result, hemlock buds are nutrient rich (Wilson et al. 2018), and elevated levels of certain soluble phenolics may be necessary to protect these tissues from early-spring folivores. Our finding of elevated early-season phenolic concentrations is consistent with work by Hatcher (1990) documenting high phenolic concentrations in western hemlock needles at budbreak, followed by decreasing concentrations as needles expanded during the growing season. The subsequently low levels of total soluble phenolics and condensed tannins observed in expanding hemlock needles between June and September may be necessary for hemlock to avoid autotoxicity. At least one study has suggested that compartmentalization problems prevent the accumulation of high amounts of tannins in new conifer foliage (Horner 1988). Evidence of low tannin concentration in expanding needles of both Scots pine (Watt 1987) and Douglas-fir (Horner 1988) during peak growth, followed by convergence with levels in mature needles for both trees after the growing season, is also consistent with this hypothesis.

Condensed tannin concentration and total soluble phenolic concentration in new foliage became indistinguishable from those of mature foliage in December (Figs. 1A and 1B). This late-season convergence indicates that these metabolites may play a role in hemlock's defense against later-arriving herbivores. In addition to phenolics directly deterring phytophagous insects, these compounds have also been shown to affect oviposition preference in certain pine-defoliating insects (Leather et al. 1987; Pasquier-Barre et al. 2000). Oviposition on plants unsuitable for larval development reduces insect fitness, and elevated foliar tannins have been shown to reduce oviposition rates (Leather et al. 1987). One destructive pest of eastern hemlock, the hemlock looper, initiates oviposition in late September and into October (Dobesberger 1989); our data suggest that during this period, newly produced hemlock needles begin to accumulate condensed tannins and other soluble phenolics. Given the shared evolutionary history of eastern hemlock and hemlock looper (Bhiry and Filion 1996), the fall or winter increase in foliar condensed tannin and total soluble phenolic concentrations may have some significance for this interaction. Adelgid also feeds on hemlock tissue from October through April. Although the two species have not coevolved, adelgid feeding has been shown to dramatically increase the concentrations of both condensed tannin (Rigsby et al. 2019) and soluble phenolics (Pezet and Elkinton 2014; Rigsby et al. 2019) in hemlock foliage. This further suggests that condensed tannins and other soluble phenolics may be an important aspect of eastern hemlock defense against late-season herbivores.

Concentrations of the structural metabolite lignin in new foliage reached levels present in mature foliage after terpenes in July (Fig. 1C). Contrary to our first hypothesis, lignin concentration in new foliage was not exceeded by any individual defensive metabolite before it reached levels found in mature foliage. Furthermore, condensed tannins and total soluble phenolics in new foliage did not reach levels found in mature foliage until well after the growing season had ended. This did not support our second hypothesis that concentrations of all metabolites would converge with levels in mature needles by the end of the spring growth period and suggests that the latter two groups of metabolites may be important against herbivores that are active outside of the spring growth period.

Terpenes

Terpene concentration in expanding needles converged with levels present in mature needles within 1 month of May budbreak (Figs. 2A–2D). We found that α-pinene and isobornyl acetate concentrations in new foliage were significantly lower than those of mature foliage in May, increased to convergence by June, and were higher than those of mature foliage in September (Figs. 2A and 2C). Similarly, resin concentration in new foliage was initially lower than that of mature foliage but reached that of mature foliage by June. Camphene concentration in new foliage was indistinguishable from that of mature foliage immediately after budbreak; in September, it was higher in new foliage than in mature foliage (Fig. 2B). The fact that terpene concentration in expanding needles rapidly converged with levels in mature needles suggests that terpenes play an important role in defense against early-season herbivores (Mumm and Hilker 2006). Elevated levels of camphene and isobornyl acetate in Douglas-fir needles, for example, have been linked to western spruce budworm resistance (Chen et al. 2002), and both terpenes are highly toxic to the insect (Zou and Cates 1997). Newly produced and expanding conifer needles are softer than mature needles (e.g., Hatcher 1990); conifers compensate for lower structural defense with toxic secondary metabolites (Mumm and Hilker 2006). As new foliage on hemlock does not fully lignify until at least July (Fig. 1C), it appears likely that terpenes fill this role.

Conclusions

In this study, we provide a first look at eastern hemlock's chemical phenology. Broadly, expanding hemlock needles had low concentrations of soluble phenolics and condensed tannins throughout the growing season and into plant dormancy, becoming indistinguishable from levels in mature foliage by December (Figs. 1A and 1B). Conversely, concentrations of the structural metabolite lignin rapidly increased in new foliage and converged with levels in mature foliage by July (Fig. 1C). Similarly, levels of α-pinene, camphene, isobornyl acetate, and resin in new foliage converged with their respective levels in mature foliage within 1 month of May budbreak (Figs. 2A–2D). Rapid convergence of terpene concentrations in new and mature foliage may implicate this class of secondary metabolites in eastern hemlock's defense against early-season herbivores, whereas soluble phenolics and condensed tannins may act in hemlock's defense against herbivores that feed during plant dormancy.

It is important to note, however, that there are limitations associated with the bulk-analysis approach to measuring plant secondary metabolites, and more refined tests will be needed to support ecological connections between the chemical patterns that we observed and the activity of hemlock herbivores. In addition to concentration, for example, the composition of terpenes and phenolics in foliage can change throughout the growing season (reviewed by Iason et al. 2012). In expanding white birch (*Betula papyrifera* Marshall) leaves, for instance, hydrolyzable tannins show distinct seasonal patterns, with certain individual tannins having an inverse time–concentration relationship (Salminen et al. 2001). Several studies have also documented this pattern for individual terpenes in conifer needles; their relative concentrations are often opposed and can vary as needles expand (see Nealis and Nault 2005; Thoss et al. 2007). Broad phenologic trends in hemlock's foliar phenolic and terpene concentrations, although useful, cannot elucidate the role of individual chemical species in this tree's herbivore defense complex. In particular, research that builds on and extends our findings by addressing the important roles of individual phenolics in mediating plant–insect interactions in this system is critical.

Since the introduction of adelgid nearly a century ago (Havill et al. 2006), there has been extensive mortality and decline of eastern hemlocks throughout eastern U.S. forests (Eschtruth et al. 2006; Orwig et al. 2002; Preisser et al. 2008). Hemlock is a late-successional species that has adapted to grow in cool understory microclimates (Hadley 2000); the loss of most canopy-dominant hemlocks in this region has inhibited seedling recruitment (Ingwell et al. 2012; Orwig and Foster 1998; Orwig et al. 2002) and virtually eliminated hemlock sapling regeneration (Preisser et al. 2011). Hemlock-associated forests are now characterized by lower densities of overstory deciduous trees, novel understory vegetation communities (Ingwell et al. 2012), and significantly reduced soil moisture and ratios of carbon to nitrogen (Orwig et al. 2008). As hemlock continues to be threatened with extirpation by adelgid, it will be important to understand the potency of individual secondary metabolites in hemlock's interactions with herbivores, in addition to understanding broad seasonal trends, to effectively conserve this species. Putatively adelgid-resistant eastern hemlocks, for instance, sustain lower adelgid densities (Ingwell and Preisser 2011), and this is likely due to their unique terpene chemistry (McKenzie et al. 2014). However, the terpene type(s) responsible for inhibiting adelgid population growth remains unknown. Such a chemical marker could provide the means to identify candidate eastern hemlocks for adelgid-resistance breeding programs and reforestation efforts aimed at not only restoring eastern hemlocks, but also maintaining the vital, broadscale ecosystem functions that this tree species provides.

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References

Appel, H.M. 1993. Phenolics in ecological interactions: the importance of oxidation. J. Chem. Ecol. **19**(7): 1521–1552. doi:10.1007/BF00984895. PMID:24249181.

Appel, H.M., Govenor, H.L., D'Ascenzo, M., Siska, E., and Schultz, J.C. 2001. Limitations of Folin assays of foliar phenolics in ecological studies. J. Chem. Ecol. **27**(4): 761–778. doi:10.1023/A:1010306103643. PMID:11446299.

Bates, D., Maechler, M., Bolker, B., and Walker, S. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. **67**(1): 1–48. doi:10.18637/jss.v067.i01.

Beckman, C.H. 2000. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiol. Mol. Plant Pathol. **57**(3): 101–110. doi:10.1006/pmpp.2000.0287.

Bhiry, N., and Filion, L. 1996. Mid-Holocene hemlock decline in eastern North America linked with phytophagous insect activity. Quat. Res. **45**(3): 312–320. doi:10.1006/qres.1996.0032.

Broeckling, C.D., and Salom, S.M. 2003. Volatile emissions of eastern hemlock, *Tsuga canadensis*, and the influence of hemlock woolly adelgid. Phytochemistry, **62**(2): 175–180. doi:10.1016/s0031-9422(02)00518-6. PMID:12482453.

Carroll, A.L. 1999. Physiological adaptation to temporal variation in conifer foliage by a caterpillar. Can. Entomol. **131**(5): 659–669. doi:10.4039/Ent131659-5.

Chen, Z., Kolb, T.E., and Clancy, K.M. 2002. The role of monoterpenes in resistance of Douglas fir to western spruce budworm defoliation. J. Chem. Ecol. **28**(5): 897–920. doi:10.1023/A:1015297315104. PMID:12049230.

Dharmadi, S.N., Elliott, K.J., and Miniat, C.F. 2019. Lack of forest tree seedling recruitment and enhanced tree and shrub growth characterizes post-*Tsuga canadensis* mortality forests in the southern Appalachians. For. Ecol. Manage. **440**: 122–130. doi:10.1016/j.foreco.2019.03.024.

Dobesberger, E.J. 1989. A sequential decision plan for the management of the eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Lepidoptera: Geometridae), in Newfoundland. Can. J. For. Res. **19**(7): 911–916. doi:10.1139/x89-138.

Ellison, A.M., Bank, M.S., Clinton, B.D., Colburn, E.A., Elliott, K., Ford, C.R., et al. 2005. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. Front. Ecol. Environ. **3**(9): 479–486. doi:10.1890/1540-9295(2005)003[0479:LOFSCF]2.0.CO;2.

Eschtruth, A.K., Cleavitt, N.L., Battles, J.J., Evans, R.A., and Fahey, T.J. 2006. Vegetation dynamics in declining eastern hemlock stands: 9 years of forest response to hemlock woolly adelgid infestation. Can. J. For. Res. **36**(6): 1435–1450. doi:10.1139/x06-050.

Fenner, M. 1998. The phenology of growth and reproduction in plants. Perspect. Plant Ecol. Evol. Syst. **1**(1): 78–91. doi:10.1078/1433-8319-00053.

Hadley, J.L. 2000. Understory microclimate and photosynthetic response of saplings in an old-growth eastern hemlock (*Tsuga canadensis* L.) forest. Ecoscience, **7**(1): 66–72. doi:10.1080/11956860.2000.11682573.

Hatcher, P.E. 1990. Seasonal and age-related variation in the needle quality of five conifer species. Oecologia, **85**(2): 200–212. doi:10.1007/BF00319402. PMID:28312556.

Havill, N.P., Montgomery, M.E., Yu, G., Shiyake, S., and Caccone, A. 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Ann. Entomol. Soc. Am. **99**(2): 195–203. doi:10.1603/0013-8746(2006)099[0195:MDFHWA]2.0.CO;2.

Heath, J.J., Kessler, A., Woebbe, E., Cipollini, D., and Stireman, J.O. 2014. Exploring plant defense theory in tall goldenrod, *Solidago altissima*. New Phytol. **202**(4): 1357–1370. doi:10.1111/nph.12755. PMID:24611577.

Horner, J.D. 1988. Astringency of Douglas-fir foliage in relation to phenology and xylem pressure potential. J. Chem. Ecol. **14**(4): 1227–1237. doi:10.1007/BF01019348. PMID:24276206.

Iason, G.R., Dicke, M., and Hartley, S.E. (*Editors*). 2012. The ecology of plant secondary metabolites: from genes to global processes. Cambridge University Press, Cambridge, UK. doi:10.1017/CBO9780511675751.

Ikeda, T., Matsumura, F., and Benjamin, D.M. 1977. Mechanism of feeding discrimination between matured and juvenile foliage by two species of pine sawflies. J. Chem. Ecol. **3**(6): 677–694. doi:10.1007/BF00988067.

Ingwell, L.L., and Preisser, E.L. 2011. Using citizen science programs to identify host resistance in pest-invaded forests. Conserv. Biol. **25**(1): 182–188. doi:10.1111/j.1523-1739.2010.01567.x. PMID:20735452.

Ingwell, L.L., Miller-Pierce, M., Trotter, R.T., III, and Preisser, E.L. 2012. Vegetation and invertebrate community response to eastern hemlock decline in southern New England. Northeast. Nat. **19**(4): 541–558. doi:10.1656/045.019.0402.

Isah, T. 2019. Stress and defense responses in plant secondary metabolites production. Biol. Res. **52**(1): 39. doi:10.1186/s40659-019-0246-3. PMID:31358053.

Koricheva, J., and Barton, K.E. 2012. Temporal changes in plant secondary metabolite production. *In* The ecology of plant secondary metabolites: from genes to global processes. *Edited by* G.R. Iason, M. Dicke, and S.E. Hartley. Cambridge University Press, Cambridge, UK. pp. 34–55. doi:10.1017/CBO9780511675751.004.

Kuznetsova, A., Brockhoff, P.B., and Christensen, R.H.B. 2017. lmerTest package: tests in linear mixed-effects models. J. Stat. Softw. **82**(13): 1–26. doi:10.18637/jss.v082.i13.

Lagalante, A.F., and Montgomery, M.E. 2003. Analysis of terpenoids from hemlock (*Tsuga*) species by solid-phase microextraction/gas chromatography/ion-trap mass spectrometry. J. Agric. Food Chem. **51**(8): 2115–2120. doi:10.1021/jf021028s. PMID:12670144.

Lagalante, A.F., Lewis, N., Montgomery, M.E., and Shields, K.S. 2006. Temporal and spatial variation of terpenoids in eastern hemlock (*Tsuga canadensis*) in relation to feeding by *Adelges tsugae*. J. Chem. Ecol. **32**(11): 2389–2403. doi:10.1007/s10886-006-9166-0. PMID:17078000.

Lagalante, A.F., Montgomery, M.E., Calvosa, F.C., and Mirzabeigi, M.N. 2007. Characterization of terpenoid volatiles from cultivars of eastern hemlock (*Tsuga canadensis*). J. Agric. Food Chem. **55**(26): 10850–10856. doi:10.1021/jf071947o. PMID:18052093.

Langenheim, J.H. 1994. Higher plant terpenoids: a phytocentric overview of their ecological roles. J. Chem. Ecol. **20**(6): 1223–1280. doi:10.1007/BF02059809. PMID:24242340.

Leather, S.R., Watt, A.D., and Forrest, G.I. 1987. Insect-induced chemical changes in young lodgepole pine (*Pinus contorta*): the effect of previous defoliation on oviposition, growth and survival of the pine beauty moth, *Panolis flammea*. Ecol. Entomol. **12**(3): 275–281. doi:10.1111/j.1365-2311.1987.tb01006.x.

Lempa, K., Loponen, J., Ossipova, S., Ossipov, V., and Pihlaja, K. 2001. Delayed greening of mountain birch leaves: ecological and chemical correlates. Ecoscience, **8**(1): 68–75. doi:10.1080/11956860.2001.11682632.

Levin, D.A. 1971. Plant phenolics: an ecological perspective. Am. Nat. **105**(942): 157–181. doi:10.1086/282712.

Mauffette, Y., and Oechel, W.C. 1989. Seasonal variation in leaf chemistry of the coast live oak *Quercus agrifolia* and implications for the California oak moth *Phryganidia californica*. Oecologia, **79**(4): 439–445. doi:10.1007/BF00378658. PMID:28313475.

McClure, M.S. 1987. Biology and control of hemlock woolly adelgid. Connecticut Agricultural Experiment Station New Haven, New Haven, Conn.

McClure, M.S., and Hare, J.D. 1984. Foliar terpenoids in *Tsuga* species and the fecundity of scale insects. Oecologia, **63**(2): 185–193. doi:10.1007/BF00379876. PMID:28311012.

McKenzie, E.A., Elkinton, J.S., Casagrande, R.A., Preisser, E.L., and Mayer, M. 2014. Terpene chemistry of eastern hemlocks resistant to hemlock woolly adelgid. J. Chem. Ecol. **40**(9): 1003–1012. doi:10.1007/s10886-014-0495-0. PMID: 25278447.

Michelozzi, M. 1999. Defensive roles of terpenoid mixtures in conifers. Acta Bot. Gall. **146**(1): 73–84. doi:10.1080/12538078.1999.10515803.

Moreira, X., Sampedro, L., Zas, R., and Pearse, I.S. 2016. Defensive traits in young pine trees cluster into two divergent syndromes related to early growth rate. PLoS One, **11**(3): e0152537. doi:10.1371/journal.pone.0152537. PMID:27028433.

Mumm, R., and Hilker, M. 2006. Direct and indirect chemical defence of pine against folivorous insects. Trends Plant Sci. **11**(7): 351–358. doi:10.1016/j.tplants.2006.05.007. PMID:16769239.

Nault, J.R. 2003. Site temperatures influence seasonal changes in terpene composition in Douglas-fir vegetative buds and current-year foliage. Can. J. For. Res. **33**(11): 2269–2273. doi:10.1139/x03-151.

Nealis, V.G., and Nault, J.R. 2005. Seasonal changes in foliar terpenes indicate suitability of Douglas-fir buds for western spruce budworm. J. Chem. Ecol. **31**(4): 683–696. doi:10.1007/s10886-005-3538-8. PMID:16124245.

Nerg, A., Kainulainen, P., Vuorinen, M., Hanso, M., Holopainen, J.K., and Kurkela, T. 1994. Seasonal and geographical variation of terpenes, resin acids and total phenolics in nursery grown seedlings of Scots pine (*Pinus sylvestris* L.). New Phytol. **128**(4): 703–713. doi:10.1111/j.1469-8137.1994.tb04034.x.

Orwig, D.A., and Foster, D.R. 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. J. Torr. Bot. Soc. **125**(1): 60–73. doi:10.2307/2997232.

Orwig, D.A., Foster, D.R., and Mausel, D.L. 2002. Landscape patterns of hemlock decline in New England due to the introduced hemlock woolly adelgid. J. Biogeogr. **29**(10–11): 1475–1487. doi:10.1046/j.1365-2699.2002.00765.x.

Orwig, D.A., Cobb, R.C., D'Amato, A.W., Kizlinski, M.L., and Foster, D.R. 2008. Multi-year ecosystem response to hemlock woolly adelgid infestation in southern New England forests. Can. J. For. Res. **38**(4): 834–843. doi:10.1139/X07-196.

Pasquier-Barre, F., Géri, C., Goussard, F., Auger-Rozenberg, M.A., and Grenier, S. 2000. Oviposition preference and larval survival of *Diprion pini* on Scots pine clones in relation to foliage characteristics. Agric. For. Entomol. **2**(3): 185–192. doi:10.1046/j.1461-9563.2000.00068.x.

Pezet, J., and Elkinton, J.S. 2014. Hemlock woolly adelgid (Hemiptera: Adelgidae) induces twig volatiles of eastern hemlock in a forest setting. Environ. Entomol. **43**(5): 1275–1285. doi:10.1603/EN13358. PMID:25259691.

Preisser, E.L., Lodge, A.G., Orwig, D.A., and Elkinton, J.S. 2008. Range expansion and population dynamics of co-occurring invasive herbivores. Biol. Invasions, **10**(2): 201–213. doi:10.1007/s10530-007-9123-z.

Preisser, E.L., Miller-Pierce, M.R., Vansant, J., and Orwig, D.A. 2011. Eastern hemlock (*Tsuga canadensis*) regeneration in the presence of hemlock woolly adelgid (*Adelges tsugae*) and elongate hemlock scale (*Fiorinia externa*). Can. J. For. Res. **41**(12): 2433–2439. doi:10.1139/x11-155.

Raffa, K.F., Mason, C.J., Bonello, P., Cook, S., Erbilgin, N., Keefover-Ring, K., et al. 2017. Defence syndromes in lodgepole–whitebark pine ecosystems relate to degree of historical exposure to mountain pine beetles. Plant Cell Environ. **40**(9): 1791–1806. doi:10.1111/pce.12985. PMID:28543133.

Rathcke, B., and Lacey, E.P. 1985. Phenological patterns of terrestrial plants. Annu. Rev. Ecol. Syst. **16**(1): 179–214. doi:10.1146/annurev.es.16.110185.001143.

R Core Team. 2018. R: a language and environment for statistical computing [online]. R version 3.5.0. R Foundation for Statistical Computing, Vienna, Austria. Available from https://cran.R-project.org/bin/windows/base/old/3.5.0/.

Rigsby, C.M., Shoemaker, E.E., Mallinger, M.M., Orians, C.M., and Preisser, E.L. 2019. Conifer responses to a stylet-feeding invasive herbivore and induction with methyl jasmonate: impact on the expression of induced defences and a native folivore. Agric. For. Entomol. **21**(2): 227–234. doi:10.1111/afe.12324.

Riipi, M., Ossipov, V., Lempa, K., Haukioja, E., Koricheva, J., Ossipova, S., and Pihlaja, K. 2002. Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? Oecologia, **130**(3): 380–390. doi:10.1007/s00442-001-0826-z. PMID:28547044.

Salminen, J.-P., Ossipov, V., Haukioja, E., and Pihlaja, K. 2001. Seasonal variation in the content of hydrolysable tannins in leaves of *Betula pubescens*. Phytochemistry, **57**(1): 15–22. doi:10.1016/S0031-9422(00)00502-1. PMID:11336256.

Sampson, D., Albaugh, T., Johnsen, K.H., Allen, H., and Zarnoch, S.J. 2003. Monthly leaf area index estimates from point-in-time measurements and needle phenology for *Pinus taeda*. Can. J. For. Res. **33**(12): 2477–2490. doi:10.1139/x03-166.

Schultz, J.C., Nothnagle, P.J., and Baldwin, I.T. 1982. Seasonal and individual variation in leaf quality of two northern hardwoods tree species. Am. J. Bot. **69**(5): 753–759. doi:10.1002/j.1537-2197.1982.tb13315.x.

Smith, H. 1982. Light quality, photoperception, and plant strategy. Annu. Rev. Plant Physiol. **33**(1): 481–518. doi:10.1146/annurev.pp.33.060182.002405.

Thoss, V., O'Reilly-Wapstra, J., and Iason, G.R. 2007. Assessment and implications of intraspecific and phenological variability in monoterpenes of Scots pine (*Pinus sylvestris*) foliage. J. Chem. Ecol. **33**(3): 477–491. doi:10.1007/s10886-006-9244-3. PMID:17268824.

Villari, C., Battisti, A., Chakraborty, S., Michelozzi, M., Bonello, P., and Faccoli, M. 2012. Nutritional and pathogenic fungi associated with the pine engraver beetle trigger comparable defenses in Scots pine. Tree Physiol. **32**(7): 867–879. doi:10.1093/treephys/tps056. PMID:22718525.

Waring, R.H., and Franklin, J.F. 1979. Evergreen coniferous forests of the Pacific Northwest. Science, **204**(4400): 1380–1386. doi:10.1126/science.204.4400.1380. PMID:17814182.

Watt, A.D. 1987. The effect of shoot growth stage of *Pinus contorta* and *Pinus sylvestris* on the growth and survival of *Panolis flammea* larvae. Oecologia, **72**(3): 429–433. doi:10.1007/BF00377575. PMID:28311141.

White, C.S. 1994. Monoterpenes: their effects on ecosystem nutrient cycling. J. Chem. Ecol. **20**(6): 1381–1406. doi:10.1007/BF02059813. PMID:24242344.

Wiggins, N.L., Forrister, D.L., Endara, M.-J., Coley, P.D., and Kursar, T.A. 2016. Quantitative and qualitative shifts in defensive metabolites define chemical defense investment during leaf development in *Inga*, a genus of tropical trees. Ecol. Evol. **6**(2): 478–492. doi:10.1002/ece3.1896. PMID:26843932.

Wilson, C.M., Schaeffer, R.N., Hickin, M.L., Rigsby, C.M., Sommi, A.F., Thornber, C.S., et al. 2018. Chronic impacts of invasive herbivores on a foundational forest species: a whole-tree perspective. Ecology, **99**(8): 1783–1791. doi:10.1002/ecy.2384. PMID:29729193.

Zou, J., and Cates, R.G. 1997. Effects of terpenes and phenolic and flavonoid glycosides from Douglas fir on western spruce budworm larval growth, pupal weight, and adult weight. J. Chem. Ecol. **23**(10): 2313–2326. doi:10.1023/B:JOEC.0000006676.68124.91.