Canadian Journal of Forest Research
Revue canadienne de recherche forestière

Do white spruce epicuticular wax monoterpenes follow foliar patterns?

Journal:	<i>Canadian Journal of Forest Research</i>
Manuscript ID	cjfr-2016-0056.R1
Manuscript Type:	Article
Date Submitted by the Author:	31-Mar-2016
Complete List of Authors:	Despland, Emma; Concordia University, Biology Bourdier, Thomas; Concordia University, Biology Dion, Emilie; Concordia University, Biology Bauce, Éric; Université Laval, Département des sciences du bois et de la forêt
Keyword:	Picea glauca, leaf surface, terpenoids, natural resistance, phytochemistry

Do white spruce epicuticular wax monoterpenes follow foliar patterns?

Emma Despland, Biology Department, Concordia University
(Emma.Despland@Concordia.ca)

Thomas Bourdier, Biology Department, Concordia University
(Thomas.Bourdier@gmail.com)

Emilie Dion, Biology Department, Concordia University
(Dion.Emilie@ymail.com) Current address: Department of Biological
Sciences, National University of Singapore

Eric Bauce, Sciences du Bois et de la Forêt, Faculté de Foresterie et
Géomatique, Laval University (Eric.Bauce@vrex.ulaval.ca)

For correspondence:

Emma Despland

Biology Department, Concordia University

7141 Sherbrooke West

Montreal H4B 1R6

514 848-2881

Emma.Despland@Concordia.ca

1 Abstract

2 We examine the extent to which foliar monoterpenes are trapped in the epicuticular waxes, as
3 part of an investigation into their role in natural defense against folivores. We monitored
4 concentrations in white spruce (*Picea glauca*) previous-year foliage and expanding foliage and
5 their epicuticular waxes, over the 2010 (14 trees) and 2011 (25 trees) growing seasons. In 2010,
6 concentrations were low in spring, and increased over the summer; in 2011 they stayed low.
7 The monoterpene profile of individual trees was similar between years, and showed a consistent
8 pattern over the growing season: in expanding foliage, δ -3-carene was only present in spring,
9 whereas bornyl acetate increased over the season. Individual wax monoterpene profiles
10 correlated with those of foliage, but the total concentration showed a different phenological
11 pattern. Total content remained constant throughout the growing season on previous-year
12 foliage, but decreased on expanding foliage. Electron microscopy suggests this is due to changes
13 in stomatal wax plugs and their role in blocking evaporation from the stomata. These findings
14 suggest that insects contacting the leaf surface will receive accurate information from the wax
15 chemical make-up about the monoterpene mix but not about overall monoterpene levels.

16 Key words: *Picea glauca*, leaf surface, terpenoids, natural resistance.

17 Introduction

18 Terpenoids are key players in the defense systems of conifers and many coniferous trees
19 produce a broad diversity of terpenoid products. Variation in monoterpene content occurs
20 between environments (e.g. it increases under drought conditions), following challenges (e.g.
21 synthesis often increases after wounding or treatment with methyl jasmonate) and between
22 tissues (e.g. levels are generally higher in stems than in needles). In addition, copious amounts
23 of monoterpenes are emitted daily by conifer foliage (Kesselmeier and Staudt 1999).

24 Monoterpene tissue pools and emissions respond differently to various challenges, and this
25 plasticity in expression presumably reflects the ecological roles of these compounds
26 (Gershenzon and Dudareva 2007, Huber et al. 2004, Keeling and Bohlmann 2006). Indeed, stem
27 monoterpenes are thought to be effective in defense against bark beetles, the most important
28 killers of conifer trees ((Phillips and Croteau 1999), but see also (Keeling 2016)) , and emitted
29 monoterpenes can act as attractants of herbivores and of their natural enemies (Phillips and
30 Croteau 1999, (Langenheim 1994), and possibly be involved in plant-plant communication
31 (Huber et al. 2004).

32 However, the ecological role of monoterpenes within needles is less clear (Martin et al. 2003b).
33 It has been suggested that monoterpenes are toxic to folivores (Kumbasli and Bauce 2013), but
34 performance experiments give ambiguous results (Mattson et al. 1991, Gershenzon and
35 Dudareva 2007). Both herbivory (Litvak and Monson 1998) and treatment with MeJA (Martin et
36 al. 2003b) lead to an increase in foliar monoterpene synthesis, but foliar pools remain stable
37 because of an increased emission of the newly produced compounds. Foliar monoterpene pools
38 are at a crossroads, where chemosynthetic pathways, damage-linked induction, stress response,
39 stomatal emission, resin duct storage and translocation to other tissues interact. The foliar pool

of monoterpenes is a transient state, very dynamic and can change over days or even hours (Martin et al. 2003). It is nonetheless ecologically relevant for organisms interacting with the leaf.

Another pool of conifer monoterpenes that has received virtually no attention is that in the epicuticular waxes on needles. Monoterpenes are synthesized during daylight and diffuse out from leaf mesophyll cells in proportion to the vapor pressure inside the needle, mostly through the stomata, but also through the cuticle (Lerdau et al. 1997). Foliar epicuticular waxes can influence monoterpene emission (Steinbauer et al. 2004), as monoterpenes are lipid soluble and can be absorbed into the waxes as they diffuse out through the stomata (Muller and Riederer 2005). Monoterpenes in epicuticular waxes can influence herbivore feeding or oviposition (Steinbauer et al. 2004; Ennis et al. 2015), as well as the invasion by plant pathogens and the growth of lichens, fungi and other epiphytic organisms (Martin et al. 2003). Patterns of foliar monoterpene synthesis in response to various environmental cues have been considered in terms of their effect on foliar pools, on translocation to other tissues and on emission, but not on epicuticular wax content. This avenue deserves further attention given the important role of epicuticular waxes in plant interactions with other organisms (Eigenbrode and Espelie 1995).

The white spruce (*Picea glauca*) genome has recently been sequenced (Birol et al. 2013). This tree exhibits at least 69 unique and transcriptionally active genes for terpene synthases, including at least 15 monoterpene synthases, and most of these are involved in the synthesis of several different terpenoid products (Keeling et al. 2011). Synthesis has been characterised in different stem tissues (Abbott et al. 2010) and monoterpenes have been detected in the needle epicuticular wax (Pruegel and Lognay 1996, Bourdier 2012). In this study, we characterize variation in white spruce foliar monoterpenes, from early to late in the growing season,

between expanding and mature foliage, and between internal needle contents and epicuticular waxes, and link this variation to wax structures on the needle surface and to functional ecological roles of these compounds.

Materials and Methods

Foliage collection

Foliage was collected from a white spruce plantation established in 1963 near Drummondville (Quebec, Canada) on four dates during spring and summer of 2010 and 2011 (May 5th and 18th, June 14th and July 12th in 2010 and May 7th and 20th, June 15th and July 12th in 2011). These dates correspond to bud flush, expanding foliage and mature foliage.

Thirteen trees were used in 2010 and 12 additional trees were added in 2011. Foliage samples were packed on dry ice and taken to the laboratory at Laval University. Twenty needles were removed from each sample, sealed in vials and preserved at -80° C.

Chemical analysis

Foliar monoterpenes were extracted by grinding 20 needles from each sample in liquid nitrogen and then extracting the contents with a 2:3 methanol:hexane solution (Daoust et al. 2010).

Two wax samples were extracted from each foliage sample: first, to measure the amount of epicuticular wax coating the needles, 200 needles were weighed and dipped in chloroform for 10 s. The solution was filtered and evaporated, and the residue weighed (Gordon et al 1998). These data are only available for the last three sampling dates of 2010. Second, to determine the monoterpenes present in the waxes, 50 needles were put into 1 ml of hexane-tetradecane (1l-120µl) solution for 30 seconds under Vortex agitation (Rivet and Albert 1990).

Both foliar and wax extracts were analyzed as per (Daoust et al. 2010) using a Varian model 3900 gas chromatograph equipped with a flame ionization detector and a SPB-5 fused silica capillary column (30 m $\times$ 0.25 mm) (Varian, Inc., Palo Alto, California, USA). Initial needle dry weight was used to estimate monoterpene concentrations.

Scanning Electron Microscopy

Whole needles were fixed on aluminium stubs with carbon double-sided tape and sputter-coated with a thin layer of platinum using a JEOL JFC 1100 ion sputter. The samples were observed in a JEOL JSM 6510 scanning electron microscope.

Photographs were taken from at least two sides of each quadrangular needle and from the base to the tip. Five needles from 3 trees and 3 sampling dates were analyzed.

Statistical analysis

We used a repeated-measures ANOVA to evaluate the overall effect of foliar age (current or previous year), phenology (early May, late May, June and July) and their interactions on variations in total monoterpene concentration. Multivariate analysis of variance (MANOVA) with the same design examined the fractional composition of different monoterpenes. Separate analyses were done for the two years of the study (2010 and 2011) and for the needles and waxes.

Spearman correlations were used to examine the relationship between monoterpene content in needles and in their overlaying wax, and in needle monoterpene content between years. Spearman's rank correlations were also used to compare the order of percent composition of individual monoterpenes between years, foliar ages and phenologies.

Results

Monoterpene composition

There were 7 major (α -pinene, camphene, myrcene, β -pinene, limonene, bornyl acetate, δ -3-carene) and 3 minor (thujone, terpinolene, α -phelladrene) monoterpene components (Figure 1).

The latter were not detected in the majority of samples and hence were excluded from analysis.

Needle monoterpene composition is correlated between the two years of study, but positive relationships are significant for limonene, myrcene and camphene only (see Figure 2).

Correlations between trees in the proportion of each monoterpene show that β -pinene and limonene are associated, and correlate negatively with myrcene, camphene and bornyl acetate (see Table 4).

Inter-annual variation

The monoterpene profiles of needles showed different patterns in 2010 and 2011 (Figure 1).

Results for 2011 were similar whether the new trees were included or not.

The lower total amount of monoterpenes observed in 2011 represented lower proportions of β -pinene, myrcene and camphene, but higher relative concentrations of α -pinene and bornyl acetate. β -pinene increased over the growing season in 2010 (contributing to the rise in total monoterpene content), but stayed constant (old foliage) or even decreased (new foliage) in 2011. By contrast, myrcene was high in expanding foliage in the spring of 2010, decreasing gradually over the season, but remained consistently low in 2011 (see Figure 1 and Table 2).

Weather data from the Environment Canada station in Drummondville show that the summer of 2010 was slightly warmer (mean temperature May-July, 2010: 18.8 °C; 2011: 18.4 °C; average 1913-2013: 16.9 °C) and considerably drier (total rain May-July, 2010: 296 mm; 2011: 357 mm,

average 1913-2013: 295 mm). In addition, the winter preceding the 2010 field season was milder (mean temperature Dec. – Apr., 2010: -0.33 °C, 2011: -3.9 °C, average 1913-2013: -5.1 °C), with less snowfall (total snow fall Dec.-Apr., 2010: 196 cm; 2011: 224 cm, average 1913-2013: 215 cm). In 2011, there were still 10 cm of snow on the ground on the last day of March but none at all in 2010.

Seasonal patterns

A repeated-measures Anova on needle total content in 2010 shows that monoterpenes increase during the year ($F_{3,91}=15.21$, $p < 0.0001$), in a similar fashion in both current-year and previous-year foliage (needle age not significant, Figure 1 a and b). In 2011, monoterpene levels were low during the entire growing season (date of harvest not significant), and lower in old foliage than in current-year needles ($F_{1,174}=8.69$, $p = 0.003$; Figure 1 c and d).

A similar Anova on wax for 2010 shows that monoterpenes in wax on current-year needles start high in May then drop in the summer (Figure 1 e), whereas levels are consistently low in the wax on old foliage (Figure 1 f) (harvest date: $F_{3,101}=11.68$, $p < 0.0001$; needle age: $F_{1,101}=14.01$, $p < 0.0001$; interaction: $F_{3,101}=13.10$, $p < 0.0001$). In 2011, there is no significant variation in levels of wax monoterpenes, but values are very low throughout, approaching the limit of detection by the instrument and hence making analysis difficult. These data are therefore not included. Repeated measures Anova on the amount of epicuticular wax in 2010 shows no significant effect of either foliage age or sampling date – see Table 1.

Multiple analysis of variance on the monoterpene composition (proportion of total represented by each of the 7 major components) of needle samples shows that the composition of monoterpenes varies significantly during the season, between current-year and previous year foliage, and between 2010 and 2011 (see Figure 1a, b, c and d). A peak of δ -3-carene occurs in

the spring in expanding foliage, and then the concentration of δ -3-carene decreases over the season as camphene and bornyl acetate increase. These seasonal changes are not observed in old foliage, where the composition of monoterpenes stays relatively constant over the growing season. This pattern was observed in both years (see Figure 1 and Table 2).

A similar pattern is observed in epicuticular waxes in 2010: δ -3-carene peaks in the spring in expanding foliage, then decreases over the growing season, while remaining low throughout the season in old foliage. Bornyl acetate and camphene increase over the growing season in current year foliage. Myrcene decreases over the growing season in expanding foliage, but remains consistently low in old foliage (see Table 3).

Wax structure and monoterpene content

Correlations between tree averages show significant positive relationships between the proportion of each monoterpene in a tree's needles and its epicuticular waxes (Figure 2).

Figure 3 shows images from two trees at the three later sampling dates in 2010: a broad band of stomata (4-7 rows) is seen on each side of the midrib. The tubular wax structure is visible between the stomatal rows, as are the crystalline wax plugs in the epistomatal chambers. The plug is eroded in the July foliage (Figure 3: C, F & I).

Discussion

Variation in monoterpene profiles

The results show different patterns in the two years of study: foliar monoterpenes increased over the growing season in the warm dry year (2010) but stayed low in the cooler, wetter year (2011). In both years, the monoterpene composition of expanding foliage changed over the

season, showing a spring peak in δ -3-carene, followed by a gradual increase in bornyl acetate over the summer. Individual trees showed a strong correlation in the blend of monoterpenes, between years and between foliage and wax, as expected since this blend is largely under genetic control (Huber et al. 2004). However, the concentration of monoterpenes in epicuticular wax decreased over the growing season, despite the fact that the amount of wax covering the needles remained constant, possibly due to lesser coverage of stomatal wax plugs.

The low monoterpene concentrations in 2011 (in needles and waxes) are presumably linked to the wet conditions. Indeed, drought and mild heat stress are linked to increased monoterpene pools (Kainulainen et al. 1992) and emissions (Niinemets 2010) in several conifers. In spruce, it is mostly the non-oxygenated compounds (β -pinene, myrcene and camphene, as observed in our study) that increase with drought (Kainulainen et al. 1992). In 2010, a warmer, drier year, foliar monoterpenes increased over the growing season. This seasonal increase has previously been observed in white spruce (Von Rudloff 1972) and other conifers (Litvak and Monson 1998), despite the fact that storage organs for monoterpenes are produced early in leaf ontogeny and rapidly filled (Lerdau et al. 1994). The levels we measured in needles are within the range measured in white spruce foliage (Mattson et al. 1991; Fuentealba and Bauce 2012).

Two compounds merit particular note for showing seasonal patterns consistent with the literature: δ -3-carene was only observed in the spring (as in another conifer species (Thoss et al. 2007)), whereas bornyl acetate increased gradually as the needles develop (as previously observed in white spruce (Von Rudloff 1972)).

Epicuticular waxes and monoterpene dynamics

We show that the monoterpene blend in epicuticular wax is similar to that in the needle, but decreases over the growing season while foliar contents increase.

194 The epidermal surface of spruce needles is covered with a layer of wax tubes, made up mostly of
195 nonacosan-10-ol that forms tubular aggregates, mixed with a variety of non-volatile organic
196 compounds including carboxyl acids, alcohols, alkanes and phenolics (Gordon et al. 1998). These
197 tubes are most dense between the rows of stomata, and the stomata themselves are blocked
198 with a plug of crystalline wax structures (Figure 3; (Percy and Baker 1990)). This wax develops
199 very early during leaf expansion: first, it fills the epistomatal chamber prior to budbreak, then
200 becomes visible on the epidermis. Within a week, needles already show a pattern of wax
201 deposition similar to fully grown needles (Percy and Baker 1990).

202 Cross-section through stomata shows that the plug consists of intermeshed wax tubes and
203 extends from just above the epidermal surface down to the upper surfaces of the guard cells, a
204 depth of 15-18 μm (Jeffree et al. 1971). This plug reduces stomatal area for diffusion; modelling
205 suggests that the presence of wax in young needles can reduce transpiration by 2/3 (Jeffree et
206 al. 1971). It seems likely that this wax also impedes diffusion of larger molecules such as
207 monoterpenes.

208 We show that the amount of wax covering needles remains the same over the growing season,
209 as seen in other spruces (Gordon et al. 1998), and as expected, since wax is formed very early in
210 needle development (Percy and Baker 1990). However, our electron micrographs suggest some
211 erosion of these stomatal plugs during the summer. Despite the fact that the relative
212 concentration of wax on the needles does not change as the needles elongate, the stomata are
213 more blocked on younger foliage. This might explain why monoterpene concentrations in waxes
214 are higher on newly formed expanding foliage.

215 Limiting water loss is most important in young foliage (Jeffree et al. 1971) and it is possible that
216 the wax plugs are most effective in occluding stomates early in the growing season.

Functional role of foliar monoterpenes

The effects of monoterpenes on folivores have been difficult to characterize (Mattson et al. 1991, Gershenzon and Dudareva 2007), due in part to the fact that monoterpenes occur in blends where it is not necessarily possible to disentangle the roles of individual compounds (Langenheim 1994).

One compound, whose effect on White spruce early spring feeders would warrant further attention, is δ -3-carene. We showed a δ -3-carene peak in the spring with concentration decreasing over the growing season. This compound is similarly expressed only during leaf expansion in Scots pine (Thoss et al. 2007) and defines two chemotypes of this species. Moreover, high and low levels of δ -3-carene in growing shoots define Sitka spruce genotypes resistant and susceptible to white pine weevil (Hall et al. 2011, Roach et al. 2014).

The epicuticular wax is the first point of contact of a folivore with a leaf, and therefore influences decisions about feeding and oviposition (Muller and Riederer 2005). For instance, female spruce budworm moths oviposit on previous year foliage in July and will therefore contact only low monoterpene content in needle waxes. These are not likely to be good predictors of the monoterpene concentrations that their offspring will be exposed to in expanding foliage in the following year, especially considering the considerable inter-annual variability in foliar monoterpene concentrations. Indeed, in our study, correlations between previous-year foliage wax in July 2010 and current-year needles in May 2011 show no significant values for any of the monoterpenes (data not shown). Previous work shows that ovipositing female budworm moths are attracted to monoterpene emissions (Grant et al. 2007) and use wax monoterpenes as a cue for host identification (Ennis et al. 2015). Similarly, ovipositing sawflies use wax monoterpenes to determine between different pine species that offer varying

quality foliage to offspring (Kazlauskas et al. 2011). However, our results suggest that, within White spruce, wax monoterpenes are not likely to provide a good cue to laying females for determining host quality for their offspring.

We still don't fully understand how monoterpenes affect the various selection pressures plants face, but they seem to contribute to alleviating several stresses. It appears that both constitutive and induced monoterpenes are important in defense against several xylophages (Huber et al. 2004, Keeling and Bohlmann 2006, Keeling 2016). Several monoterpenes, notably δ -3-carene and bornyl acetate, have been suggested to be toxic to the spruce budworm at high concentrations (Kumbasli and Bause 2013, Fuentealba and Bause 2012). Monoterpenes are also known to be active against fungi and gram-negative bacteria (Novak et al. 2014), and could protect against pathogens both at the leaf surface and within leaf airspaces (Martin et al. 2003). Monoterpenes might also have an internal physiological role, e.g. increasing thermal tolerance of photosynthesis, decreasing evapotranspiration or limiting UV damage (Holopainen and Gershenzon 2010).

Increasingly, molecular studies are unravelling the biochemical and genomic architecture underlying monoterpene production in conifers (e.g. (Keeling et al. 2011, Hall et al. 2013, Roach et al. 2014, Bohlmann et al. 2015)). However, establishing ecological roles of monoterpenes is hard (Gershenzon and Dudareva 2007, Keeling 2016). Our results demonstrate a hitherto ignored pool of conifer monoterpenes: that in the epicuticular waxes. We also show considerable seasonal and interannual variation in monoterpenes, both in foliar pools and in epicuticular waxes. Wax monoterpenes deserve further attention for their effects on organisms interacting with the foliage, for their potential role as cues to these organisms, for their impact

on monoterpene emissions and for their potential role as an endpoint for monoterpene production.

Acknowledgements

Thanks to Paule Huron with help with field work, to Martin Charest for chemical analyses, to Chris Daignault for wax measurements, to Darragh Ennis for discussions and to two anonymous reviewers for helpful suggestions and comments. This work was funded by a Canadian Natural Science and Engineering Research Council Collaborative Research and Development Grant to the iFOR consortium.

References

- Bohlmann, J., Gesell, A., Blaukopf, M., Madilao, L., Macaire, M.S., Withers, S., Mattson, J., and Russel, J.H. 2015. The gymnosperm cytochrome P450 CYP750B1 catalyzes stereospecific monoterpene hydroxylation of (+)-sabinene in thujone biosynthesis in *Thuja plicata*. *Plant Physiology*
- Bourdier, T. 2012. Behavioral adaptations of the Eastern spruce budworm (Lepidoptera: Tortricidae) to natural White spruce (*Picea glauca* (Moench)) resistance. M.Sc.: Concordia University
- Daoust, S., Mader, B., Bauce, E., Dussutour, A., Despland, E., and Albert, P.J. 2010. Influence of epicuticular wax composition on the feeding pattern of a phytophagous insect: implications for host resistance. *Canadian Entomologist* **142**: 261-270.
- Ennis, D., Despland, E., Chen, F., Forgione, P., and Bauce, E. 2015. Spruce budworm feeding and oviposition are stimulated by monoterpenes in white spruce epicuticular wax. *Insect Science*
- Fuentealba, A., and Bauce, E. 2012. Carryover effect of host nutritional quality on performance of spruce budworm progeny. *Bulletin of Entomological Research* 102: 275-284.
- Gershenzon, J., and Dudareva, N. 2007. The function of terpene natural products in the natural world. *Nature Chemical Biology* **3**: 408-414.

- 289 Gordon, D., Percy, K., and Riding, R. 1998. Effects of u.v.-B radiation on epicuticular
290 wax production and chemical composition of four *Picea* species. *New Phytologist* **138**:
291 441-449.
- 292 Grant, G.G., Guo, J., MacDonald, L., and Coppens, M.D. 2007. Oviposition response of
293 spruce budworm (Lepidoptera: Tortricidae) to host terpenes and green-leaf volatiles.
294 *Canadian Entomologist* **139**: 564-575.
- 295 Hall, D.E., Robert, J.A., Keeling, C.I., Domanski, D., Quesada, A.L., Jancsik, S., Kuzyk,
296 M.A., Hamberger, B., Borchers, C.H., and Bohlmann, J. 2011. An integrated genomic,
297 proteomic and biochemical analysis of (+)-3-carene biosynthesis in Sitka spruce (*Picea*
298 *sitchensis*) genotypes that are resistant or susceptible to white pine weevil. *Plant Journal*
299 **65**: 939-948.
- 300 Hall, D.E., Yuen, M.M.S., Jancsik, S.J., Quesada, A.L., Dullat, H.K., Li, M., Henderson,
301 H., Arango-Velez, A., Liao, N.Y., Docking, R.T., Chan, S.K., Cooke, J.E.K., Breuil, C.,
302 Jones, S.J.M., Keeling, C.I., and Bohlmann, J. 2013. Transcriptome resources and
303 functional characterization of monoterpene synthases for two host species of the
304 mountain pine beetle, lodgepole pine (*Pinus contorta*) and jack pine (*Pinus banksiana*).
305 *BMC Plant Biology* **13**: 80.
- 306 Holopainen, J.K., and Gershenzon, J. 2010. Multiple stress factors and the emission of
307 plant VOCs. *Trends in Plant Science* **15**: 176-184.
- 308 Huber, D.P.W., Ralph, S., and Bohlmann, J. 2004. Genomic hardwiring and phenotypic
309 plasticity of terpenoid based defenses in conifers. *Journal of Chemical Ecology* **30**: 2399-
310 2418.
- 311 Jeffree, C.E., Johnson, P.G., and Jarvis, P.G. 1971. Epicuticular wax in the stomatal
312 antechamber of Sitka spruce and its effects on the diffusion of water vapour and carbon
313 dioxide. *Planta* **98**: 1-10.
- 314 Kainulainen, P., Oksanen, J., Palomäki, V., Holopainen, J.K., and Holopainen, T. 1992.
315 Effect of drought and waterlogging stress on needle monoterpenes of *Picea abies*.
316 *Canadian Journal of Botany* **70**: 1613.
- 317 Keeling, C.I. 2016. Bark beetle research in the postgenomic era. *Advances in Insect*
318 *Physiology*
- 319 Keeling, C.I., Weisshaar, S., Ralph, S.G., Jancsik, S., Hamberger, B., Dullat, H.K., and
320 Bohlmann, J. 2011. Transcriptome mining, functional characterization, and phylogeny of
321 a large terpene synthase gene family in spruce (*Picea* spp.). *BMC Plant Biology* **11**: 43.
- 322 Keeling, C.I., and Bohlmann, J. 2006. Genes, enzymes and chemicals of terpenoid
323 diversity in the constitutive and induced defence of conifers against insects and
324 pathogens. *New Phytologist* **170**: 657-675.

- 325 Kesselmeier, J., and Staudt, M. 1999. Biogenic Volatile Organic Compounds (VOC): An
326 Overview on Emission, Physiology and Ecology. *Journal of Atmospheric Chemistry* **33**:
327 23-88.
- 328 Kumbasli, M., and Bauce, E. 2013. Spruce budworm biological and nutritional
329 performance responses to varying levels of monoterpenes. *iForest* **6**: 310-314.
- 330 Langenheim, J. 1994. Higher plant terpenoids: a phytocentric view of their ecological
331 role. *Journal of Chemical Ecology* **20**: 1223-1280.
- 332 Ler dau, M., Litvak, M., and Monson, R. 1994. Plant chemical defense: monoterpenes and
333 the growth-differentiation balance hypothesis. *Trends in Ecology & Evolution* **9**: 58.
- 334 Ler dau, M., Litvak, M., Palmer, P., and Monson, R. 1997. Controls over monoterpene
335 emissions from boreal forest conifers. *Tree Physiology* **17**: 563-569.
- 336 Litvak, M.A., and Monson, R.K. 1998. Patterns of induced and constitutive monoterpene
337 production in conifer needles in relation to insect herbivory. *Oecologia* **114**: 531-540.
- 338 Martin, D.M., Gershenzon, J., and Bohlmann, J. 2003. Induction of Volatile Terpene
339 Biosynthesis and Diurnal Emission by Methyl Jasmonate in Foliage of Norway Spruce.
340 *Plant Physiology* **132**: 1586.
- 341 Mattson, W.J., Haack, R.A., Lawrence, R.K., and Slocum, S.S. 1991. Considering the
342 nutritional ecology of the spruce budworm and its management. *Forest Ecology and*
343 *Management* **39**: 183-210
- 344 Muller, C., and Riederer, M. 2005. Plant surface properties in chemical ecology. *Journal*
345 *of Chemical Ecology* **31**: 2621-2651.
- 346 Niinemets, U. 2010. Mild versus severe stress and BVOCs: thresholds, priming and
347 consequences. *Trends in Plant Science* **15**: 145-153.
- 348 Novak, M., Krajnc, A.U., Lah, L., Zupanec, N., Kraševac, N., Križman, M., Bohlmann,
349 J., and Komel, R. 2014. Low-density *Ceratocystis polonica* inoculation of Norway spruce
350 (*Picea abies*) triggers accumulation of monoterpenes with antifungal properties.
351 *European Journal of Forest Research* **133**: 573-583.
- 352 Percy, K.E., and Baker, E.A. 1990. Effects of simulated acid rain on epicuticular wax
353 production, morphology, chemical composition and on cuticular membrane thickness in
354 two clones of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). *New Phytologist* **116**: 79-87.
- 355 Phillips, M.A., and Croteau, R.B. 1999. Resin-based defenses in conifers. *Trends in Plant*
356 *Science* **4**: 184-190.

- 357 Pruegel, B., and Lognay, G. 1996. Composition of the cuticular waxes of *Picea abies* and
358 *P. sitchensis*. *Phytochemical Analysis* **7**: 29-36.
- 359 Rivet, M.P., and Albert, P.J. 1990. Oviposition behavior in spruce budworm
360 *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). *Journal of Insect Behavior*
361 **3**: 395-400.
- 362 Roach, C.R., Hall, D.E., Zerbe, P., and Bohlmann, J. 2014. Plasticity and evolution of
363 (+)-3-carene synthase and (-)-sabinene synthase functions of a sitka spruce monoterpene
364 synthase gene family associated with weevil resistance. *Journal of Biological Chemistry*
365 **289**: 23859-23869.
- 366 Staudt, M., Bertin, N., Frenzel, B., and Seufert, G. 2000. Seasonal Variation in Amount
367 and Composition of Monoterpenes Emitted by Young *Pinus pinea* Trees– Implications for
368 Emission Modeling. *Journal of Atmospheric Chemistry* **35**: 77-99.
- 369 Steinbauer, M.J., Schiestl, F.P., and Davies, N.W. 2004. Monoterpenes and epicuticular
370 waxes help female autumn gum moth differentiate between waxy and glossy *Eucalyptus*
371 leaves of different ages. *Journal of Chemical Ecology* **30**: 1117-1142.
- 372 Thoss, V., O'Reilly-Wapstra, J., and Iason, G.R. 2007. Assessment and Implications of
373 Intraspecific and Phenological Variability in Monoterpenes of Scots Pine (*Pinus*
374 *syvestris*) Foliage. *Journal of Chemical Ecology* **33**: 477-491.
- 375 Von Rudloff, E. 1972. Seasonal variation in the composition of the volatile oil of the
376 leaves, buds, and twigs of white spruce (*Picea glauca*). *Can. J. Bot.*, **50**:1595-1603

Tables

Table 1 : Amount of epicuticular wax (mg/g dry mass) present on foliage in 2010.

Foliage year	Date	Wax (mg/g)
current	18 May 2010	11.86 +/-3.62
current	14 June 2010	10.53 +/-1.79
current	12 July 2010	10.37 +/-2.68
previous	18 May 2010	10.81 +/-3.15
previous	14 June 2010	11.14 +/-2.39
previous	12 July 2010	11.51 +/-2.98

Table 2: Output of a Manova on proportion of each monoterpene in needles, showing p-values for all effects tested. Predictors included in the model include main effects of year (Y, 2010 vs 2011, d.f. = 1), foliar age (A, current vs previous year, d.f. =1) and date of harvest (D, early may, late mate, june or july, treated as a regression, d.f. = 1) as well as the interactions between them. Residual d.f = 241. = 241. Effects significant at the p=0.01 level are highlighted.

	αpinene	βpinene	Limonene	Myrcene	Camphene	Bornyl	Carene	total
Date	0.018	0.337	0.501	0.120	<0.0001	<0.0001	<0.0001	<0.0001
Year	<0.0001	<0.0001	0.158	0.002	0.003	0.001	0.547	<0.0001
Age	0.011	0.004	0.996	0.334	<0.0001	<0.0001	<0.0001	<0.0001
D *Y	0.28	0.004	0.008	0.014	0.582	0.205	0.263	0.007
D *A	0.013	<0.0001	0.977	0.115	<0.0001	<0.0001	<0.0001	<0.0001
Y * A	0.294	0.9	0.103	0.32	0.907	0.008	0.628	0.15
D*Y*A	0.363	0.001	0.002	0.021	0.479	0.753	0.684	0.005

Table 3 : Output of a Manova on the proportion of each monoterpene in wax in 2010. Predictors included in the model include main effects of foliar age (A, current vs previous year, d.f. =1) and date of harvest (D, early may, late may, june or july, treated as a regression, d.f. = 1) as well as the interactions between them. Residual d.f = 241. Effects significant at the $p=0.01$ level are highlighted.

	α pinene	β pinene	Limonene	Myrcene	Camphene	Bornyl	Carene	total
Date	0.997	0.319	0.0690	0.0002	0.0003	0.023	0.009	<0.0001
Age	0.347	0.035	0.5377	0.019	0.671	0.854	0.156	<0.0001
D*A	0.109	0.356	0.1892	0.0002	0.016	0.049	0.006	<0.0001

Table 4: Pearson correlations between proportions of different monoterpenes in needles in 2010. Current-year and previous-year foliage pooled. Values significant at the $p=0.05$ level ($|r| > 0.5$) in bold.

	α -pinene	β -pinene	Limonene	Myrcene	Camphene	Bornyl	Carene
α -pinene	1	-0.56	-0.72	0.15	0.38	0.03	0.42
β -pinene		1	0.70	-0.61	-0.85	-0.55	-0.28
Limonene			1	-0.14	-0.52	-0.34	-0.32
Myrcene				1	0.46	0.32	-0.18
Camphene					1	0.63	0.22
Bornyl						1	0.20
Carene							1

List of figures

Figure 1 : Monoterpene concentrations measured on four sampling dates in 2010 (14 trees) and 2011 (25 trees), ppm per mass of foliage assayed in current and previous-year needles (a-b: 2010; c-d: 2011) and in epicuticular waxes coating those needles (e-f: 2010).

Figure 2: Correlations in proportion of monoterpene content represented by each compound: a) between needles and waxes in 2010 (α -pinene $r = 0.66$, $p = 0.01$; β -pinene $r = 0.89$, $p < 0.001$; limonene $r=0.72$, $p = 0.003$; myrcene $r=.066$, $p=0.01$; camphene $r=0.48$, $p=0.08$; bornyl acetate $r=0.58$, $p=0.03$; carene $r=0.90$, $p < 0.001$; N=14 trees), b) between needles in 2010 and 2011 (α -pinene $r = 0.45$, $p = 0.12$; β -pinene $r = -0.19$, $p = 0.52$; limonene $r=0.76$, $p = 0.002$; myrcene $r=0.74$, $p=0.003$; camphene $r=0.55$, $p=0.05$; bornyl acetate $r=0.46$, $p=0.12$; carene $r=0.47$, $p = 0.10$; N= 13 trees). Current-year and previous-year foliage pooled for both analyses.

Figure 3 : Scanning electron micrographs of needles from two individual trees (panels A-F, tree A10; G-I: tree A2) on 3 sampling dates (panels A, D, G: 18 May 2010; B, E, H: 14 June 2010; C, F, I: 12 July 2010). Panels A-C show rows of stomata on either side of the leaf midrib. The density of wax on the leaf surface is greater in the zone between stomata than outside. Plugs of crystalline wax tubes are visible in epistomatal chambers; they appear more eroded as the season progresses, but maintain their crystalline structure.

Draft

(a)

New needle monoterpenes

(b)

Old needle monoterpenes

Compound

- apinene
- camphene
- bpinene
- myrcene
- d.3.carene
- limonene
- bornyl.acetate

(c)

(d)

(e)

Wax monoterpenes, new foliage

(f)

Wax monoterpenes, old foliage

18 May

14 June

12 July