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1 **Fading of wound-induced volatile release during**
2 ***Populus tremula* leaf expansion**

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13
14 **Abstract**

15 The release of stress-driven volatiles throughout leaf development has been little studied.
16 Therefore, we subjected poplar leaves during their developmental stage (from two days to two weeks
17 old) to wounding by a single punch hole, and measured online the wound-induced volatile organic
18 compound emissions. Our study shows that the emission of certain volatile compounds fades with
19 increasing leaf age. Among these compounds we found lipoxygenase products (LOX products),
20 acetaldehyde, methyl benzoate, methyl salicylate, and mono- and sesquiterpenes.

21 In parallel, we studied the fading of constitutive emissions of methanol during leaf maturation, as
22 well as the rise in isoprene constitutive emission during leaf maturation and its relationship to leaf
23 photosynthetic capacity. We found highly significant relationships between leaf chlorophyll content,
24 photosynthetic capacity, and leaf size during leaf ageing.

25 As the level of constitutive defences increases with increasing leaf age, the strength of the volatile
26 signal is expected to be gradually reduced. The higher elicitation of volatile organic compound
27 emissions (especially LOX products) in younger leaves could be an evolutionary defence against
28 herbivory, given that younger leaves are usually more subjected to infestation and herbivory.

29
30 **Key-words:**

31 isoprene; lipoxygenase products; methanol; leaf age; wounding;

32

33 **1. Introduction**

34 Plants release a part of their assimilated carbon into constitutive (not related to periods of stress)
35 volatile organic compound (VOC) emissions (Grote et al. 2013b; Niinemets et al. 2013). Isoprene is the
36 major compound emitted in mature leaves of many tree species, including *Populus tremula*. Isoprene is
37 constitutively synthesized as a product of the methylerythritol phosphate (MEP) pathway in isoprene-
38 emitting plants (Sharkey et al. 2008), which is directly related to photosynthetic activity through the
39 Calvin cycle and DMADP (dimethylallyl diphosphate) pool. Based on the close relationship between
40 isoprene synthesis, isoprene emission, and photosynthesis (Loreto and Sharkey 1990; Monson and Fall
41 1989), leaf isoprene emissions may then vary within the same shoot together with leaf age because of
42 the increasing photosynthetic capacity (Centritto et al. 2004) and chlorophyll content during leaf growth
43 (Rasulov et al. 2014). By extension, the constitutive emission of other volatiles may also vary during
44 leaf development.

45 *Populus* genus has acropetally development, which means that the youngest leaves are found at the
46 tip of the shoot, and the leaf age and size increase as we move downwards. Therefore, another important
47 factor for leaf constitutive emissions would be the fact that cell elongation and division occurs more
48 intensively in younger and expanding leaves than in more mature and fully grown leaves. Pectin
49 demethylation during cell wall expansion is a likely source of methanol emission (Fall and Benson
50 1996; Galbally and Kirstine 2002), and therefore we may find differences in constitutive methanol
51 emission levels in neighbouring leaves within the same shoot.

52 Plants also emit VOCs when subjected to biotic and abiotic stress, so-called stress-driven emissions
53 (Grote et al. 2013b). For example, as a consequence of mechanical damage, poplar leaves emit a rapid
54 burst of volatiles lasting ca. 5 min (Portillo-Estrada et al. 2015b). The volatile blend is mainly composed
55 of green leaf volatiles, methanol and acetaldehyde. The damage leads one to assume a loss of carbon to
56 the plant as well as a decrease in the photosynthetic activity and a loss of water balance in the leaf.
57 There are studies on the quantitative effect of the degree of damage to wound-induced volatile emissions
58 in *P. tremula* mature leaves (Brilli et al. 2011; Fall et al. 1999; Portillo-Estrada et al. 2015b), but up to
59 present, there is little experimental data on leaf constitutive volatile emissions linked to the leaf
60 ontogenetic level, and no studies yet on the potentially different wound-induced volatile emissions
61 throughout young leaf development.

62 We used *P. tremula* shoots with leaves of increasing age during leaf expansion. We measured both
63 online leaf photosynthetic capacity and constitutive volatile emissions in leaves of increasing age under
64 optimal growth conditions. This was followed by mechanical wounding using a hole puncher to measure
65 the wound-induced responses as a function of leaf age.

66 We hypothesize that: (1) constitutive isoprene emission is positively related to photosynthetic
67 activity, which also means that it increases with leaf growth; (2) constitutive methanol emission is
68 maximum in younger and smaller leaves; and (3) wound-induced volatile emissions will be linked to
69 leaf age during expansion presumably because of the different physiological activity during leaf
70 development.

71 **Material and methods**

72 *Plant material*

73 We used root suckers of 15-20 leaves from a naturally established *Populus tremula* population at
74 the campus of the Estonian University of Life Sciences (58.39° N, 26.70° E, elevation 41 m). All of the
75 shoots used for this experiment are clones from the same tree, thus minimizing genetic variation effect
76 among replicates. The shoots, grown in the field in natural conditions and natural soil, were cut under
77 water and always transferred to the laboratory in the morning around 9:00. The shoots underwent a
78 period of adaptation to the measurement conditions by keeping them at room temperature beneath a 500
79 W halogen lamp providing a quantum flux density of ca. 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at leaf level. Measurements
80 were performed in leaves that had an area ranging between 3.2 to 65.2 cm^2 and corresponded to the first
81 (ca. 1-2 days old) to the ninth (ca. 14 days old) leaf position from the tip of the shoot, respectively (Fig.
82 1b).

83 *Experimental setup*

84 The experimental setup and procedure were similar to the experiment in Portillo-Estrada et al.
85 (2015b). We measured leaf net CO_2 assimilation rate by enclosing the leaves in a standard 8 cm^2 leaf
86 cuvette (3010-S of Walz GFS-114 3000) of a GFS-3000 gas-exchange system (Walz GmbH, Effeltrich,
87 Germany). A LED array/PAM-fluorimeter 3055-FL (Walz GmbH) was used for leaf illumination with
88 a saturating quantum flux density of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Leaf temperature was kept constant at 25 °C. The
89 cuvette was flushed with ambient air at a flow rate of 750 $\mu\text{mol s}^{-1}$. Air humidity was maintained at a
90 constant level (16000 ppm H_2O , approx. 60% relative humidity) and CO_2 concentration was 400 μmol
91 mol^{-1} .

92 Simultaneously with photosynthesis, constitutive leaf volatile emissions were measured online by
93 a PTR-TOF-MS (proton-transfer-reaction time-of-flight mass spectrometer) model 8000 (Ionicon
94 Analytik GmbH, Innsbruck, Austria). The details of the measurement principle, parameters and
95 calibration are described in Portillo-Estrada et al. (2015b). We measured 26 relevant volatile molecules
96 emitted by poplar leaves constitutively and after mechanical wounding (see the detailed list in Table 1
97 in Portillo-Estrada et al. (2015b)). Among these, the group of lipoxygenase products (LOX products)
98 included C_5 and C_6 compounds derived from the oxidation of linoleic acid, present in the cell
99 membranes (see biosynthetic pathways in Fall et al. (1999) and Fall et al. (2001)).

100 Once the leaves had reached steady levels of CO_2 exchange, water vapour, and isoprene emission
101 at saturating light level, we averaged three consecutive measurements of CO_2 exchange as an estimate
102 of photosynthetic capacity (A_{max}). Isoprene, methanol, and acetaldehyde emission levels at maximum
103 photosynthetic capacity were estimated by averaging a series of data of ca. 2-3 minutes during the steady
104 state. A total of 45 leaves of different age were measured for constitutive VOC emissions.

105 The dataset of constitutive isoprene emission through leaf expansion was fitted to an “exponential
106 rise to maximum” equation where the theoretical values would achieve a maximum during leaf maturity
107 (Eller et al. 2012; Sun et al. 2013). Following the same principle (Rasulov et al. 2014; Sun et al. 2013),
108 photosynthetic capacity was fitted to the same function type. As for constitutive methanol emission, we
109 used a negative exponential equation knowing that during leaf maturity there is also a basal level of

110 methanol emission (Eller et al. 2012) and that other authors found a higher emission at the top of poplar
111 shoots (Nemecek-Marshall et al. 1995).

112 *Leaf wounding*

113 Volatile emissions induced by wounding with a hole puncher were tested in leaves of increasing
114 age: first nine leaves from the shoot top. The punch hole area was $19.07 \pm 0.15 \text{ mm}^2$, and its perimeter
115 $15.48 \pm 0.06 \text{ mm}$, which was used to express the volatile emission rates per unit wound length (mm).
116 We used the punch hole procedure to perform the mechanical damage because of the rapidness of the
117 wounding treatment and its high replicability in producing wound edges of given length (Portillo-
118 Estrada et al. 2015b).

119 The volatile emissions induced during the few minutes following the wounding were integrated
120 and expressed by wound length. Seven minutes of emission data were enough to record the first
121 emission burst of volatiles related to the wounding, after which the emission levels came back to pre-
122 wounding values.

123 The datasets of wound-induced volatile emission though leaf expansion were fitted to a negative
124 exponential equation assuming that the emission of the volatiles of study was positive or close to zero
125 during leaf maturity, as shown by some studies (Brilli et al. 2011; Portillo-Estrada et al. 2015b).

126 *Leaf area and leaf age*

127 Once leaf cuvette measurements were finished, the leaf petiole was removed and the leaf area
128 measured by scanning the leaf blade. Leaf area picture processing occurred as in Portillo-Estrada et al.
129 (2015a) to minimize errors in the leaf area estimation due to shadows in the leaf picture.

130 Leaf age was estimated from leaf area after modelling the data published by Rasulov et al. (2015)
131 on the evolution of the leaf area during leaf expansion of clonal *P. tremula* individuals used for this
132 experiment. The data was fitted to a sigmoidal function ($r^2 = 0.998$, $P < 0.001$):

$$133 \quad L_{area} = 1.062 + \left(\frac{52.167}{1 + e^{-\left(\frac{L_{age} - 7.575}{2.399}\right)}} \right), \quad (\text{Eqn 1})$$

134 and then inversed to a logit function to estimate the leaf age (L_{age} , in days) based on the leaf area
135 (L_{area} , in cm^2):

$$136 \quad L_{age} = 7.575 - 2.399 \times \ln \left(\frac{52.167}{L_{area} - 1.062} - 1 \right). \quad (\text{Eqn 2})$$

137 *Leaf chlorophyll content*

138 Circular leaf discs of 1 cm diameter were taken by a cork borer and stored at $-80 \text{ }^\circ\text{C}$ for further
139 analysis of leaf chlorophyll content. Leaf discs were ground with Precellys 24 tissue homogenizer
140 (Bertin Technologies, France) in Precellys lysing kits for soft tissue CK 14 (2 mL plastic tubes with 1
141 mm diameter plastic balls inside) at $0 \text{ }^\circ\text{C}$ in 100 % acetone with added calcium carbonate. The extracts
142 were then centrifuged and filtered through a $0.45 \text{ }\mu\text{m}$ PTFE membrane filter. Leaf chlorophyll content
143 was determined with an Agilent Technologies 1200 Series HPLC system (Agilent Technologies, Santa
144 Clara, CA, USA) using a linear gradient of acetone concentration in water as in Opris et al. (2013).

145 Leaf chlorophyll content (mmol m^{-2}) increase through leaf age was fitted with the same equation
146 type than photosynthetic capacity, knowing that leaves will achieve a maximum of chlorophyll content
147 at maturity (Rasulov et al. 2014).

Results

Photosynthesis and constitutive volatile emissions throughout leaf expansion

We confirmed a highly significant ($r^2 = 0.88$ and $P < 0.001$; multiple linear regression analysis) three-way interaction between leaf age (L_{age} , in days), leaf chlorophyll content (L_{Chl} , in mmol m^{-2}) and net photosynthetic capacity (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$) at a constant saturating photosynthetic photon flux density:

$$A_{\text{max}} = -1.38 + 0.54 \times L_{\text{age}} + 17.88 \times L_{\text{Chl}}. \quad (\text{Eqn. 3})$$

The equation was also significant ($r^2 = 0.84$ and $P < 0.001$) when accounting for leaf area (L_{area} , in cm^2) instead of leaf age:

$$A_{\text{max}} = -2.72 + 0.0451 \times L_{\text{area}} + 27.36 \times L_{\text{Chl}}. \quad (\text{Eqn. 4})$$

As leaves expanded, leaf chlorophyll content raised to a level of 0.4 to 0.5 mmol m^{-2} (ca. 40 to 50 $\mu\text{g cm}^{-2}$) (Fig. 1a) and leaf photosynthetic capacity attained a level of 10-15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 1c) following the trend of an “exponential rise to maximum” function in both cases. Photosynthetic capacity linearly increased as leaf chlorophyll content increased during leaf development (Fig. 1d).

When applying the same light and temperature conditions to *P. tremula* leaves of increasing age, the constitutive volatile emissions of isoprene increased exponentially to a maximum, reached after 10-15 days of development (Fig. 2a). Leaf isoprene emission positively correlated with the net CO_2 assimilation rate (Fig. 2b). Methanol emission rates were higher as leaves were younger and smaller (Fig. 2c). We also found a vague relationship ($r^2 = 0.31$; $P = 0.001$) between leaf methanol emission rate and leaf photosynthesis (Fig. 2d).

We could visually identify a threshold at one week of leaf development (typically a leaf blade of 5.5 cm in length at the midrib level, maximum 5 cm in width, leaf area of ca. 20 cm^2 , and 7 days of development) where the exponential character of the relationships (Fig. 1a and c) turned to an almost steady response against the x-axis (leaf area) also showing larger variability in leaf chlorophyll content and photosynthetic capacity. In accordance with that, leaf chlorophyll a+b content of the youngest leaves (smaller than 20 cm^2) was smaller, $0.307 \pm 0.032 \text{ mmol m}^{-2}$ (or $27.4 \pm 2.9 \mu\text{g cm}^{-2}$, average \pm SE), than for the more mature leaves (larger than 20 cm^2), $0.466 \pm 0.010 \text{ mmol m}^{-2}$ (or $41.6 \pm 0.9 \mu\text{g cm}^{-2}$) ($P < 0.0001$, two-tailed Student's *t*-test) (Fig. 1a). Leaf net CO_2 assimilation was also significantly ($P < 0.0001$) smaller in younger leaves, $5.7 \pm 0.7 \mu\text{mol m}^{-2} \text{s}^{-1}$, than in larger and more mature leaves, $10.88 \pm 0.39 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 1c).

Concerning the constitutive volatile emissions, isoprene emission rates were lower ($P < 0.0001$) in younger leaves, $2.60 \pm 0.49 \text{ nmol m}^{-2} \text{s}^{-1}$, than in older leaves, $6.24 \pm 0.43 \text{ nmol m}^{-2} \text{s}^{-1}$ (Fig. 2a). Contrarily, leaf methanol emission rate during the first week of leaf expansion (leaf area smaller than 20 cm^2) was significantly ($P < 0.0001$) higher, $5.43 \pm 0.17 \text{ nmol m}^{-2} \text{s}^{-1}$, than in more mature leaves, $4.09 \pm 0.13 \text{ nmol m}^{-2} \text{s}^{-1}$ (Fig. 2c).

The changes of leaf chlorophyll content, photosynthetic capacity, and constitutive emissions of

185 isoprene and methanol with increasing leaf area (cm²) can be found in Figure S1, Suppl. 1.

186 *Wound-induced volatile emissions throughout leaf expansion*

187 Mechanical damage (leaf punch) was performed to leaves of increasing leaf age (i.e. increasing
188 leaf size), and the subsequent induced volatile emissions measured.

189 The sum of all volatiles emitted during the post-wounding period was larger in younger leaves than
190 in further developed leaves (Fig. 3a), and similarly occurred in LOX products (Fig. 3b) and acetaldehyde
191 (Fig. 3c) emissions. Contrarily, leaf age did not have an effect on methanol wound-induced emission
192 (Fig. 3d). At a much smaller level (lower than 1 pmol mm⁻¹ cut length, i.e. three orders of magnitude
193 smaller than LOX products), the wound-induced emissions of methyl benzoate (Fig. 3e), methyl
194 salicylate (Fig. 3f), monoterpenes (Fig. 3g), and sesquiterpenes (Fig. 3h) presented significant
195 relationships with the leaf age, following an exponential decay function in all cases.

196 The relationships of leaf wound-induced emissions with leaf area can be found in Figure S2, Suppl.
197 1.

198 **Discussion**

199 *Leaf chlorophyll content, photosynthesis, and isoprene emission throughout leaf ontogeny*

200 In previous studies on hybrid poplar, leaf chlorophyll content and photosynthetic capacity were
201 observed to increase rapidly during early leaf development (Rasulov et al. 2014; Reich 1983), both
202 parameters being positively interrelated (Reich 1983). Similarly to Reich (1983), we found that linear
203 fitting was the best option to model the increase of the light-saturated photosynthesis over rising
204 chlorophyll content (Fig. 1d).

205 Leaf net photosynthesis raised exponentially during leaf ageing and reached a maximum value at
206 ca. 10 and 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in mature leaves. These CO_2 assimilation levels as well as the concomitant
207 isoprene emissions during leaf ontogeny (Fig. 2a) are in accordance with the findings of Sun et al.
208 (2013) during poplar canopy development, confirming Hypothesis 1. Moreover, the observed isoprene
209 emission levels (Fig. 2a) were comparable to those reported by Loreto et al. (2007) at different leaf
210 positions from the apex (also studying the effects of leaf ontogeny) at ambient CO_2 concentration and
211 at a temperature of 25 °C.

212 The correlation between isoprene emission and photosynthesis rates (Fig. 2b) is well known
213 throughout scientific literature, especially when one considers the instantaneous responses of isoprene
214 emission and photosynthesis to changes in light intensity (e.g., Grote et al. 2013a; Monson 2013).
215 Contrarily to light manipulation experiments where isoprene emission is related to photosynthesis,
216 different relationships can be observed across long-term environmental gradients (Rasulov et al. 2015)
217 and throughout leaf ontogeny (Niinemets et al. 2015; Rasulov et al. 2014). In fact, throughout leaf
218 lifespan, isoprene emission is typically induced somewhat later than positive net photosynthesis rates
219 are observed (Harley et al. 1994; Rasulov et al. 2014). In our case, we measured the maximum
220 photosynthetic capacity at saturating light level and associated maximum isoprene emissions at a given
221 leaf age (Fig. 2a, b). Clearly, isoprene emission and photosynthesis rate were positively correlated, but
222 we nevertheless want to emphasize that using leaves of different age and shoots integrates random
223 effects that make the observed relationship somewhat scattered (Fig. 2b).

224 Despite the precise knowledge on the isoprene biosynthetic pathways, the evolutionary and
225 ecological reasons of leaf isoprene emission are still unclear, but different plausible hypotheses have
226 been proposed (Sharkey et al. 2008; Vickers et al. 2009). Isoprene emission is responsive to leaf
227 temperature and sensitive to light input, but ultimately related to isoprene synthase activity (Kuzma and
228 Fall 1993) and dimethylallyl diphosphate pool size (Niinemets and Sun 2015), that we hypothesize
229 smaller in younger leaves, as observed by Rasulov et al. (2014).

230 *Constitutive methanol emissions during leaf development*

231 Methanol emission has been shown as plant waste product that, due to its solubility in water and
232 high volatility, is unavoidably emitted (Peñuelas and Llusà 2004). Despite that, Peñuelas et al. (2005)
233 suggested a potential ecological role in mediating plant-insect interactions after measuring large
234 emission amounts caused by caterpillar feeding (Peñuelas et al. 2005). In addition, a controlled
235 mechanical wounding experiment on mature poplar leaves showed that methanol emission can
236 constitute 15% of wound-induced emissions and is highly correlated with the degree of damage

237 (Portillo-Estrada et al. 2015b).

238 As proposed by several authors, constitutive methanol emission peak is closely related to leaf
239 expansion. This is due to pectin demethylation during cell wall expansion (Harley et al. 2007; Karl et
240 al. 2003). In our study, constitutive methanol emission consequently peaked at the earliest
241 developmental stage (Fig. 2c), when the growth rate was presumably at its highest (Sun et al. 2013).
242 This has been also found by other studies (Fares et al. 2010) and confirms Hypothesis 2. There was also
243 a weak relationship between constitutive methanol emission and photosynthesis (Fig. 2d), most
244 probably driven by the simultaneous leaf expansion and net assimilation rate (Fig. 1c).

245 *Wound-induced volatile emissions related to leaf age*

246 Total volatile emissions after wounding were constituted mainly by LOX products, acetaldehyde
247 and methanol emissions (Fig. 3). The level of wound-induced emissions was dependent on leaf
248 ontogenetic level in many cases, which confirms Hypothesis 3.

249 Methanol, as well as emitted as a product of leaf expansion, it can be emitted due to oxidative
250 stress. Such conditions occur upon leaf wounding (Brilli et al. 2011; Loreto et al. 2006; Portillo-Estrada
251 et al. 2015b). However, we did not find a higher elicitation of methanol emission in younger leaves and
252 it was steady throughout leaf expansion (Fig. 3c). The cause could be related to the previously
253 mentioned methanol high volatility and solubility in water. For that reason, methanol emission is
254 directly related to stomatal conductance, preventing the formation of a stock within the leaf. Therefore
255 methanol pool size in the cytosol and interstitial spaces of leaf mesophyll could be always small
256 regardless of leaf age. This makes inevitably high methanol emission in young leaves certainly *de novo*
257 emission (not originated from a stock). In conclusion, upon leaf wounding and breakage of leaf tissues,
258 no additional methanol would be released from any stock in younger leaves.

259 Acetaldehyde is one of the most frequently identified oxygenated compounds emitted from leaves
260 (Monson 2013). It could be related to leaf wounding in the way that the pool of acetyl-CoA may react
261 with C₆ aldehydes synthesized upon wounding (in the LOX product blend) to form C₆ acetates, and
262 acetaldehyde could leak from the acetyl-CoA pool during this reaction (Graus et al. 2004). The question
263 lies in why younger leaves could hold a bigger acetyl-CoA or pyruvate pool, which are precursors of
264 acetaldehyde emission. Young leaves have been associated with higher growth respiration (Loreto et al.
265 2007), dark respiration (Rasulov et al. 2014) and mitochondrial activity (Dickmann et al. 1975) than
266 older leaves. Therefore the higher activity could explain a higher acetyl-CoA and pyruvate content in
267 younger poplar leaves, making the response to wounding greater through acetaldehyde emission.

268 LOX products were emitted in higher amounts by younger leaves. Linoleic acid is the precursor of
269 LOX products (Fall et al. 1999; Fall et al. 2001), and it is found in cell membranes. In principle, young
270 leaves could have smaller content of linoleic acid per unit area in comparison to older leaves, which are
271 thicker and supposedly have more membranes per area unit. However, the content of lipoxygenase
272 isozymes in young leaves is by far higher than in older leaves (Saravitz and Siedow 1995). This suggests
273 that the higher enzymatic activity may therefore drive the higher response in younger leaves, as we
274 observed (Fig. 3b). In ecological terms, a higher LOX product emission is beneficial for younger leaves
275 in keeping herbivores away, because young leaves with thinner cell walls (Tosens et al. 2012) lack

276 constitutive mechanical defences and also contain less non-volatile defensive metabolites such as
277 condensed tannins (Kursar and Coley 1991). In addition, young leaves have been shown to be more
278 heavily consumed by herbivores (Dudt and Shure 1994; Johnson et al. 1984; Southwood et al. 1986).
279 Nevertheless, we must acknowledge that the results of our study cannot be fully extrapolated to a
280 herbivory situation. Bricchi et al. (2010) found significant differences in leaf responses to mechanical
281 wounding in comparison to real herbivory: membrane polarization and volatile blend composition; most
282 probably due to the lack of herbivore-derived oral secretions in mechanical wounding.

283 At a much lower level, monoterpenes, sesquiterpenes, and methylated benzenoids as MeBe and
284 MeSa were emitted after leaf wounding. Their emission level depended also on leaf age. The emission
285 of LOX products is an ubiquitous response across species and stresses, whereas the emission of volatile
286 mono- and sesquiterpenes and benzenoids such MeSa are stress dependent, reflecting selective
287 activation of genes after the stress event (Copolovici et al. 2014; Possell and Loreto 2013).
288 Monoterpenes and sesquiterpenes were most likely emitted as a result of the exposure of the non-
289 specific pools of these volatiles in leaf lipid and liquid phases (Niinemets and Reichstein 2002) to the
290 ambient upon leaf wounding, and therefore should not reflect *de novo* emissions. Newly synthesized
291 isoprenoids normally peak in the subsequent hours to days after wounding (Erb et al. 2015). Their
292 accumulation in leaf structures (especially in conifer needles) protects the plant against herbivory by
293 decreasing leaf palatability (Dicke and Baldwin 2010) and may help to seal leaf wounds (Loreto et al.
294 2008). But poplar leaves do not have specialized structures to store terpenoids. However, a basal
295 synthesis level could happen in poplar leaves, and according to our results, could be greater in younger
296 leaves.

297 The synthesis and emission of volatile hormones such as MeBe and MeSa have been found to
298 attract natural predators during plant feeding as a defence response to plant herbivory (War et al. 2011;
299 Zhao et al. 2010) as well as repel parasites themselves (Snoeren et al. 2010). However, the peak of
300 synthesis of MeBe and MeSa normally occurs hours to days after the wounding (Niinemets and Monson
301 2013; Staudt et al. 2010), but our results then suggest that there could be a minimal basal synthesis
302 forming a small pool of these compounds in younger leaves. Our results are in agreement with findings
303 that in general, young leaves of *Populus* spp. have a greater capacity for biotic stress-dependent
304 induction of monoterpene emission than older leaves (Brilli et al. 2009).

305 Fares et al. (2010) also found a higher level of stress-induced volatile emission in younger leaves
306 when exposed to oxidative stress (2-week exposure to 80 ppb of ozone). In particular, they found a
307 higher amount of LOX products in younger leaves. In accordance to our results, the level of stress-
308 induced methanol emission did not depend on leaf age during poplar leaf expansion. Our study
309 evidences an effect of leaf age on stress-induced volatile emissions in poplar. More research is needed
310 to characterize this relationship across other species.

311 **Conclusion**

312 This study provides evidence of major variation in wound-induced volatile release through leaf
313 expansion. However, the key implication of the study is that even for the same species and tree shoot,
314 volatiles released in response to the elicitor of given strength can vastly vary in dependence on leaf
315 ontogeny. As the level of constitutive defences increases with leaf age, the strength of the volatile signal
316 is expected to be gradually reduced. The higher elicitation of BVOC emissions (especially LOX
317 products) in younger leaves could be an evolutionary defence against herbivory, given that younger
318 leaves are usually more subject to infestation and herbivory. Further studies are needed to test whether
319 herbivory also causes similar BVOC emission level changes through leaf development.

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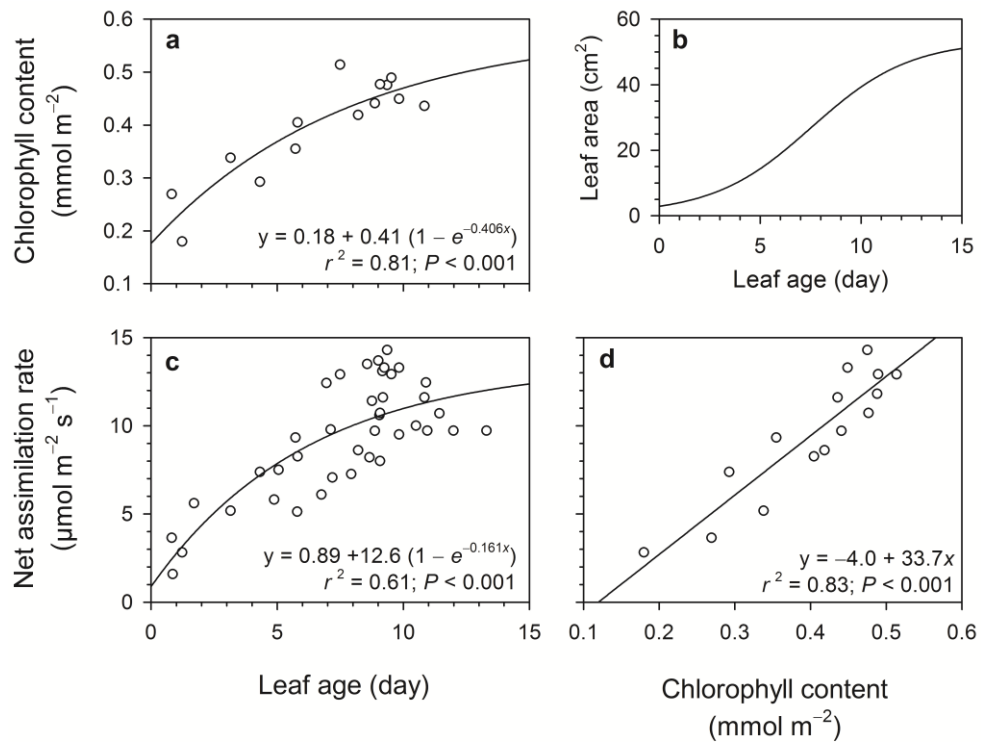
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470 **Figures:**

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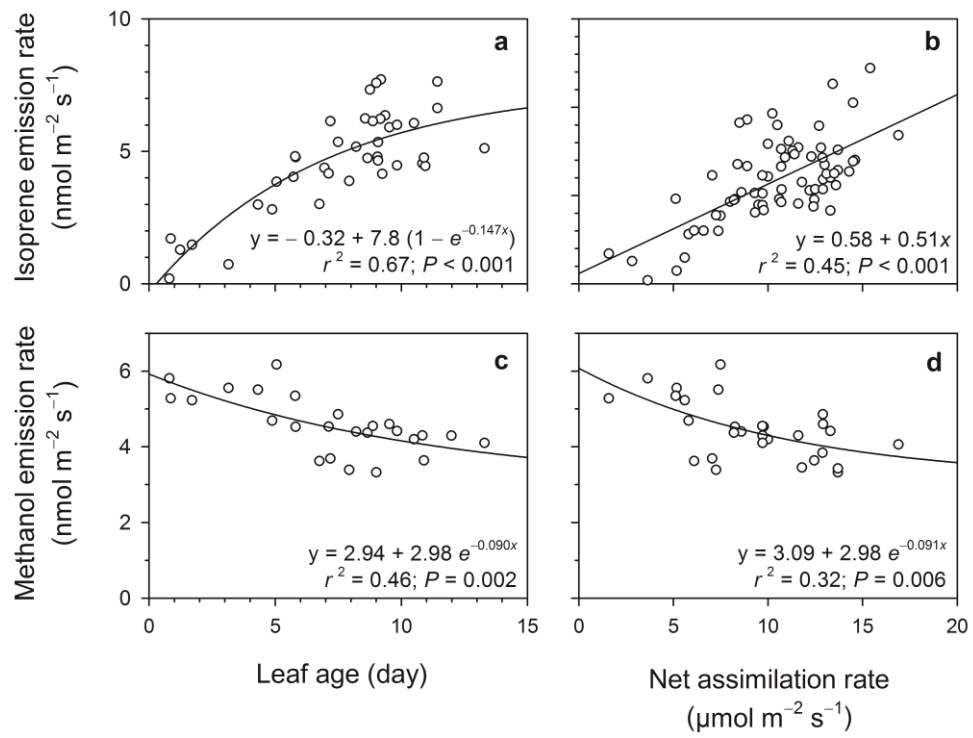
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474 **Figure 1**

475 Ontogenetic changes in (a) the chlorophyll a+b content, (b) leaf area, and (c) photosynthetic capacity in
476 leaves of temperate deciduous broad-leaved poplar (*Populus tremula*). Panel (d) shows the linear
477 relationship between leaf photosynthetic capacity and chlorophyll content. In (b), leaf size increases
478 with increasing leaf age according to a logistic (or sigmoidal) relationship (see Sun et al. (2013) and
479 Rasulov et al. (2015) for characteristic growth curves of poplar leaves with 5 cm^2 leaves being ca. 2
480 days old, and 50 cm^2 leaves 14 days old.

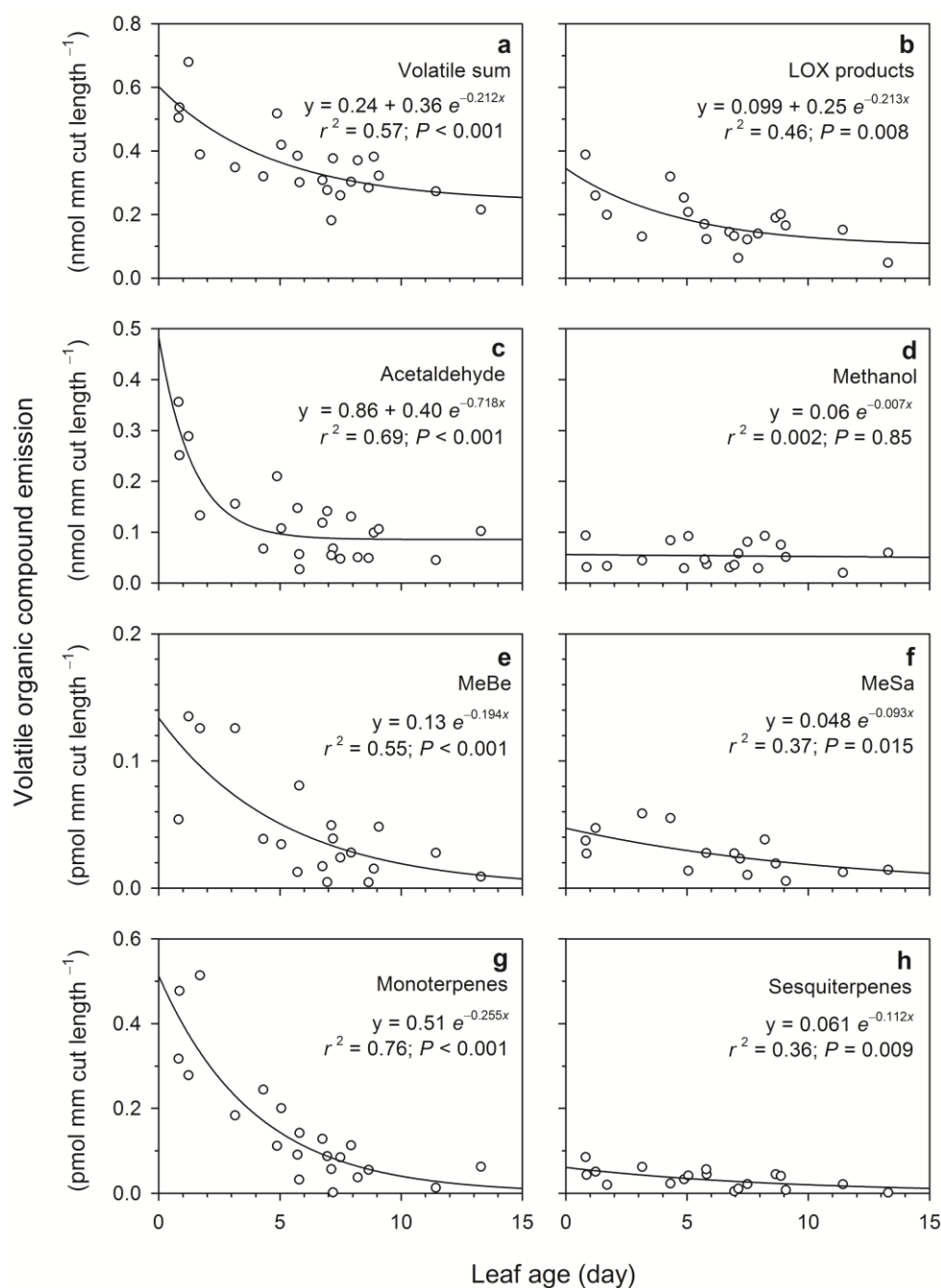


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484 **Figure 2**

485 Leaf isoprene and methanol emission rates in relation to (a, c) the leaf age and (b, d) leaf photosynthetic
 486 capacity in leaves of *Populus tremula*.

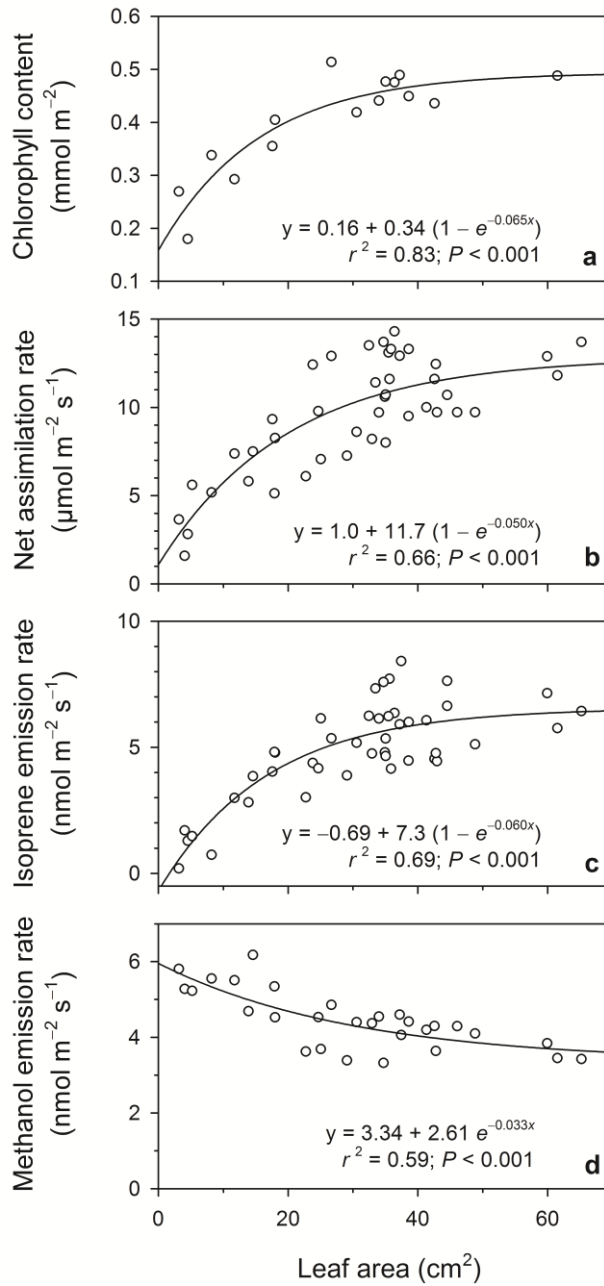


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Figure 3

490 Correlations of the integrated volatile emissions released by leaf wounds to leaf age *Populus tremula*
 491 leaves. The wounds were produced by a single punch hole (15.48 mm edge length). In (a), the sum of
 492 volatiles includes 26 relevant compounds and in (b), LOX products include C₅ (pentenyl family) and C₆
 493 (hexenal and hexanal families). For full list of compounds we refer to Portillo-Estrada et al. (2015b).
 494 MeBe (e) stands for methyl benzoate, and MeSa (f) for methyl salicylate.

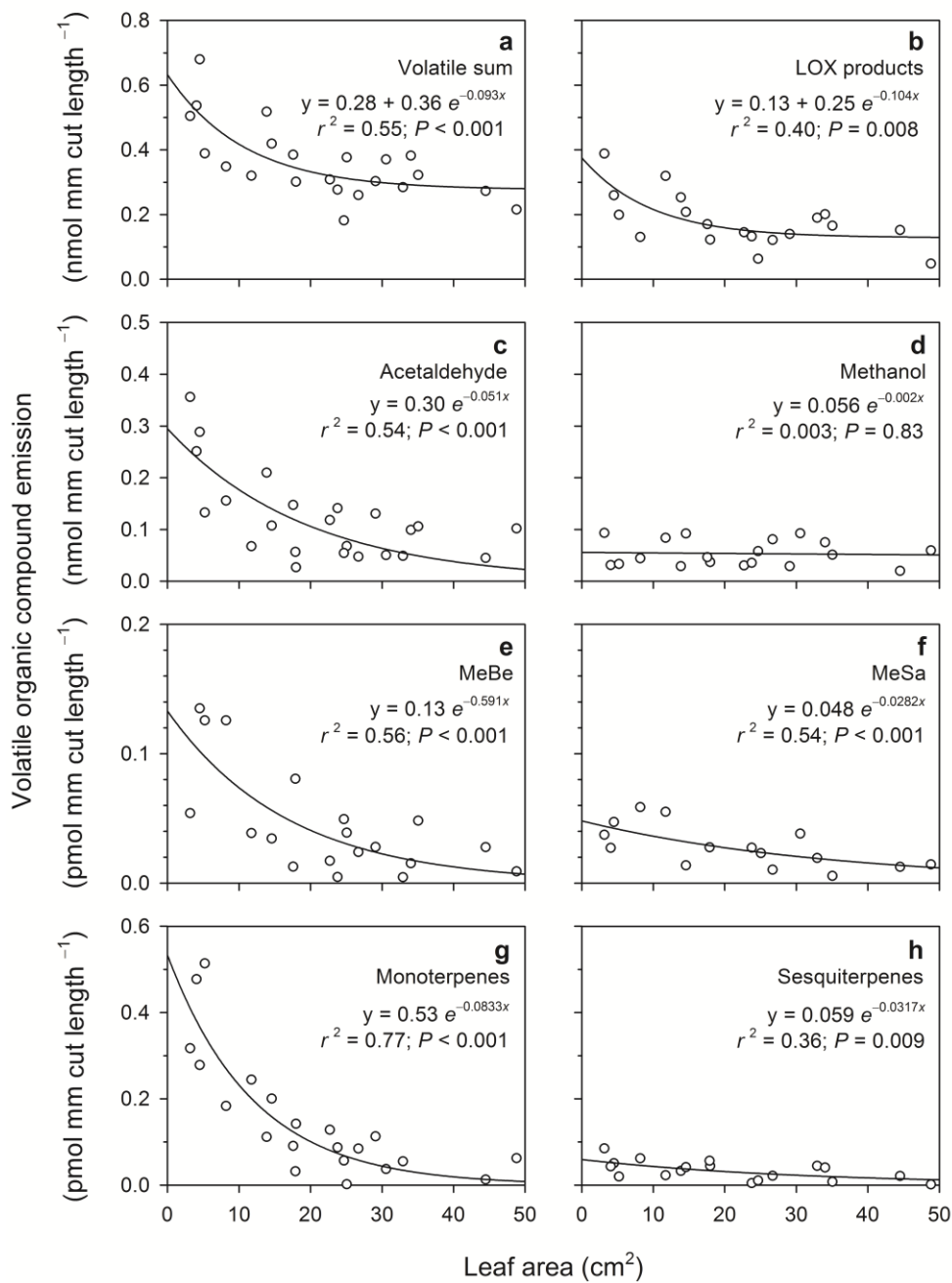


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498 **Figure S1**

499 Leaf (a) chlorophyll a+b content, (b) photosynthetic capacity, and constitutive (c) isoprene and (d)
500 methanol emissions during *Populus tremula* leaf expansion.

501



502

503

Figure S2

504

Integrated volatile emissions released by leaf wounds in *Populus tremula* leaves during leaf expansion.

505

The wounds were produced by a single punch hole (15.48 mm edge length). See Figure 3 for further

506

description of volatiles measured.