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1 **Simulated herbivory affects the volatile emissions of oak saplings,**
2 **while neighbourhood affects flavan-3-ols content of their leaves**

3
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27 Polish Academy of Sciences in Kórnik.
28

29 **Abstract**

30 To what extent particular plant defences against herbivorous insects are constitutive or
31 inducible will depend on the costs and benefits in their neighbourhood. Some defensive
32 chemicals in leaves are thought to be costly and hard to produce rapidly, while others, including
33 volatile organic compounds that attract natural enemies, might be cheaper and can be released
34 rapidly. When surrounding tree species are more closely related, trees can face an increased
35 abundance of both specialist herbivores and their parasitoids, potentially increasing the benefits
36 of constitutive and inducible defences. To test if oaks (*Quercus robur*) respond more to
37 herbivore attacks with volatile emission than with changes in leaf phenolic chemistry and
38 carbon to nitrogen ratio (C: N), and whether oaks respond to the neighbouring tree species, we
39 performed an experiment in a forest in Poland. Oak saplings were placed in neighbourhoods
40 dominated by oak, beech, or pine trees, and half of them were treated with the phytohormone
41 methyl jasmonate (elicitor of anti-herbivore responses). Oaks responded to the treatment by
42 emitting a different volatile blend within 24 hours, while leaf phenolic chemistry and C: N
43 remained largely unaffected after 16 days and multiple treatments. Leaf phenolics were subtly
44 affected by the neighbouring trees with elevated flavan-3-ols concentrations in pine-dominated
45 plots. Our results suggest that these oaks rely on phenols as a constitutive defence and when
46 attacked emit volatiles to attract natural enemies. Further studies might determine if the small
47 effect of the neighbourhood on leaf phenolics is a response to different levels of shading, or if
48 oaks use volatile cues to assess the composition of their neighbourhood.

49

50

51 **Key Words-**, VOCs, Intra-specific variation, Methyl jasmonate, Tritrophic interactions,
52 Polyphenols.

53

55 Plant defences against herbivorous insects are tailored to counter herbivore pressure and can
56 also respond to herbivory by induction and upregulation of various traits. Plant defences can
57 prevent insect herbivory altogether when they repel foraging insects (Bernays and Chapman
58 1994; Gripenberg et al. 2010). For example, ovipositing Lepidoptera may avoid leaves with
59 dense trichomes (Kumar 1992; War et al. 2013), high concentrations of defensive chemicals
60 (Renwick and Chew 1994), predatory ants (Freitas and Oliveira 1996), or signs of existing
61 herbivore infestation (De Moraes et al. 2001). Subsequently, plant defences can hamper feeding
62 or digestion, or otherwise harm insects, thus lowering the survival and growth of insect
63 herbivores (Feeny 1968; Forkner et al. 2004; Frost and Hunter 2008; Nykänen and Koricheva
64 2004). Plants can also defend themselves against insect herbivores indirectly by increasing the
65 natural enemy pressure on their herbivores by slowing insect growth (Kondoh and Williams
66 2001), hosting natural enemies with shelter or food (Wäckers et al. 2005; Walter 1996), or
67 signalling to natural enemies that herbivores are present (Amo et al. 2013; Pearse et al. 2020;
68 Price et al. 1980). These signals usually include volatile organic compounds (VOCs) that attract
69 natural enemies of herbivores such as parasitoids, predatory insects, and birds (Amo et al. 2013;
70 Dicke 2016; Janssen et al. 2002). How such defence traits are integrated depends on their costs
71 and benefits in their ecological context (Agrawal and Fishbein 2006).

72 Constitutive plant defences have different benefits and costs than induced defences.
73 Constitutive defences are present regardless of insect attacks, while induced defences are
74 generated or upregulated when a plant is attacked (Tollrian and Harvell 1999). Constitutive
75 defences are effective at repelling foraging herbivores as these insects tend to avoid better-
76 defended or less nutritious plants (e.g. ovipositing insects; Gripenberg et al. 2007).
77 Subsequently, constitutive defences reduce the growth and survival of herbivores (Nykänen
78 and Koricheva 2004). However, maintaining high levels of constitutive defences can be
79 energetically costly for the plant, resulting in trade-offs with other physiological processes
80 (Monson et al. 2022; Moreira et al. 2014; Stamp 2003). Induced defences can be less costly, as
81 defence investments are only higher during actual herbivore attacks (Karban 2011).

82 The speed of induction is a key factor for such defences, as they must increase rapidly to ensure
83 efficiency. Defences that take relatively long to accumulate in sufficient concentrations are not
84 likely candidates for induction by herbivore attacks. However, delayed induction of these types
85 of defences can still be adaptive when current herbivory predicts future herbivory (Mertens et
86 al. 2021; Rieske and Dillaway 2008). This can lead to current herbivory having knock-on
87 effects on future herbivores (Ekholm et al. 2020; Karban and Baldwin 2007; Karban and
88 Takabayashi 2018). In contrast, VOCs are usually of lower molecular weight and may peak
89 immediately after herbivory (e. g. green leaf volatiles (GLV); Hoballah et al. 2004; Robert et
90 al. 2013) or in the next photosynthetic cycle (e.g. terpenes; Robert et al. 2013). This makes
91 them a particularly fast response against herbivores. Fast induction has also been reported for
92 non-volatile chemical defences, such as terpenoids and lipophilic flavonoids (e.g. Keefover-
93 Ring et al. 2016; Martemyanov et al. 2012).

94 The inducibility of many VOCs and their upregulation in response to external stimuli is linked
95 to at least two ecological factors. First, signalling to natural enemies of herbivores will only
96 work if the signal is a reliable indicator of current or recent herbivory. Second, VOCs can also
97 have an ecological cost when volatiles attract herbivores rather than repel them (Ghirardo et al.
98 2012; Heil 2004). This could potentially explain the extensive variation in VOC production
99 among individual plants within populations (Bertić et al. 2021).

100 The efficacy and relative importance of various plant defences depend on the composition of
101 local communities of herbivores and their natural enemies, and by the predictability of
102 herbivory. When herbivore pressure is low and unpredictable, inducible defences are favoured,
103 since they are produced only when needed and do not require a constitutive investment into
104 their maintenance (Åström and Lundberg 1994; Mertens et al. 2021). This may in part explain
105 why the relative importance of constitutive and induced plant defences differs between habitats
106 that differ in herbivore pressure (Agrawal and Hastings 2019; Pellissier et al. 2016).

107 Due to their size and longevity, trees encounter numerous insects during their lifetimes, which
108 selects for strong constitutive defences (Feeny 1976). However, growing far from conspecifics
109 or other phylogenetically closely related individuals can reduce the apparency of small trees
110 (circumference < 1m) to specialised herbivores (Vialatte et al. 2010). Therefore, the rate of
111 insect attacks on small trees may be reduced or less predictable when they are in a
112 neighbourhood consisting mainly of distantly related species (Mallick et al. 2023; Yguel et al.
113 2011; but see opposite trend on larger trees Molleman et al. 2022). Specifically, oak trees
114 surrounded by distantly related neighbours experience less herbivory than oak trees that are
115 surrounded by conspecifics and closely related tree species (Yguel et al. 2011). In addition, the
116 efficacy of VOCs in attracting natural enemies of herbivores may depend on the neighbourhood
117 because the neighbourhood affects the natural enemy pool and ambient VOCs (Jactel et al.
118 2011; Janssen et al. 2002). However, the effect of neighbourhood on the effectiveness of plant
119 defences has rarely been studied (Dicke and Hilker 2003).

120 Since neighbouring trees can affect pressure by insect herbivores, plants might benefit from
121 tailoring their defences to their neighbours. Populations of oaks in which individuals tend to be
122 surrounded by conspecifics appear to have strong constitutive defences against insects
123 (provenance experiment; Pihain et al. 2019). Similarly, we can expect evolution of defence
124 induction in response to neighbourhood composition. Such a plastic response in defence
125 strategies to neighbourhood composition requires interaction among plants, which can be
126 mediated by ambient VOCs or through underground connections (Brosset and Blande 2021;
127 Karban 2021; Simard 2018). Most studies on such interactions focus on within-species
128 interactions where plants without herbivores are exposed to volatiles from plants with
129 herbivores, and then often show priming to respond to herbivory or induction of defences (e.g.
130 Soler et al. 2007; Tschardt et al. 2001). However, plants can also respond to herbivore-
131 induced VOCs from distantly related plant species (Karbon et al. 2000; Kessler et al. 2006).
132 Studies addressing how neighbourhood might affect plant defence without induction of
133 neighbours are rare (Glinwood et al. 2011; Ninkovic et al. 2016). We might expect that trees
134 will always host some herbivores so that on average neighbourhoods will be reflected in

135 ambient herbivore-induced VOCs. As a result, lower abundance of conspecifics in a
136 neighbourhood might translate into less priming or induction from conspecifics. Notably, the
137 identity of neighbours can additionally affect the light environment and micro-climate, and this
138 can also affect leaf chemistry (Barbier et al. 2008; Kovács et al. 2017). In summary, 1)
139 neighbourhood can select for different levels of investment in constitutive defences, 2) plants
140 can plastically upregulate defences, and 3) there are signals informing plants on their
141 neighbourhood. We hypothesise that trees can plastically increase investment in leaf defences
142 (higher concentration of defensive chemicals, reduced nutritional value) when they are placed
143 among closely related neighbours where herbivore pressure is high.

144 We studied constitutive and induced chemical defences of oak saplings (*Quercus robur*) placed
145 in different neighbourhoods in a forest in Poland. Oaks are important components of temperate
146 forests in the northern hemisphere and harbour a high diversity of species of herbivorous insect
147 (Southwood 1961). They have a rich secondary chemistry, including various tannins that affect
148 insect herbivores through oxidative activity and protein precipitation (Barbehenn et al. 2006;
149 Bertić et al. 2021; Feeny 1970; Forkner et al. 2004; Salminen and Karonen 2011; Volf et al.
150 2022). When induced by (simulated) caterpillar feeding, the oak species that we studied emit
151 VOCs that are associated with the attraction of natural enemies of caterpillars (Mrazova et al.
152 2023; Volf et al. 2021). Oaks also show genetically-determined intraspecific variation in
153 defences that have consequences for their susceptibility to insect herbivores (Ghirardo et al.
154 2012; Pihain et al. 2019; Roslin et al. 2006; Valdés-Correcher et al. 2021). However, the
155 possible plastic response to neighbourhood composition has not been studied. To test oak
156 responses to insect herbivory and neighbourhood composition, we placed potted oak saplings
157 in three types of neighbourhood (dominated by either oak, beech, or pine), and treated half of
158 the saplings with methyl jasmonate (MeJA). MeJA is a phytohormone that is central in
159 mediating the response of plants to insect herbivory and is commonly used in ecological studies
160 to mimic plant responses to leaf-chewing herbivores (e.g. Amo et al. 2022; Mrazova and Sam
161 2019; Semiz et al. 2012). After applying MeJA, we sampled VOCs, and intermittently
162 monitored leaf traits using a leafclip sensor. After 16 days, we determined the carbon-nitrogen
163 ratio (C: N), phenolic composition, and antioxidant activity of the leaves. We tested the
164 following hypotheses: 1) leaf phenolics and C: N are mostly constitutive, while VOCs are
165 rapidly induced by the MeJA treatment, and 2) oaks among closely related neighbours
166 upregulate their phenolic defensive leaf chemistry.

167

168

170 *Experimental design.* We carried the study out in Puszcza Zielonka forest near Kamińsko
171 village in Western Poland, using potted saplings of *Q. robur* (purchased from Szkółki
172 Kórnickie, Kórnik, Poland) in June 2022. On June 2nd, we placed 17 pairs of potted saplings
173 (within-pair distance 10 meters) in 10 sites that varied in neighbourhood. After excluding three
174 saplings eaten by deer, we had ten oak saplings in four areas dominated by oaks (*Q. petraea*),
175 seven in two areas dominated by beeches (*Fagus sylvatica*), and twelve in four areas dominated
176 by pines (*Pinus sylvestris*). The undergrowth in these neighbourhoods was sparse due to
177 browsing wildlife, and no other oak saplings were present. We planted the saplings levelled
178 with the surface, but they remained in the pots and were watered as needed.

179 Two days after placing the saplings in the field (June 4th), we sprayed one individual within
180 each pair with 2mM of the plant hormone MeJA, and the other with a control solution (distilled
181 water with molecular-grade ethanol and triton X-100). After the solution had dried, we sampled
182 VOCs from the oaks following Kallenbach *et al.* (2014). Specifically, we placed two 1.5 cm
183 polydimethylsiloxane tubes (PDMS; Carl Roth GmbH, Karlsruhe, Germany) on a stainless-
184 steel wire attached to the measured branch, and enclosed them in a polyamide bag (Stella Pack
185 S. A. 35 x 43 cm). We attempted to enclose similar-sized twigs. The VOCs from the headspace
186 were passively adsorbed onto the PDMS tubes that were collected after 24 hours.

187 We then repeated the MeJA and control treatments every four days (June 8th, 12th, and 16th),
188 and we switched the position of saplings within pairs twice during the experiment. To gauge
189 how rapidly MeJA treatment and neighbourhood affect leaf chemistry over time, we took
190 measurements using the Dualex+ (METOS Pessl Instruments GmbH, Weiz, Austria) five times
191 during the study period (June 4th, 5th, 10th, 12th, and 16th). For each sapling, we measured four
192 leaves in two places. The Dualex+ is a leafclip sensor that non-invasively measures the nitrogen
193 balance index (NBI), and indices of chlorophyll, flavonol, and anthocyanin content in leaves
194 (Goulas *et al.* 2004). Chlorophyll content and NBI can be expected to reflect light conditions
195 (Šušić *et al.* 2023) and might also respond to herbivory (Visakorpi *et al.* 2020). Sixteen days
196 after the start of the experiment (June 20th), we harvested all the leaves for analysis of phenolics
197 and C: N. Leaves were dried at 35 °C for three days and then chopped in a blender and then
198 milled using an IKA mill MF 10.1 (IKA-Werke GmbH, Staufen, Germany). Sub-samples for
199 C: N analysis were dried at 65 °C for 24 hours.

200 *Volatile emission measurements.* The VOCs absorbed in the PDMS tubes were analysed using
201 gas chromatography-mass spectrometry (GC/MS) using a Thermal Desorption (TD) TD100-
202 xr (Markes International, Bridgend, UK) installed on a Trace 1300 gas chromatograph coupled
203 to an ISQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The
204 PDMS tubes were inserted into metal tubes and placed in the TD instrument. The tube was
205 dried with a helium flow (20 mL/min) for 5 minutes. Then it was heated to 200 °C for 5 minutes
206 (the helium flow was 60 mL/min). The desorbed VOCs were captured in a cold trap (0 °C),
207 which was then heated to 300 °C for 4 minutes. The split flow was 10 mL/min. GC separation
208 was performed using a VF-5MS capillary column (30 m × 0.25 mm id, 0.25 µm film thickness,
209 Agilent Technologies, Santa Clara, CA, USA) and helium as the carrier gas at a flow rate of 2

210 mL/min, following the temperature conditions: 39 °C for 1 minute, increasing at a rate of 8
211 °C/min to 95 °C, then increasing to 300 °C at a rate of 20 °C/min. Finally, the temperature was
212 held for 2 min. VOCs were detected using an electron ionisation (EI) ion source (70 eV, 230
213 °C) and a single quadrupole in a scan range of 33-500 m/z. The Thermo Xcalibur 2.0 software
214 (Qual Browser, Quan Browser, Thermo Fisher Scientific) was used for data processing, method
215 calibration, and validation. VOC emissions were quantified as the area under the
216 chromatographic peak.

217 *Leaf phenolics and C: N analysis.* We measured total phenolic content using the Folin–
218 Ciocalteu method (Sokół-Łętowska et al. 2020; details in Supplementary Information 1) and
219 antioxidant activity as Trolox equivalents (Gao 2000, Re 1999; details in Supplementary
220 Information 1). The content of individual (classes of) phenolics and their total content were
221 determined using an optimized high-performance liquid chromatography coupled photodiode
222 array (HPLC-PDA) method (Sokół-Łętowska et al. 2020; details in Supplementary Information
223 1). Carbon and nitrogen contents (% d.m.) were determined using a 2400 CHN Elemental
224 Analyzer (Perkin Elmer, Waltham, MA, USA).

225 *Data analysis.* For VOC emissions, we log-transformed the data to approach a normal
226 distribution of residuals. We used concentration of phenolics as mg/g of the dry leaf tissue. We
227 analysed the composition of VOCs and leaf phenolics using non-metric multidimensional
228 scaling (NMDS) and PERMANOVA in the R package vegan (Oksanen et al. 2007;
229 R_Core_Team 2023). As predictors in PERMANOVA models, we used treatment (sprayed
230 with MeJA vs. control), and neighbourhood (dominated by oak, beech, or pine), and the
231 interaction between treatment and neighbourhood.

232 We compared the log of total VOC emission, C: N, sums of classes of phenolics, and
233 antioxidant activity across treatments using ANOVA analysis. When a predictor affected VOC
234 or phenolic composition, we further explored which VOCs or phenols most responded to the
235 predictor using ANOVAs. We used the Bonferroni method to correct for multiple testing within
236 each set of ANOVAs.

237 To test if treatment, or neighbourhood and their interaction affected changes over time in NBI,
238 chlorophyll content, flavanol index, and anthocyanin index (data from Dualex+ leafclip
239 sensor), we implemented mixed models with sapling ID as a random effect in R. As predictors
240 we used day, treatment (sprayed with MeJA vs. control), and neighbourhood (dominated by
241 oak, beech, or pine), and the interaction between treatment and neighbourhood. We then
242 performed model selection by step-wise eliminating non-significant predictors.

243

245 *VOC emissions*. In total, 18 VOCs were identified in *Q. robur* saplings (Table 1). These belong
 246 to aldehydes (1), alkanes (4), GLVs (1), monoterpenes (6), and sesquiterpenes (6). The oak
 247 saplings treated with MeJA had on average much higher total VOC emissions than controls
 248 (5.5 times, $N = 30$, $F = 6.89$, $p = 0.014$). The VOC composition was also clearly affected by
 249 the MeJA treatment (Table 2, Figure 1a). At least four individual *Q. robur* saplings appeared
 250 not to have responded to MeJA treatment (placed among control saplings in Figure 1a).

251

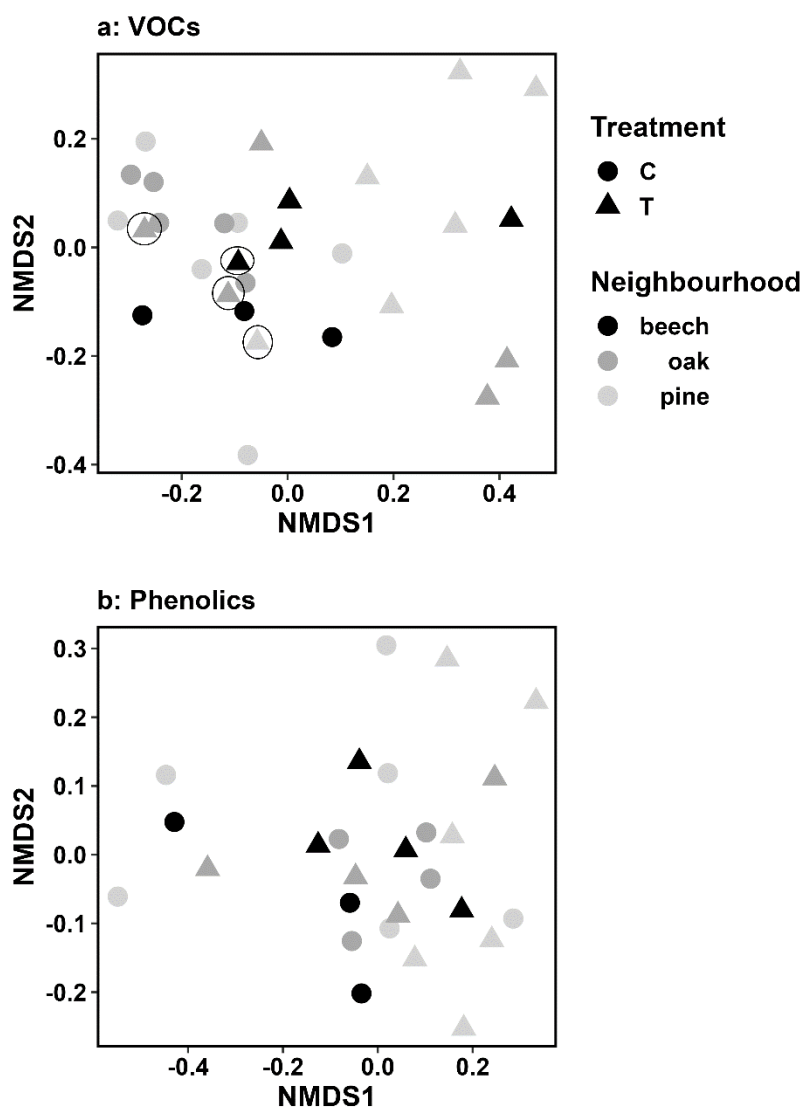


Figure 1: NMDS plots of a) VOC emissions, and b) phenolic composition by MeJA treatment (C = control, T = MeJA treatment) and neighbourhood for *Q. robur* saplings. Individuals that appeared not to have responded to MeJA treatment are encircled. Statistical results are in Table 2.

268 Specifically, oak saplings treated with MeJA emitted significantly elevated amounts of six
 269 VOCs: the monoterpenes Eucalyptol, Linalool, o-Cymene, β -Ocimene, β -cis-Ocimene and the
 270 sesquiterpene α -Copaene (after correcting for multiple testing; Table 1).

271

272 Table 1: Results of One-way ANOVAs for the effect of Treatment (Control vs. MeJA
 273 application) on the log of VOC emissions of *Q. robur* saplings, with means $p < 0.05$ in bold
 274 font. Corrected values denote Bonferroni-corrected p-values. All effects of MeJA were
 275 positive. See Supplementary Information 2 for details.

Class	variable	MeJA	Control	F-value	p-value	corrected
Aldehydes						
	Nonanal	17.4	16.4	5.788	0.023	0.419
Alkanes						
	Eicosane	16.8	16.4	1.153	0.292	1
	Hexadecane	15.9	15.7	0.227	0.638	1
	Pentadecane	15.9	15.7	0.227	0.638	1
	Tridecane	15.6	15.1	1.657	0.209	1
Green Leaf Volatiles						
	Methyl salicylate	17.1	16.1	4.202	0.050	0.904
Monoterpenes						
	Eucalyptol	16.9	14.6	21.857	<0.001	0.001
	Limonene	15.0	14.6	4.280	0.048	0.869
	Linalool	16.7	14.7	36.715	<0.001	<0.001
	o-Cymene	15.5	13.6	16.896	<0.001	0.006
	β -Ocimene	18.3	14.8	28.030	<0.001	<0.001
	β -cis-Ocimene	15.7	13.6	25.563	<0.001	<0.001
Sesquiterpenes						
	(<i>Z,E</i>)- α -Farnesene	18.2	16.6	6.210	0.019	0.344
	Caryophyllene	17.9	17.1	3.372	0.077	1
	α -Copaene	15.6	14.4	31.089	<0.001	<0.001
	α -Cubebene	15.6	14.8	3.553	0.070	1
	α -Farnesene	18.2	16.6	6.305	0.018	0.330
	δ -Cadinene	17.1	15.7	8.782	0.006	0.113

276

277

278 Table 2: PERMANOVA results for log VOC emissions, and Phenolics of leaves of *Q. robur*
 279 saplings. Treatment = MeJA application or control, Neighbourhood = neighbouring trees are
 280 beeches, oaks, or pines. Interaction terms were not significant and were removed from the
 281 models.

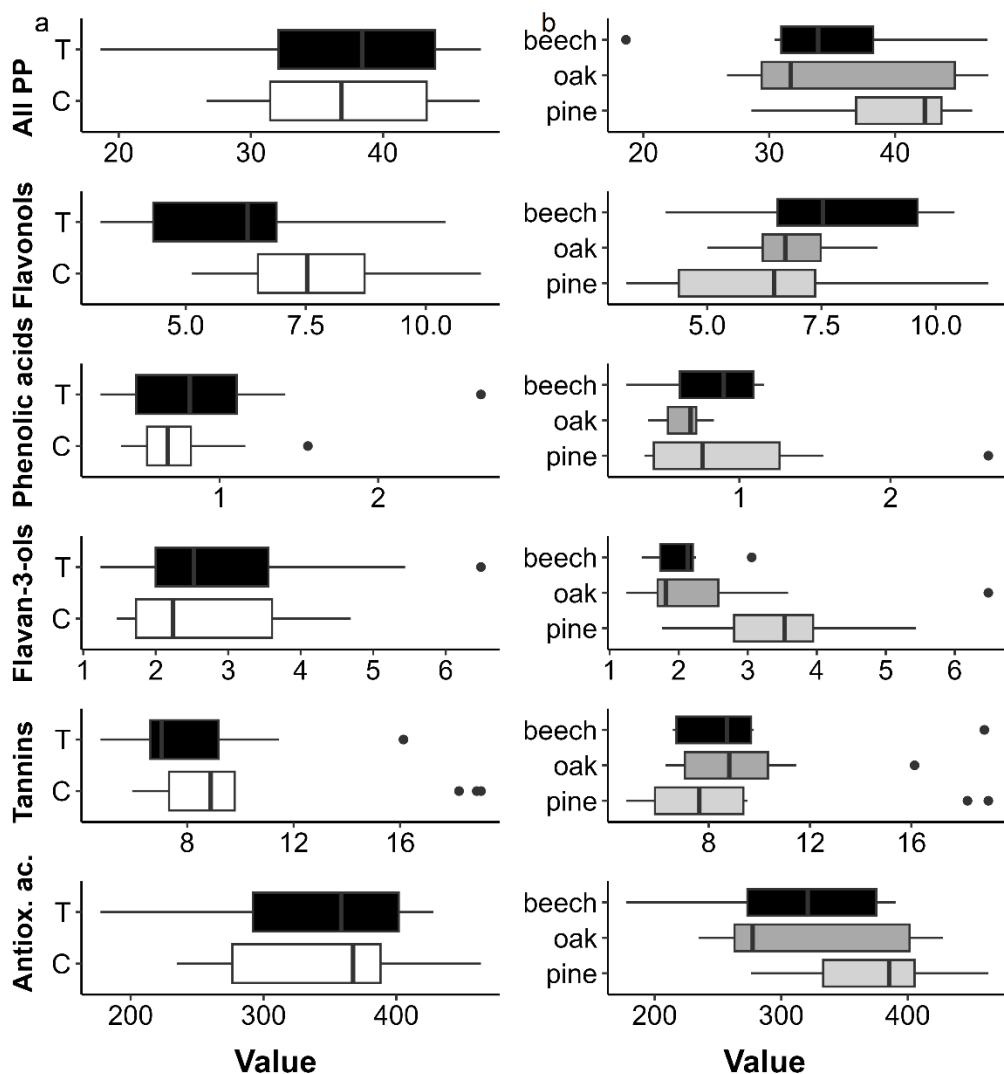
	DF	SoS	R ²	F-value	p-value
VOCs					
Treatment	1	1.23	0.19	6.42	0.001
Neighbourhood	2	0.41	0.06	1.08	0.329
Residual	25	4.79	0.74		
Total	28	6.43	1		
Phenolics					
Treatment	1	0.07	0.05	1.49	0.169
Neighbourhood	2	0.14	0.10	1.36	0.190
Residual	23	1.14	0.85		
Total	26	1.35	1		

282

283

284 *Phenolics in leaves.* The treatment with MeJA did not affect the phenolic composition of leaves
 285 (Figure 1b, Table 2). Neighbourhood also did not have an overall significant effect on phenolic
 286 composition (Figure 1b, Table 2). The total concentrations of all major classes of phenolics
 287 were not significantly higher in saplings that received the MeJA treatment (Table 3a, Figure
 288 2a). Flavonols showed significantly lower concentrations in induced trees (Table 3a; Figure
 289 2a). Antioxidant activity and C: N also did not differ significantly between the MeJA and
 290 control treatments (Figure 2a, Table 3a).

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Figure 2. Effects of a) MeJA (T = treated, C = control), and b) neighbourhood (dominated by beech, oak, or pine) for all phenolics (All PP) in mg gallic acid equivalent/g, classes of phenolics as mg/g of the dry leaf tissue (summing concentrations within classes), and antioxidant activity (Antiox. ac.) expressed as Trolox equivalent in $\mu\text{M Tx/g}$ for *Q. robur* saplings. The total flavonol concentration was significantly lower for MeJA-treated *Q. robur* saplings (Table 3a), and the total flavan-3-ols concentrations differed marginally significantly between neighbourhoods (Table 3b).

302 Table 3. One-way ANOVA results for the effect of a) MeJA treatment, and b) Neighbourhood
 303 on C to N ratio, concentrations of phenolics, and the antioxidant activity per tree species, with
 304 means. Units for All phenolics are mg gallic acid equivalent/g, individual (groups of) phenolics
 305 are in mg/g of the dry leaf tissue, summing concentrations within classes to calculate Sums per
 306 class, and antioxidant activity (Antiox. ac.) are expressed as Trolox equivalent in uM Tx/g. p
 307 < 0.05 is in bold font and p between 0.05 and 0.10 is underlined. Corrected values denote
 308 Bonferroni-corrected p-values. Significant effects of MeJA application were all negative
 309 (reduced concentration in leaves of MeJA treated saplings). Flavan-3-ols were elevated in
 310 neighbourhoods dominated by pines, specifically catechin (Cat). See Supplementary
 311 Information 1 for full names of individual compounds and categories.

a: MeJA treatment						b: Neighbourhood						
Class	variable	MeJA	Control	F-value	p-value	corrected	Oak	Beech	Pine	F-value	p-value	corrected
Sums												
	C to N ratio	21.15	20.26	0.63	0.435	1	19.03	21.10	21.63	2.238	0.129	1
	All phenolics	37.41	37.04	0.015	0.904	1	35.81	34.04	40.03	1.656	0.212	1
	Flavonols	6.01	7.77	5.953	0.022	1	6.89	7.76	6.30	1.132	0.339	1
	Phenolic acids	0.91	0.73	0.859	0.363	1	0.64	0.82	0.96	1.012	0.379	1
	Flavan3-ols	3.01	2.63	0.575	0.455	1	2.56	2.08	3.44	3.072	<u>0.065</u>	1
	Tannins	8.19	10.50	2.272	0.144	1	9.39	9.57	9.08	0.032	0.968	1
	Antioxidant activity	343.1	339.1	0.021	0.887	1	318.7	312.2	373.0	2.416	0.111	1
Flavonols												
	Qpent	0.032	0.015	0.772	0.388	1	0.00	0.009	0.049	3.363	<u>0.052</u>	1
	Qpent-hex	0.317	0.357	0.396	0.535	1	0.336	0.272	0.374	0.879	0.428	1
	Qhex-rham+PGG	0	0.123	5.022	0.034	1	0.090	0.000	0.074	0.716	0.499	1
	Qrut+Qgal	0.439	0.446	0.005	0.946	1	0.433	0.455	0.440	0.013	0.987	1
	Qgrm+Qglu+Irhhex-rham	1.969	2.137	0.29	0.595	1	2.131	2.240	1.884	0.480	0.624	1
	Qahex+Krut	0.365	0.456	0.748	0.395	1	0.382	0.407	0.428	0.064	0.938	1
	Irpent-hex	0.046	0.043	0.012	0.915	1	0.038	0.041	0.051	0.07	0.932	1
	Kglu	1.478	1.992	2.168	0.153	1	1.72	2.468	1.296	4.482	0.022	1
	Qrham	0.286	0.488	3.766	<u>0.064</u>	1	0.336	0.481	0.358	0.551	0.583	1
	Irgal	0.482	0.754	3.855	<u>0.061</u>	1	0.692	0.63	0.551	0.326	0.725	1
	Irgm+Irgal	0.149	0.141	0.025	0.877	1	0.189	0.174	0.100	1.301	0.291	1
	Kahex	0.087	0.441	5.133	0.032	1	0.2	0.108	0.383	0.981	0.390	1
	Irahex	0.047	0.099	3.241	0.084	1	0.061	0.055	0.09	0.528	0.596	1
	Kder	0.09	0.101	0.136	0.716	1	0.083	0.079	0.114	0.624	0.544	1
	K	0.217	0.176	0.533	0.472	1	0.202	0.339	0.111	8.628	0.002	<u>0.069</u>
Phenolic acids												
	3CQA	0.119	0.045	0.736	0.399	1	0.037	0.046	0.136	0.583	0.566	1
	p-coum der 1	0.540	0.437	0.337	0.567	1	0.414	0.412	0.587	0.47	0.631	1
	5CQA	0.030	0.028	0.022	0.883	1	0.016	0.04	0.031	3.855	0.035	1
	4CQA	0.221	0.222	0.001	0.978	1	0.169	0.317	0.201	2.298	0.122	1
	p-coum der 2	0.054	0.035	0.725	0.403	1	0.023	0.069	0.045	1.168	0.328	1
Flavan-3-ols (flavan-3-ols, procyanidins, and condensed tannins)												
	pr unid 1	1.751	1.670	0.203	0.656	1	1.613	1.697	1.787	0.337	0.717	1
	Cat	0.802	0.822	0.004	0.951	1	0.429	0.268	1.384	6.992	0.004	0.186
	pr unid 2	0.459	0.134	2.105	0.159	1	0.521	0.110	0.269	0.923	0.411	1
Tannins (elagitanins, gallotannins, elagic acid and elagic acid derivatives)												
	Ve	2.849	4.798	2.497	0.127	1	3.601	3.960	3.811	0.021	0.979	1
	diHHDP 1	0.353	0.344	0.049	0.827	1	0.377	0.328	0.341	0.467	0.633	1
	Ca	0.399	0.391	0.023	0.880	1	0.451	0.357	0.38	1.042	0.368	1
	diHHDP 2	0.006	0.007	1.044	0.317	1	0.006	0.007	0.006	0.424	0.659	1
	ET unid 1	0.029	0.028	0.001	0.979	1	0.013	0.046	0.028	0.457	0.638	1
	DGHHDP hex	0.188	0.195	0.078	0.782	1	0.194	0.203	0.183	0.267	0.768	1
	HHDP hex	0.177	0.15	0.587	0.451	1	0.159	0.137	0.183	0.592	0.561	1
	ET unid 2	0.186	0.196	0.113	0.739	1	0.215	0.190	0.176	0.574	0.571	1
	TGG 1	1.680	1.626	0.045	0.833	1	1.662	1.707	1.618	0.04	0.961	1
	Ca or Ve	1.265	1.73	3.224	<u>0.085</u>	1	1.522	1.582	1.412	0.134	0.876	1
	TGG 2	0.093	0.053	2.556	0.122	1	0.085	0.047	0.082	0.738	0.488	1
	EA der 1	0.071	0.061	0.679	0.418	1	0.07	0.062	0.066	0.103	0.902	1
	ET unid 3	0.036	0.037	0.031	0.861	1	0.033	0.033	0.04	0.775	0.472	1
	Cas	0.228	0.225	0.003	0.955	1	0.296	0.208	0.191	1.88	0.174	1
	EA der 2	0.443	0.461	0.136	0.715	1	0.480	0.491	0.409	1.267	0.300	1
	EA	0.184	0.196	0.212	0.649	1	0.223	0.214	0.153	4.577	0.021	0.953

312

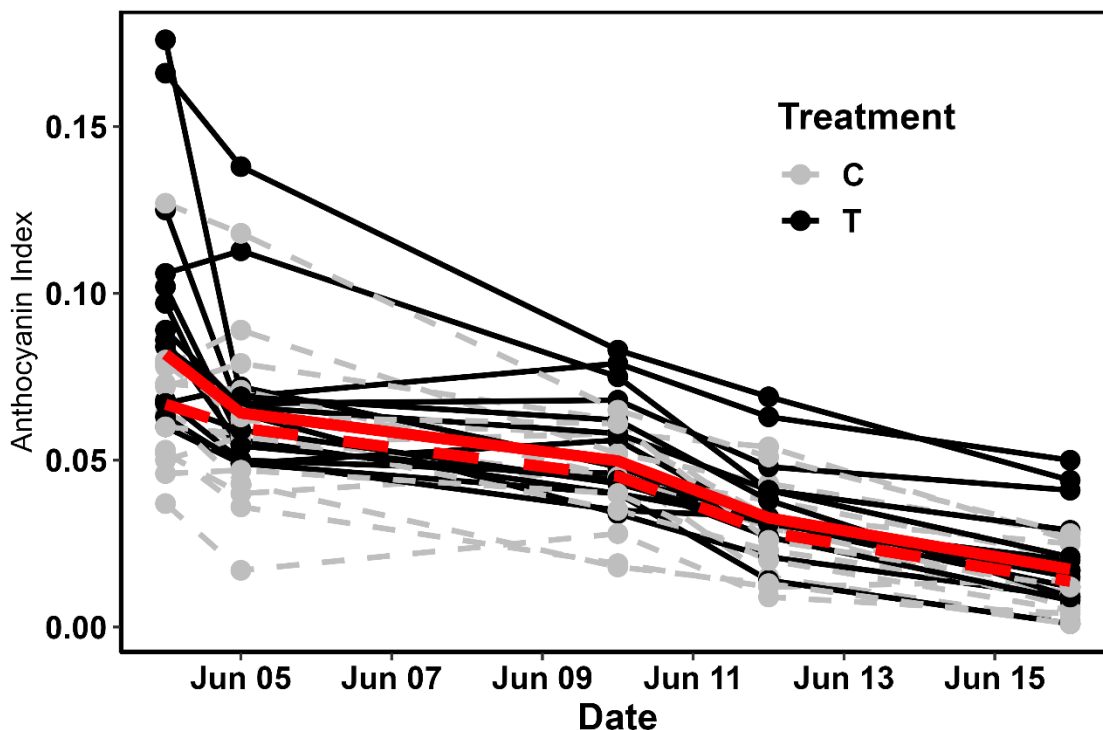
313 The total concentrations of phenolics in leaves of oak saplings were not significantly affected
314 by neighbourhood (Table 3b). Only the sum of flavan-3-ols concentrations was significantly
315 different between neighbourhoods (Table 3b), with higher concentrations in neighbourhoods
316 dominated by pines (Figure 2b). C: N and antioxidant activity did not differ significantly
317 between neighbourhoods (Table 3b).

318

319 After correction for multiple testing, no phenolic compound differed significantly in
320 concentration between saplings placed in different neighbourhoods (Table 3b). Without this
321 correction, five of 39 phenolic compounds had significantly different concentrations among
322 neighbourhoods (Table 3b).

323

324 *Temporal changes in leaf chemistry.* To gauge the speed of leaf phenolics induction and
325 changes in NBI and chlorophyll concentrations, we measured leaf parameters with the leafclip
326 Dualex+ at intervals during the study. All measured parameters changed over time (Table 4).
327 For anthocyanin we found significantly lower values for oak saplings treated with MeJA , but
328 the difference between treatment and control saplings decreased rather than increased over time
329 despite multiple applications of MeJA (Table 4, Figure 3).



330

331 Figure 3. The Anthocyanin index averaged per sapling as measured on live oak leaves using
332 the leafclip sensor. The red lines denote the averages for MeJA treated (solid) and control
333 saplings (dashed).

334

335

336 Table 4. Results of mixed effects models for leafclip-sensor parameters with sapling ID as a
 337 random effect. On the right are models selected by backward elimination of non-significant
 338 predictors, on the left are full models. All parameters changed over time, and only anthocyanin
 339 index was related to MeJA treatment.

	Full model					Selected model				
	Estimate	Std.Error	DF	t-value	p-value	Estimate	Std.Error	DF	t-value	p-value
Chlorophyll ($\mu\text{g}/\text{cm}^2$)										
Intercept	-15044.26	1016.87	113	-14.795	<0.001	-15347.12	708.42	114	-21.664	<0.001
Date	0.00	0.00	113	14.821	<0.001	0.00	0.00	114	21.701	<0.001
TreatmentT	-591.65	1422.34	25	-0.416	0.681					
Neighbour oak	1.19	1.76	25	0.675	0.506					
Neighbour pine	-0.52	1.70	25	-0.306	0.762					
Date * Treatment	0.00	0.00	113	0.415	0.679					
Flavonols Index										
Intercept	171.02	48.61	129	3.518	0.001	202.04	34.01	130	5.941	<0.001
Date	0.00	0.00	129	-3.494	0.001	0.00	0.00	130	-5.908	<0.001
TreatmentT	60.58	68.08	29	0.890	0.381					
Neighbour oak	-0.05	0.07	29	-0.772	0.446					
Neighbour pine	-0.04	0.07	29	-0.590	0.560					
Date * Treatment	0.00	0.00	129	-0.891	0.375					
Anthocyanins Index										
Intercept	83.39	7.84	113	10.640	<0.001	83.39	7.84	113	10.641	<0.001
Date	0.00	0.00	113	-10.635	<0.001	0.00	0.00	113	-10.635	<0.001
TreatmentT	24.82	10.96	25	2.264	0.033	24.82	10.96	27	2.265	0.032
Neighbour oak	0.00	0.01	25	-0.165	0.870					
Neighbour pine	0.00	0.01	25	0.134	0.894					
Date * Treatment	0.00	0.00	113	-2.263	0.026	0.00	0.00	113	-2.263	0.026
Nitrogen Balance Index										
Intercept	-18029	1535	121	-11.746	<0.001	-18081.22	1033.25	122	-17.499	<0.001
Date	0.00	0.00	121	11.761	<0.001	0.00	0.00	122	17.523	<0.001
TreatmentT	-92.89	2082.87	27	-0.045	0.965					
Neighbour oak	1.84	2.04	27	0.903	0.375					
Neighbour pine	0.77	1.98	27	0.388	0.701					
Date * Treatment	0.00	0.00	121	0.045	0.965					

340

341

342

343

DISCUSSION

344 To test our hypotheses on effects of herbivory and neighbourhood on plant defences, we placed
 345 oak saplings in neighbourhoods dominated by oak, beech, or pine trees, and then treated half
 346 with the phytohormone MeJA. We showed that VOCs were induced with MeJA application
 347 but leaf phenolics, C: N, NBI, and chlorophyll concentration were not changed. Leaf phenolic
 348 composition did not differ significantly between saplings surrounded by oaks, beeches, and
 349 pines, but flavan-3-ols were elevated in neighbourhoods dominated by pines. These results
 350 show that VOCs can rapidly be induced, while phenolic composition and leaf nutritional value
 351 do not respond rapidly to a chemical mimic of herbivory. Our findings also suggest that the
 352 composition of neighbouring trees can induce rapid changes in leaf chemistry in oak saplings.

353

354 **Response to MeJA.** Many inducible VOCs serve as infochemicals, attracting natural enemies
 355 of herbivores (Dicke and Hilker 2003; Karban 2021). The significant increases in emission of
 356 three VOCs within 24 hours after induction provide evidence that VOCs are readily inducible
 357 in oak saplings. The significantly increased emission of monoterpenes and a sesquiterpene
 358 corroborates other studies on oaks (Mrazova et al. 2023; Semiz et al. 2012). The emission of
 359 these VOCs indicates signalling to natural enemies (Rosenkranz et al. 2021). In particular,

360 terpenoids often act as signals to natural enemies (Rosenkranz et al. 2021). Moreover, emission
361 of these volatiles by oak saplings has been shown to be associated with the recruitment of
362 natural enemies of caterpillars (Mrazova et al. 2023).

363

364 In contrast to VOC emissions, phenolics and nutritional quality of leaves (C: N and NBI)
365 showed no response to MeJA induction over 16 days, although the leafclip measurements
366 indicated slightly higher anthocyanin levels in MeJA-treated saplings. This weak or absent
367 induction of phenolics corroborates delayed or weak responses to MeJA in leaf phenolic
368 chemistry in mature oaks (Ekholm et al. 2020; Volf et al. 2021), and may be typical for woody
369 plants (Haukioja and Hanhimäki 1985; Nykänen and Koricheva 2004). This difference in
370 inducibility between VOC emissions and phenolics was expected based on purported benefits
371 and costs of polyphenols such as tannins and volatiles. In short, recruiting natural enemies
372 using VOC signalling probably needs to be rapid to be effective. VOC productions might be
373 facilitated by the possibly low cost of production (Robert et al. 2013). In contrast, upregulating
374 defensive leaf chemistry may have little effect on herbivores that have already established
375 (Nykänen and Koricheva 2004), while producing such defences may be costly for some
376 defensive chemicals (Stamp 2003).

377

378 *Effect of neighbourhood.* We found some effects of neighbourhood on concentrations of
379 flavan-3-ols and a few other polyphenols in oak leaves. This effect occurred over the
380 experimental period of 18 days, which suggests that concentrations of some phenolics can
381 change over relatively short periods, if triggered by stimuli other than MeJA induction.
382 Phenolic compounds serve multiple functions in plants, including defence against herbivores
383 and UV protection (Salminen and Karonen 2011). Since we used potted plants that were not
384 affected by soil conditions at the given sites, the main factors in play could primarily involve
385 either shading and the resulting reductions in UV and desiccation stress, or stimulation by
386 VOCs emitted by the neighbouring trees. The shading gradient decreases from beech trees
387 through oaks to pines (Barbier et al. 2008; Kovács et al. 2017). Indeed, in saplings surrounded
388 by pines, we found the highest levels of flavan-3-ols, chemicals that can play important roles
389 in protection against radiation (Hernández et al. 2011; Šamec et al. 2021; Zhang et al. 2013).
390 However, if shading was the dominant factor affecting leaf chemistry of studied saplings, we
391 would expect to also find differences between neighbourhoods in chlorophyll content or NBI
392 (Šušić et al. 2023). No such effects were detected with the leafclip sensor. Perhaps chlorophyll
393 and NBI take more time to respond to shading. The leaf phenolic composition may also be a
394 plastic response to prime the plant for increased herbivore pressure in particular
395 neighbourhoods (similar to inter-population difference; Pihain et al. 2019). In this case, the
396 effect of pine VOCs on leaf chemistry may reflect lower abundance of parasitoids that are
397 specialised on insects that feed on oaks in pine-dominated forests. In turn, this may select oak
398 trees to rely more on defensive leaf chemistry than on emitting signals to parasitoids (no such
399 reduction was apparent in our data but exposure to neighbourhood was only two days before
400 VOC sampling). Elevated leaf phenolic concentrations would not be needed in oak-dominated
401 neighbourhoods where VOCs reduce herbivore pressure by attracting parasitoids. While
402 airborne communication between plants appears widespread (Karban 2021), further studies are
403 needed to verify if ambient VOCs in the forest understory can induce changes in leaf chemistry

404 of saplings, and if this correlates with herbivore pressure or effectiveness of VOC emission in
405 recruiting natural enemies.

406

407 *Individual differences.* We found individual differences in response to MeJA treatment among
408 *Q. robur* saplings. Three MeJA-treated *Q. robur* saplings clustered with controls. The
409 unresponsiveness to MeJA of these individuals indicates that there is variation among
410 individuals in the response to induction, as shown before in the same species (Ghirardo et al.
411 2012). Specifically, Ghirardo et. al (2012) found that oaks that were classified as ‘susceptible’
412 to defoliation by the moth *Tortrix viridana* (often a major pest on oaks) emit a strong VOC
413 signal that attracted *T. viridana* females, while ‘resistant’ oaks tended have low volatile
414 emission. Therefore, not emitting VOCs may be adaptive in the face of insect herbivores that
415 use the VOCs to locate profitable trees. This ecological cost of VOC emission may also vary
416 with neighbourhood. Such alternative defence strategies emphasize the importance of
417 considering not only differences between species (Mrazova et al. 2023) and populations (Pihain
418 et al. 2019; Valdés-Correcher et al. 2021), but also variation within plant populations.

419

420 **Conclusions**

421 We found that most *Q. robur* saplings induced with the phytohormone MeJA emitted a
422 significantly increased and altered VOC blend (six out of 18 identified VOCs were elevated),
423 but some individuals maintained low volatile emissions. In contrast, responses to MeJA in
424 terms of leaf phenolics and nutritional quality were absent or small. The neighbourhood in
425 which saplings were placed affected flavan-3-ol concentration within 18 days of exposure. Our
426 results suggest that oak saplings rely mostly on constitutive leaf chemistry to defend directly
427 against insect herbivores, and most (but not all) oak saplings emit VOCs, some of which are
428 known to attract that natural enemies of insect herbivores. The effect of the neighbourhood on
429 phenolics might be a response to variation in levels of abiotic stress, in particular the light
430 environment, or a response to the way herbivore pressure and their natural enemies are affected
431 by the neighbourhood of a tree.

432

433

434

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660

661 **Competing Interests**

662 The authors have no relevant financial or non-financial interests to disclose.
663

664 **Author contributions**

665 Freerk Molleman developed the initial concept of the study, led the fieldwork, analysed the data and
666 wrote the first draft of the manuscript, Manidip Mandal participated in the planning and fieldwork,
667 performed induction, collected the volatile samples, and participated in the VOC analysis, Anna Sokół-
668 Łętowska performed phenolics analyses, Urszula Walczak participated in developing the concept,
669 planning of the study, and in fieldwork, Martin Volf participated in developing the concepts and
670 planning of the study, Soumen Mallick participated in fieldwork and collection of volatile samples,
671 Martin Moos and Petr Vodrážka advised on volatile sample collection and performed volatile
672 measurements, Andreas Prinzing participated in developing the concept, Priscila Mezzomo participated
673 in developing the concept, provided training in VOC sample collection and interpretation of VOC
674 measurements. All authors contributed to the manuscript writing.
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