



## Volatile production differs between oak leaves infested by leaf-miner *Phyllonorycter harrisella* (Lepidoptera: Gracillariidae) and galler *Neuroterus quercusbaccarum* (Hymenoptera: Cynipidae)

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**Abstract.** Plants defend themselves by producing various volatile organic compounds (VOCs) that have direct and indirect effects on insect herbivores. Their production is often specific to the plant and herbivore species involved, with some herbivores being able to manipulate their production. Here, we used passive volatile sampling using polydimethylsiloxane (PDMS) tubing to compare VOCs produced by control, mined and galled oak leaves in the field. Leaves mined by a microlepidopteran leaf-miner (*Phyllonorycter harrisella*) produced a lower amount of two sesquiterpenes and an increased amount of eucalyptol. In contrast, leaves galled by the gall wasp (*Neuroterus quercusbaccarum*) did not produce a lower amount of any of the VOC measured when compared to the control. They produced a higher amount of farnesene,  $\beta$ -bourbonene and eucalyptol. Some of these VOC are known for their anti-herbivore function. In a second experiment, we treated the experimental leaves with the phytohormone methyl jasmonate (MeJA) to determine if leaf-miners or gallers reduced the overall inducibility of infested leaves. MeJA induced a sixteen-fold increase in VOC production. However, there was no difference in VOC production of control, mined and galled leaves treated with MeJA. Our results show that up- and down-regulation of VOCs can vary among leaves infested by different herbivores. More experiments are needed to determine if this is due to manipulation by the herbivores themselves or due to a defensive response of the plant.

### INTRODUCTION

With approximately 6.1 million estimated species, arthropods are a highly successful group (Hamilton et al., 2013). Their diversity is most commonly ascribed to the diversity of their feeding strategies, of which herbivory is the most widespread (Slansky & Rodriguez, 1987). In response to herbivory, plants have developed various defensive strategies, including a huge variety of secondary metabolites with both direct and indirect effects on herbivores (Degen et al., 2004; Wink, 2006). Direct chemical defences are targeted directly at the herbivore, affecting its behaviour or physiology. On the other hand, indirect chemical defences, such as herbivore induced plant volatiles (HIPVs), attract the predators and parasitoids of the herbivore (Aljibory & Chen, 2018). For example, blends of volatile organic compounds (VOCs) emitted by herbivore-

damaged leaves stimulate parasitoid ovipositional probing behaviour (Dutton et al., 2000) or attract birds (Mäntylä et al., 2008; Amo et al., 2013).

HIPVs are highly specific to particular species of herbivores (Danner et al., 2018) and act by attracting predators or parasitoids, which makes them an efficient form of defence against a diverse set of arthropod herbivores (De Moraes et al., 1998; Turlings & Erb, 2018). In response to the proliferation of plant defences, arthropod herbivores have evolved mechanisms like detoxification and sequestration (Heckel, 2014). Many guilds of arthropod herbivores, such as sap-suckers, leaf-chewers, leaf-miners or gallers, have even evolved an ability to manipulate plant metabolic pathways to their own advantage (Mapes & Davies, 2001; Tooker & De Moraes, 2011; Chung et al.,

2013; Savchenko et al., 2013; Giron et al., 2016; Zhang et al., 2016; Zhang, 2017).

In particular, gall-inducing arthropods are an excellent example of host manipulation. This group of herbivores is able to alter the metabolic pathways of the host and manipulate them to produce galls, which provide both shelter and highly nutritive food for the herbivore (Stone & Schönrogge, 2003). In addition, gallers stimulate the indole-3-acetic acid (IAA) pathway in plants, which can result in the downregulation of both the salicylic acid (SA) and the jasmonic acid (JA) pathways, by their negative crosstalk (Tooker & Helms, 2014). As a result, gallers not only manipulate the nutritive quality of plant tissues, but also downregulate defences of their hosts that depend on SA and JA pathways (Tooker et al., 2008; Hall et al., 2017). Many gallers are reported manipulating host VOCs (Tooker & De Moraes, 2007; Tooker et al., 2008; Torres-Gurrola et al., 2011). The specific effects, however, seem to depend on the herbivore-plant system studied (Hall et al., 2017; Borges, 2018). In some cases, the synthesis of VOCs does not differ in galled and intact plants (Hall et al., 2017), whereas in other cases VOCs that attract natural enemies of gallers are downregulated in the abovementioned fashion (Tooker & De Moraes, 2007).

Leaf-miners are another herbivore guild known to manipulate the nutritive value and defences of their hosts (Dutton et al., 2000; Vogler et al., 2010; Zhang et al., 2016; Zhang, 2017). Leaf-miners are known to maintain nutrient-rich green tissues in senescent and even fallen leaves, termed the green island phenotype (Giron et al., 2016). Leaf-miners induce cytokinins (CKs), phytohormones that regulate plant growth and affect senescence (Zhang et al., 2018). Recently, it was shown that a certain leaf-mining species (*Borborystis euryae*: Gracillariidae) is able to actively induce callus proliferation (Guiguet et al., 2018). Consequently, such a manipulation can hamper direct and indirect chemical defences of the plant (Giron et al., 2007). In addition, leaf-miners are known to induce changes in the VOC profiles of plants (Johne et al., 2006; Vogler et al., 2010; De Backer et al., 2017). Detailed understanding of the manipulation of VOCs by leaf-miners is currently lacking, but downregulation of VOCs is recorded (Vogler et al., 2010; Zhang et al., 2016) and different mechanisms proposed (Zhang, 2017). For example, by interfering with CK production, leaf-miners may be able to disrupt synthesis of sesquiterpenes, as both supposedly originate from the mevalonate (MVA) pathway. In addition, they may affect HIPV synthesis by manipulating the JA-pathway (Zhang, 2017).

To test if VOC emissions from leaves infested by leaf-miners and gallers differ, we compared VOCs emitted by control, mined and galled leaves of *Quercus robur*. In addition, in a second experiment, we treated the experimental leaves with methyl jasmonate (MeJA), which is known to trigger strong VOC production (Rodriguez-Saona et al., 2001). We compared whether the inducibility of leaves infested by gallers and leaf-miners differed from the control. We expected: (i) VOC production would be reduced or un-

changed in both galled and mined leaves in comparison to healthy leaves, and (ii) the inducibility of VOC production would be reduced in galled leaves as well as in mined leaves.

## METHODS

### Experimental site and organisms

We conducted the field part of this study from July 24<sup>th</sup> to July 27<sup>th</sup> 2018 at a study site of Leipzig University, the Arboretum Großpösna (51°40'N, 12°30'E).

We used *Quercus robur* as the host species for our experiment. *Q. robur* harbours a high diversity of gallers and leaf-miners. It is an ideal species for exploring responses to such herbivores. Oak VOCs include mainly monoterpenes, sesquiterpenes or fatty acid derivatives that can supposedly serve both as direct and indirect chemical defences (Pearse et al., 2013). We focused our observational study on a single mature *Q. robur* tree (diameter at breast height = 27.7 cm). This design has limitations in terms of generalising our results. However, it excluded other potential sources of variation such as the genetic background of the host or environmental conditions.

We selected 10 healthy, 10 galled and 10 mined leaves on this tree. Average size of the selected leaves was  $42.43 \text{ cm}^2 \pm 2.68$ . The selected leaves were E or SE oriented and ca 1.0–1.8 m above ground. All sampled leaves were mature and fully developed. The control leaves were undamaged by herbivores or pathogens and did not show any signs of mechanical damage. On the galled leaves there was an average of  $32 \pm 6.32$  galls. The average area of a single gall was  $0.0785 \text{ cm}^2 \pm 0.0064$ . Leaf area covered by galls averaged  $6.62\% \pm 1.65$ . In mined leaves there was an average of  $1.8 \pm 0.25$  mines. The average mine area was  $0.1081 \text{ cm}^2 \pm 0.0215$ . Leaf area mined averaged  $5.59\% \pm 0.75$ .

The leaf miner studied was *Phyllonorycter harrisella* (Linnaeus, 1761), the larvae of which feed exclusively on *Quercus* species. They go through three different developmental stages: an early serpentine phase, intermediate blotch phase and late tentiform phase. All selected mines were in the blotch phase.

The gall studied was *Neuroterus quercusbaccarum* (Linnaeus, 1758), which only galls oaks. *N. quercusbaccarum* has both agamic and bisexual generations. The agamic generation appears in spring. Unfertilized eggs are laid in the buds of *Quercus* leaves, where the larvae induce red, spherical galls. From these galls, the bigamic adults emerge in June, which lay fertilized eggs on the underside of oak leaves. The larvae induce flat, reddish disks, which were present when we conducted our experiment. All the galls were in a similar developmental stage.

We identified the gallers in the field based on gall morphology. The leaf-miner identity was confirmed by DNA barcoding using COI and standard primers and protocols (Folmer et al., 1994).

### VOC trapping

We sampled oak VOCs by passive trapping with polydimethylsiloxane (PDMS) tubes following a modified protocol of that used by Kallenbach et al. (2015). This method was previously successfully used for sampling VOCs in the field (Nordström et al., 2017). It reliably captures monoterpenes and sesquiterpenes (Kallenbach et al., 2014), which are the main constituents of oak VOC blends (Pearse et al., 2013).

Using the PDMS tubes, we sampled VOCs from individual leaves. First, we attached two PDMS tubes (technical replicates) by wire to each leaf. We then enclosed individual leaves and the PDMS tubes in polyamide (PA) oven bags. We closed the bags as tight as possible without damaging the leaf. We sampled VOCs from the headspace for approximately 24 h, then removed the

tubes and brought them to the laboratory for further analysis. Hereafter we refer to this dataset as “pre-treatment”.

After we sampled the VOCs for the first time, we treated the experimental leaves with MeJA that is commonly used to simulate herbivory and induce plant defences in a standard way (Degenhardt & Lincoln, 2006). We treated the leaves by spraying them with a 20 mM MeJA mixture (20 mM MeJA in 1% EtOH and 0.1% Triton-X), following Mrazova & Sam (2018) and Belhadj et al. (2006), who used MeJA concentrations of up to 15 mM and 30 mM, respectively, in their experiments on willows and grapevine. After approximately 90 min the plastic bags were closed again and VOCs were passively sampled for 24 h as described above. Hereafter we refer to this dataset as “post-treatment”.

Once the sampling was finished, we harvested and photographed the leaves. The images were processed and analysed in imageJ (Abramoff et al., 2004) to obtain the area of individual leaves and area occupied by herbivores.

### Chemical analysis

We used gas chromatography to quantify the sampled VOCs. The PDMS tubes were analysed using a thermal desorption-gas chromatograph-mass spectrometer (TD-GC-MS) consisting of a thermodesorption unit (MARKES, Unity 2, Llantrisant, United Kingdom) equipped with an autosampler (MARKES, Ultra 50/50). PDMS tubes were transferred to empty stainless steel tubes (MARKES) and desorbed with helium as the carrier gas and a flow path temperature of 150°C using the following conditions: dry purge 5 min at 20 ml/min, pre purge 2 min at 20 ml/min, desorption 8 min at 280°C with 20 ml/min, pre trap fire purge 1 min at 30 ml/min, trap heated to 300°C and hold for 4 min. The VOCs were separated on a gas chromatograph (Bruker, GC-456, Bremen, Germany) connected to a triple-quadrupole mass spectrometer (Bruker, SCION) equipped with DB-WAX column: (30 m × 0.25 mm inner diameter × 0.25 µm film thickness, Restek). The temperature program was: 60°C (hold 2 min), 30°C/min to 150°C, 10°C/min to 200°C and 30°C/min to 230°C (hold 5 min). Helium was used as the carrier gas at a constant flow rate of 1 ml/min. MS conditions were set at a 40°C manifold, 240°C transfer line and 220°C for the ion source. The scan-range was 33–500 m/z for a full scan and scan-time was 250 ms. We selected the most prominent peaks in the chromatograms (signal to noise ratio > 10). Peaks that were also present in air blanks were regarded as systemic contamination and were excluded from further analysis. VOCs were tentatively identified by comparison with the NIST database and comparison with retention indices in the literature. The peak areas of these compounds were calculated using the Bruker Workstation software (v8.0.1).

### Statistical analysis

VOC concentration was standardised for leaf area by calculating VOC production per cm<sup>2</sup> to account for variation in leaf size. First, we analysed if the total production of VOCs, their diversity (measured as Shannon-Index) and production of individual VOCs differed between control, mined and galled leaves using ANOVA. Total VOC production (summarised as the total peak area) and the area of peaks of individual compounds were log transformed. When a significant effect of herbivore infestation on the production of VOCs was recorded, we performed a post-hoc Tukey HSD test to compare the individual treatments. Pre- and post-treatment samples were analysed separately. The analyses were performed in R (R core team, Version 3.4.3, 2017).

Second, we compared the overall VOC profiles of control, mined and galled leaves using multivariate statistical methods. Areas of peaks of individual compounds were log transformed and used as response variables in the analyses. We visualised the

differences in VOC production of individual leaves using Principal Component Analysis (PCA). Then we analysed the effect of herbivore infestation using Redundancy Analysis (RDA). We used herbivore presence/absence as explanatory variables in the first analysis. As there was some variation in the area occupied by the herbivores, we then ran a second analysis using the quantitative data on the leaf area affected by the herbivores to confirm the results. The significance of the constrained axes was tested using Monte-Carlo test with 9999 permutations. Pre- and post-treatment samples were analysed separately. All multivariate analyses were conducted in CANOCO 5 (ter Braak & Šmilauer, 2012).

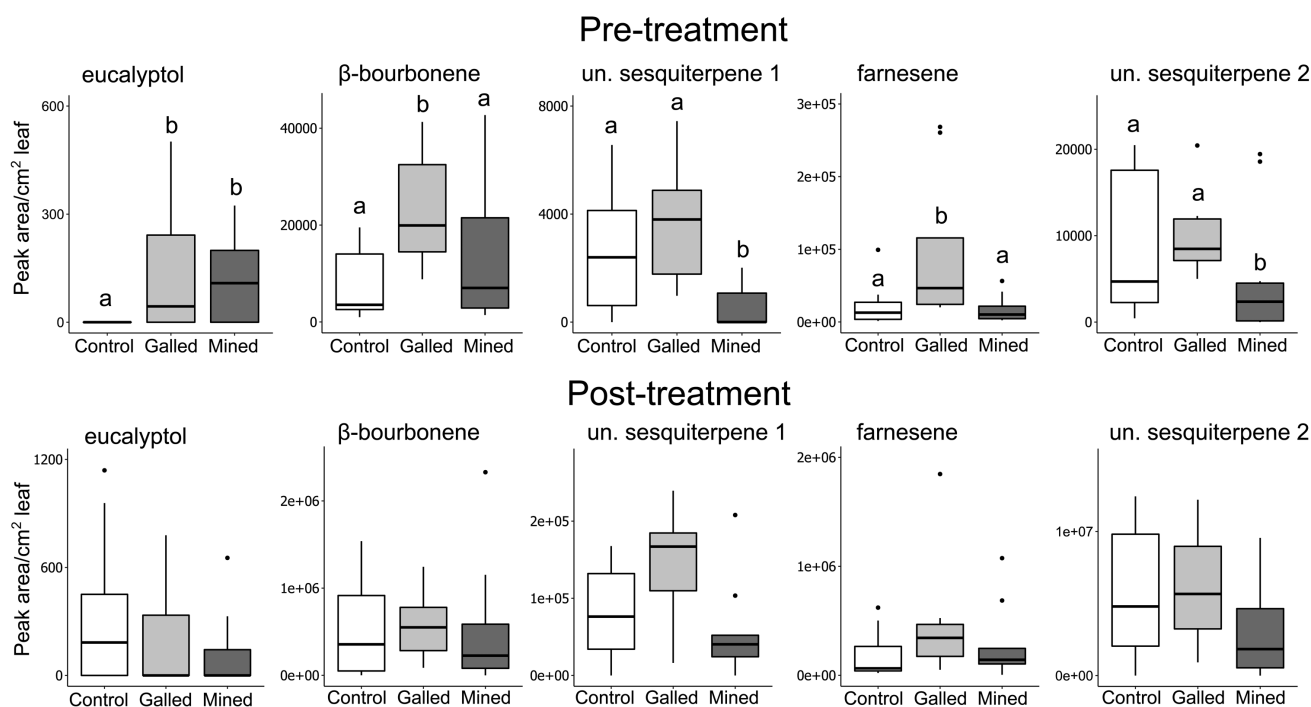
Finally, we tested if the VOC production increased after the MeJA treatment and if the response in terms of the total production of VOCs and production of individual VOCs differed between control, mined and galled leaves using repeated measures ANOVA. The total VOC production (summarised as the total peak area) and area of peaks of individual compounds were log transformed. MeJA treatment and herbivore infestation were used as explanatory variables. Leaf identity was used as a random factor. The analyses were performed in R (R core team, Version 3.4.3, 2017). Only the leaves for which the data from both pre- and the post-treatment were available, were included in this analysis. Therefore, the number of galled leaves in this analysis was reduced to eight (one leaf had to be replaced before the induction experiment and one died due to mechanical damage during the induction experiment).

## RESULTS

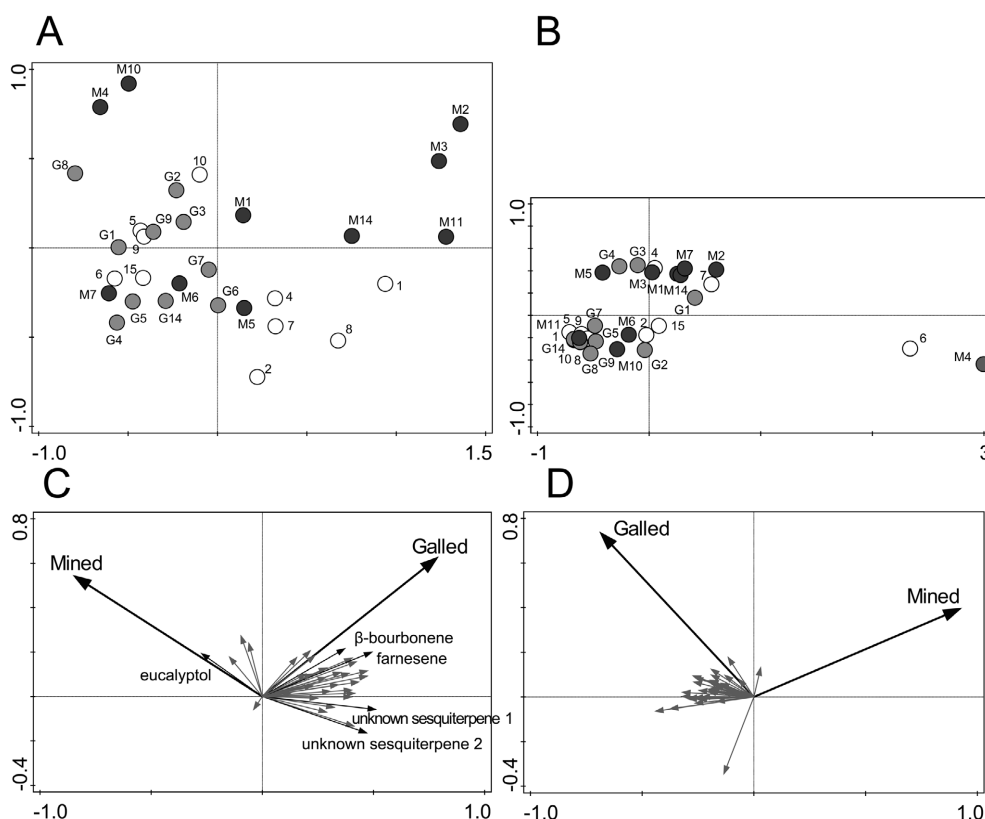
In total, we quantified 36 VOC compounds produced by the leaves (Table S1). Herbivore infestation did not have a significant effect on the total production of VOCs or their diversity in the pre- or post-treatments (Table 1). However, herbivore infestation affected production of five VOCs significantly when we analysed the production of individual compounds in the pre-treatment dataset. The production of eucalyptol (1-,8-cineol) was upregulated in galled and mined leaves (not detected in control leaves; peak area  $122.89 \pm 41.00$  in mined leaves and  $135.50 \pm 64.75$  in galled leaves;  $F_{(2,27)} = 5.506$ ,  $p = 0.010$ ). The production of  $\beta$ -bourbonene and farnesene was upregulated in galled leaves only. The production of  $\beta$ -bourbonene was 3× higher in galled leaves when compared to control leaves ( $23,486.71 \pm 4,294.22$  in galled leaves vs.  $7,874.61 \pm 2,226.76$  in the control;  $F_{(2,27)} = 4.795$ ,  $p = 0.017$ ). The production of farnesene was 4.2× higher compared to the control ( $94,646.76 \pm 37,688.91$  in galled leaves vs.  $22,451.98 \pm 9,359.14$  in the control;  $F_{(2,27)} = 5.906$ ,  $p = 0.015$ ). Mined leaves did not significantly differ from control leaves in terms of  $\beta$ -bourbonene or farnesene ( $13,010.74 \pm 4,478.86$  for  $\beta$ -bourbonene,  $17,593.86 \pm 5,801.01$  for farnesene). The production of unknown sesquiterpenes 1 and 2 was

**Table 1.** Differences in total volatile production (in terms of the total peak area per cm<sup>2</sup> of the leaf sampled) and Shannon-Diversity before (“pre-treatment”) and after the MeJA-treatment (“post-treatment”) as measured using a one-way ANOVA.

Measure	Treatment	$F_{(2,27)}$	p
Total VOC production	pre	2.522	0.099
	post	1.793	0.186
Shannon-Diversity	pre	0.290	0.751
	post	0.473	0.629



**Fig. 1.** Comparison of peak areas (standardised per cm<sup>2</sup> of leaf area; n = 10 for mined leaves and control, n = 8 for galled leaves) of compounds significantly up- or downregulated in the galled and mined leaves in pre-treatment (above) and post-treatment (below) as identified by ANOVA and Tukey HSD tests. Boxes are quartiles 2 and 3, bars IQR 1.5, thick line the median. Significant differences between control, galled and mined leaves identified by post-hoc tests are indicated by lowercase letters.



**Fig. 2.** Results of the PCA (above) and the RDA (below) of the pre-treatment (left) and post-treatment (right). A – variability in the volatile profiles emitted by the control, mined and galled leaves in the pre-treatment phase. First two unconstrained axes account for 51.78% of the variation. B – variability in volatile profiles emitted in the post-treatment phase. First two unconstrained axes account for 77.90% of the variation. C – the effects of herbivore infestation on the volatile profiles emitted by the control, mined and galled leaves in the pre-treatment phase. The constrained axes account for 14.1% of the total variation (pseudo-F = 2.2, p = 0.0128). Compounds that showed significant individual trends are in black and named. All other compounds are in grey. D – the effects of herbivore infestation on the volatile profiles emitted by the control, mined and galled leaves in the post-treatment phase. The constrained axes account for 6.9% of the total variation (pseudo-F = 1.2, p = 0.3047).

downregulated in mined leaves ( $530.03 \pm 237.77$  in mined leaves vs.  $2,556.28 \pm 746.90$  in control,  $F_{(2,27)} = 7.934$ ,  $p = 0.002$ , for unknown sesquiterpene 1; and  $5,185.83 \pm 2,363.90$  in mined leaves vs.  $8,877.92 \pm 2,587.23$  in control,  $F_{(2,27)} = 4.099$ ,  $p = 0.042$ , for unknown sesquiterpene 2). Galled leaves did not significantly differ in the production of these two compounds ( $3,701.44 \pm 772.11$  for unknown sesquiterpene 1 and  $9,983.86 \pm 1,746.14$  for unknown sesquiterpene 2; Fig. 1 above). No individual compound showed a significant response to herbivore infestation in post-treatment; Fig. 1 below).

The results of multivariate analyses were broadly consistent with the results of the ANOVA. The PCA for pre-treatment samples showed high variability in VOC profiles among mined leaves (Fig. 2A). Galled leaves had more uniform VOC profiles, but still did not form a very clearly defined cluster. There were no clearly defined clusters in the PCA analysis of post-treatment samples (Fig. 2B). The RDA revealed that the herbivore infestation accounted for 14.1% of the total variation in pre-treatment samples (pseudo- $F = 2.2$ ,  $p = 0.0128$ ; Fig. 2C). After treatment with MeJA, the significant effect disappeared (pseudo- $F = 1.2$ ,  $p = 0.3047$ ; Fig. 2D). The results remained largely unchanged when the quantitative data on the area occupied by the galls and mines of the herbivores were used. The herbivore infestation accounted for 12.6% of the total variation in pre-treatment samples (pseudo- $F = 2.2$ ,  $p = 0.0318$ ). After treatment with MeJA, the effect was no longer significant (pseudo- $F = 1.0$ ,  $p = 0.4019$ ).

The total VOC concentration increased sixteen-fold after treatment with MeJA (mean total peak area in pre-treatment  $809,662.21$  vs.  $133,314,94.40$  in post-treatment,  $F_{(1,53)} = 90.063$ ,  $p < 0.001$ ). Regarding individual compounds, all of them except three, one of which was eucalyptol ( $F_{(1,49)} = 0.519$ ,  $p = 0.4746$ ), were emitted in a significantly higher concentration after treatment with MeJA. There was no significant interaction between the treatment and the herbivore infestation on the total production of VOCs ( $F_{(2,49)} = 0.086$ ,  $p = 0.9173$ ) or production of individual compounds.

## DISCUSSION

We examined the effects of infestations of a galler and leaf-miner on the production of leaf VOCs in *Quercus robur*. We did not find a decrease in total production or diversity of VOCs emitted by infested leaves. Neither did we detect any downregulation of individual terpenoid VOCs in galled leaves as reported in some previous studies (Tooker & De Moraes, 2007; Torres-Gurrola et al., 2011; Jiang et al., 2018). However, we recorded that the production of two unidentified sesquiterpenes was downregulated only in mined leaves. In addition, three compounds were upregulated in both galled and mined leaves or only in galled leaves. These results confirm findings of previous studies and illustrate that VOC production can vary between leaves infested by different herbivores (Vogler et al., 2010; Clavijo McCormick et al., 2014; Jiang et al., 2018).

Leaf-miners are able to alter metabolic pathways of their hosts and modulate the production of major phytohormones

(Body et al., 2013; Zhang et al., 2016). This includes changes in biosynthesis of jasmonic acid, salicylic acid, abscisic acid or cytokinins (Giron et al., 2016; Zhang, 2017). Such changes can positively affect the nutritive value of the tissue the leaf-miner feeds on and can hamper the direct and indirect chemical defences of the host (Zhang et al., 2016; Guiguet et al., 2018). For example, apple plants infested with *Phyllonorycter blancardella* emit less sesquiterpenes than healthy ones (Vogler et al., 2010). Potentially, this could benefit the miner as some sesquiterpenes can attract parasitoids or repel ovipositing females when produced in larger quantities in that system (Dorn & Hern, 1999; Vogler et al., 2010). Here we recorded a similar trend as oak leaves infested with *P. harrisella* produced a lower amount of two unknown sesquiterpenes. Although this may suggest a possible manipulation of oak sesquiterpenes by *P. harrisella*, it is not possible to conclude whether the lower production of these VOCs is a by-product of induced changes in metabolic pathways or whether their downregulation possibly benefits the leaf-miner. Identifying the precise structures and conducting manipulative experiments on the bioactivity of these compounds would be needed to confirm this.

None of VOCs were produced in lower amounts by the leaves infested with the studied gallers. Gallers are known for their ability to manipulate their hosts (Giron et al., 2016). Such manipulation can also involve downregulating the production of VOCs in some cases, as reported for the dipteran *Mayetiola destructor* (Tooker & De Moraes, 2007) and the hemipteran *Trioza anceps* (McKinnon et al., 1999; Tooker & De Moraes, 2007; Torres-Gurrola et al., 2011; Jiang et al., 2018). However, this does not always seem to be the case. In their review, Hall et al. (2017) show that galling insects downregulate mainly the direct chemical defences of their hosts, while the production of many VOCs does not differ between galled and control plants. They attribute this to the different function of direct and indirect chemical defences. Downregulating chemical defences with direct negative effects is usually beneficial for the galler (Sarmiento et al., 2011). On the other hand, maintaining VOC constitutive levels may be a more beneficial strategy than their downregulation as this could mask the presence of the galling insect from its natural enemies (Hall et al., 2017).

Infestation with the galling insect, and to a lesser extent the leaf-miner, also led to an upregulation of several VOCs (eucalyptol,  $\beta$ -bourbonene and farnesene) and to VOC profiles that significantly differed from that of the control leaves. This is similar to the findings of Jiang et al. (2018), who report an increase in VOC production by *Quercus robur* leaves infested with the gall wasps *Cynips spp.* and *Neuroterus albipes*. Compounds upregulated in the leaves infested by galling and mining insects differed slightly in our study. Eucalyptol was upregulated in both mined and galled leaves. Eucalyptol is known for its insect-repellent (Edwards et al., 1993; Sfara et al., 2009) and insecticide effects (Sabraoui et al., 2016). Its production is often stimulated by herbivory (Torres-Gurrola et al., 2011; Santos et al., 2016). On the other hand,  $\beta$ -bourbonene and farnesene

were upregulated only in galled leaves. Farnesenes are present in VOC blends emitted by a wide range of species of plants in response to herbivory (Dorn & Hern, 1999; Kigathi et al., 2009; Allmann & Baldwin, 2010; Pierre et al., 2011). They seem to act as both direct defences, by repelling herbivores, and as indirect defences, in attracting their natural enemies (De Moraes et al., 1998; Wei & Kang, 2006). Vogler et al. (2010) record a lower production of farnesenes in miner-infested leaves of apple plants. Although we did not record a similar trend, it is interesting that while the leaves galled by the galling insect produced a higher amount of farnesene those infested by miners did not differ in farnesene production from the control.

The compound  $\beta$ -bourbonene was previously detected in VOC bouquets of *Quercus robur* (Ghirardo et al., 2012), where it is upregulated in response to herbivory by the leaf-rolling microlepidopteran *Tortrix viridana*. Its upregulation is also reported in other plant species, typically in response to damage by chewing insect herbivores (Mäntylä et al., 2008; Gossner et al., 2014). Together with our results, this indicates that the production of  $\beta$ -bourbonene can be upregulated in leaves attacked by various herbivores, or by leaf-chewing and galling herbivores. Although some results indicate that  $\beta$ -bourbonene might be involved in attracting predators (Mäntylä et al., 2008), more information on its specific bioactivity is needed to fully explore its role. This is also the case of several of the compounds mentioned above. Yet, having information on the bioactivity of VOCs is crucial for interpreting similar results. This is because upregulation of various metabolites and pathways can have differential effects on herbivores. For example, the Colorado potato beetle (*Leptinotarsa decemlineata*) and its symbiotic bacteria elicit the SA pathway and its dependent defences while suppressing the induction of JA dependent defences by SA and JA negative crosstalk (Chung et al., 2013). In turn, herbivory leads to upregulation of plant defences but not the ones efficient against the respective herbivore, which benefits the beetle.

We found no difference in VOC production between control, mined and galled leaves once they were treated with MeJA. This was probably due to the high concentration of MeJA we used and its strong effect, which may have saturated the plant's response. We were thus unable to address our second hypothesis that the inducibility will differ between mined, galled and control leaves. MeJA is commonly used in ecological studies as it triggers a massive and unspecific response in VOC production (Franceschi et al., 2002; Kost & Heil, 2008; Mäntylä et al., 2014). High concentrations of MeJA are used when assessing general patterns such as the attraction of predators by plant VOCs (Belhadj et al., 2006; Mrazova & Sam, 2018). However, the effects of highly concentrated MeJA can mask responses to naturally occurring herbivory as illustrated by our study. Using lower concentrations of MeJA, such as in Rigsby et al. (2019), would be advisable for experiments similar to the one we carried out. Alternatively, using real herbivores, such as caterpillars, could be an even better way to

investigate the changes in VOC inducibility under natural conditions (Peñaflor et al., 2017).

More surveys are needed to explore the diversity of plant VOCs, their responses to herbivory under natural conditions and their bioactivity. One of the main limitations of our study is the small sample size. Methods of passive VOC sampling should facilitate a significant increase in sample size in future studies. As demonstrated by Kallenbach et al. (2014) and Chalal et al. (2015), PDMS tubes are a labour- and cost-efficient as well as easy-to-handle method of passive sampling of VOCs. We showed that this method is able to detect differences in VOC production between leaves infested by different herbivores. In combination with recent advances in the -omic approaches, mass spectrometry and gas chromatography (Wu et al., 2013; Dyer et al., 2018), such field surveys supported by manipulative experiments are likely to greatly contribute to our comprehension of complex trophic interactions between plants and insects.

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**Table S1.** Summary of average peak areas of all compounds detected by TD-GC-MS. The peak areas for the upper five compounds recorded in the mined, galled and control leaves were significantly different.

No.	Compound	RT (min)	RI Calculated	RI Literature	Average peak area/cm <sup>2</sup> pre-treatment			Average peak area/cm <sup>2</sup> post-treatment		
					Control	Mine	Gall	Control	Mine	Gall
1	eucalyptol	3.54	1228	1224	n.d	122.89	135.50	334.51	117.20	183.32
8	β-bourbonene <sup>a,e</sup>	5.21	1551	1585	7,874.61	13,010.74	23,486.71	531,655.65	519,375.10	580,922.44
12	unknown sesquiterpene 1	5.57	1616		2,556.28	530.03	3,701.44	82,420.14	55,520.19	142,814.45
19	farnesene <sup>a,e</sup>	6.36	1734	1725	22,451.98	17,593.86	94,646.76	180,572.19	282,557.57	485,766.86
20	unknown sesquiterpene 2	6.45	1746		8,877.92	5,185.83	9,983.96	5,822,889.29	3,007,836.70	6,129,445.43
2	β-ocimene <sup>a,e</sup>	3.72	1262	1242	68,086.71	74,026.94	123,691.60	738,905.02	522,452.55	1,174,649.30
3	unknown 1	4.00	1316		164,666.05	199,200.31	232,291.61	1,252,787.37	616,764.72	1,132,417.07
4	unknown 2	4.03	1323		927.79	3,093.55	966.82	49,177.24	24,691.47	37,904.48
5	α-cubebene <sup>b,e</sup>	4.84	1481	1463	1,463.67	1,909.40	2,894.94	168,011.03	87,742.18	144,193.40
6	unknown sesquiterpene 3	4.90	1492		n.d	n.d	n.d	28,641.48	10,695.47	50,728.89
7	unknown sesquiterpene 4	5.06	1522		6,484.39	7,224.92	9,012.21	64,332.78	43,496.04	74,071.41
9	unknown sesquiterpene 5	5.29	1566		1,810.60	1,923.21	2,376.69	123,875.62	65,336.22	141,246.44
10	unknown sesquiterpene 6	5.39	1582		108.57	207.07	83.72	167,858.45	77,500.77	89,115.24
11	β-copaene <sup>c,e</sup>	5.54	1608	1585	591.82	739.87	1,307.94	50,742.95	31,583.64	59,489.65
13	unknown sesquiterpene 7	5.65	1627		1,711.26	2,494.76	3,631.96	126,217.45	86,267.53	158,447.42
14	caryophyllene <sup>a,e</sup>	5.70	1633	1663	6,616.95	6,255.08	10,353.10	270,435.20	161,675.81	234,033.88
15	unknown sesquiterpene 8	5.78	1646		664.19	512.77	1,656.93	12,208.15	n.d	1,904.07
16	methyl benzoate <sup>d,e</sup>	5.82	1654	1635	323.26	880.56	997.74	43,018.71	25,035.20	25,294.26
17	γ-murolene <sup>b,e</sup>	6.17	1706	1684	7,100.86	6,405.23	10,511.43	266,855.74	170,196.22	276,165.54
18	unknown sesquiterpene 9	6.25	1718		5,197.89	5,813.73	8,168.66	149,050.59	81,892.85	195,811.66
21	unknown sesquiterpene 10	6.54	1759		315,081.33	443,999.05	693,291.04	4,885,167.34	4,490,019.77	9,235,564.28
22	unknown sesquiterpene 11	6.60	1767		37,980.93	45,833.13	85,839.14	87,804.25	23,719.60	91,854.49
23	unknown sesquiterpene 12	6.71	1883		21,656.28	18,737.43	26,252.05	275,570.97	146,826.73	368,282.89
24	unknown sesquiterpene 13	6.77	1890		4,789.29	5,275.57	6,973.86	191,513.83	101,639.33	243,827.39
25	unknown 2	6.87	1904		2,267.82	8,850.39	3,390.82	274,229.28	164,119.21	229,489.54
26	unknown sesquiterpene 14	6.93	1912		416.11	878.93	737.39	37,762.47	21,345.35	51,132.15
27	unknown sesquiterpene 15	7.00	1921		1,646.18	1,524.32	2,302.38	51,844.16	30,960.68	79,152.65
28	unknown 3	7.07	1931		414.73	1,759.41	874.36	61,301.49	34,398.37	49,912.74
29	unknown 4	7.27	1956		76.94	837.95	165.63	30,209.79	17,033.54	25,919.77
30	unknown sesquiterpene 16	7.34	1965		152.87	81.23	118.58	39,980.28	32,710.07	81,837.98
31	unknown 5	7.70	2008		454.81	546.36	2,167.47	24,118.19	12,989.26	17,277.92
32	unknown 6	8.85	2025		2,231.31	2,920.67	3,344.97	46,871.55	79,981.66	80,426.60
33	hexenol benzoate <sup>e</sup>	9.66	2079		15,332.19	19,677.09	21,939.34	59,442.48	80,395.46	95,819.47
34	unknown sesquiterpene 17	9.92	2095		5,136.49	5,087.44	6,312.59	8,422.44	8,060.22	15,927.51
35	α-cadinol <sup>b,e</sup>	10.317	2119	2211	4,883.35	4,602.08	6,125.24	11,919.25	9,813.74	21,308.71
36	unknown 7	10.44	2126		n.d	n.d	n.d	13,041.85	8,827.29	17,375.40

<sup>a</sup> Chung et al., 1993; <sup>b</sup> Choi, 2003; <sup>c</sup> Sylvestre et al., 2007; <sup>d</sup> Ferreira et al., 2001; <sup>e</sup> NIST, 2011.