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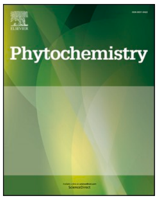

Variation in induced responses in volatile and non-volatile metabolites among six willow species: Do willow species share responses to herbivory?

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ABSTRACT

Chemical variation is a critical aspect affecting performance among co-occurring plants. High chemical variation in metabolites with direct effects on insect herbivores supports chemical niche partitioning, and it can reduce the number of herbivores shared by co-occurring plant species. In contrast, low intraspecific variation in metabolites with indirect effects, such as induced volatile organic compounds (VOCs), may improve the attraction of specialist predators or parasitoids as they show high specificity to insect herbivores. We explored whether induced chemical variation following herbivory by various insect herbivores differs between VOCs vs. secondary non-volatile metabolites (non-VOCs) and salicinoids with direct effects on herbivores in six closely related willow species. Willow species identity explained most variation in VOCs (18.4%), secondary non-VOCs (41.1%) and salicinoids (60.7%). The variation explained by the independent effect of the herbivore treatment was higher in VOCs (2.8%) compared to secondary non-VOCs (0.5%) and salicinoids (0.5%). At the level of individual VOCs, willow species formed groups, as some responded similarly to the same herbivores. Most non-VOCs and salicinoids were upregulated by sap-suckers compared to other herbivore treatments and control across the willow species. In contrast, induced responses in non-VOCs and salicinoids to other herbivores largely differed between the willows. Our results suggest that induced responses broadly differ between various types of chemical defences, with VOCs and non-VOCs showing different levels of specificity and similarity across plant species. This may further contribute to flexible plant responses to herbivory and affect how closely related plants share or partition their chemical niches.

1. Introduction

Plants produce various defensive metabolites, many of which can be induced by insect herbivory (Janz, 2011; Wetzel and Whitehead, 2020). Induced chemical defences differ in their roles in plants (Kessler and Baldwin, 2001). For example, chemical defences, such as various non-volatile plant metabolites (non-VOCs), can directly impair herbivores' feeding preferences and performance (Agrawal et al., 2000; Johnson et al., 2009). In contrast, many volatile organic compounds (VOCs) serve as indirect defences and attract predators and parasitoids of insect herbivores (Kessler and Baldwin, 2001; Fatouros et al., 2012; Pellissier et al., 2016). Herbivory by different insect species can trigger contrasting chemical responses that differ in specificity between VOCs and non-VOCs (Danner et al., 2018; Mezzomo et al., 2023). Theoretically, such specificity in induced responses can improve their efficacy as different herbivores and their natural enemies respond to different plant metabolites (De Moraes et al., 1998; Mumm and Dicke, 2010; Li et al., 2022). Understanding trends in intra- and interspecific variation in induced responses is thus a critical step towards unravelling their roles in plant defence.

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Induced direct defences seem to have especially pronounced effects on generalist herbivores. For example, these defences can affect larval growth or impair herbivore feeding (Ali and Agrawal 2012). In contrast, many specialised insect herbivores evolved adaptations that allow them to partly avoid such adverse effects or even use lineage-specific metabolites from their host plants as feeding or oviposition cues (Ali and Agrawal 2012). On the community scale, this could lead to chemical divergence in direct defences among co-occurring plants in a manner that contributes to maintaining their chemical diversity (Sedio and Ostling, 2013). Indeed, previous studies found high variation and low phylogenetic signals in direct chemical defences (Kursar et al., 2009; Carrillo-Gavilán et al., 2015). Such trends seem particularly pronounced in the case of closely related sympatric species that appear to partition their chemical niches to avoid sharing specialised herbivores adapted to a particular combination of plant traits (Becerra 2007; Sedio et al., 2018a,b; Leong et al., 2024).

Inducible VOCs play various roles in plant defences. Some of them directly affect plant-herbivore interactions or mediate plant-plant communication. Simultaneously, other VOCs attract predators and parasitoids, serving as infochemicals mediating tri-trophic interactions (Turlings and Erb, 2018). The upregulation of VOCs upon herbivory is typically faster than in the case of non-VOCs (Volf et al., 2021; Mezzomo et al., 2023). VOCs also exhibit relatively high specificity as their upregulation differs between plants attacked by herbivores from different taxa and feeding guilds (Danner et al., 2018; Mezzomo et al., 2023). Emitting specific signals, such as particular VOCs or blends of VOCs as a response to specific herbivory, can enhance the attraction of specialised parasitoids or predators (Turlings and Erb, 2018). Potentially, it may also improve plant-plant communication between conspecific or congeneric hosts, which leads to more efficient induction of defences with direct effects on herbivores (Hughes et al., 2015). However, to ensure efficient communication, one might expect that the signals emitted by co-occurring plants attacked by the same herbivores would exhibit some conservatism. Otherwise, the signals might be too diverse to convey information reliably (Zu et al., 2020).

The differential functional roles of VOCs and non-VOCs may support contrasting trends in their induced variation among co-occurring plant species. While induced responses in non-VOCs might primarily reflect the chemical niche partitioning between plant species, VOCs may follow the trends expected under the ’mutual benefit hypothesis’, which predicts the effects of defences shared among different co-occurring hosts (Volf et al. 2019). Such patterns in responses may further contribute to differential trends, resulting in either a low or negative correlation between non-VOCs and VOCs, as observed by previous studies (Rasmann et al., 2015; Volf et al., 2021; Mezzomo et al., 2023).

Willows (*Salix* L.) include multiple species growing in sympatry and are ideal models for exploring the trends in specificity and intraspecific variation of induced plant responses. Willows produce diverse defensive chemistry (Volf et al., 2015). Their nonvolatile leaf defences are largely phenolic-based and include various tannins or flavonoids. Additionally, they include salicinoids, which are phenolic glycosides characteristic of the Salicaceae family (Volf et al., 2015, 2023). Salicinoids can affect generalist herbivores by acting as feeding deterrents, retarding larval growth, or increasing mortality (Kolehmainen et al., 1995). However, several specialised herbivores (e.g., leaf beetles) evolved counter-adaptations to overcome direct chemical defences in willows (Peacock et al. 2001; Unsicker et al. 2015). The pressure by these specialised herbivores may have promoted divergence in chemical defences among co-occurring willow species, leading to pronounced differences in their non-VOCs (Volf et al., 2023). Willows also produce various inducible VOCs with direct and indirect effects on herbivores (Hughes et al., 2015; Mrazova and Sam, 2018). For example, the monoterpene alpha-pinene was positively correlated with increased bird predation rates in the grey willow (Mrazova and Sam, 2018). VOCs in willows and other Salicaceae species seem to be particularly strongly induced by herbivory from specialised insects such as leaf beetles (Peacock et al., 2001; Unsicker et al., 2015).

We studied six willow species commonly occurring in sympatry to explore their induced responses in VOCs and non-VOC secondary metabolites (non-VOCs). Among non-VOCs, we focused on induced responses in salicinoids that play a key role in willow defence against herbivores (Volf et al., 2015). We measured the response variation induced by different herbivore treatments consisting of plants exposed to different herbivore species and guilds. We compared the induced variation with the interspecific variation between the willow species. We expect that: I) The herbivore treatments will induce responses in the plants, with VOCs showing stronger responses than non-VOCs. II) The induced variation in VOCs will mainly reflect the herbivore identity, with closely related willow species responding similarly to individual herbivores. III) The induced variation in non-VOCs and salicinoids will mainly reflect willow species identity, with closely related species responding differently. IV) Induced responses in VOCs vs. non-VOCs and salicinoids will be largely independent, resulting in no correlation between them. By testing these hypotheses, we further explore how variation in different chemical defences may contribute towards defence in co-occurring plant species.

2. Results

Trends were quantified in three sets of metabolites: i) VOCs, ii) nonvolatile secondary metabolites (non-VOCs), and iii) salicinoids plus their derivatives.

2.1. VOCs

We detected 24 VOCs emitted by the plants upon herbivory (Appendix S2 – Table S1). Most of them belonged among green-leaf-volatiles (GLVs), monoterpenes, and sesquiterpenes, although other classes of VOCs, such as alkanes and aldehydes, were also detected. The samples clustered according to the willow species, but clustering according to the treatment was also visible (Fig. 1). The results of the RDA-based variation partitioning supported these findings. The plants significantly responded to the herbivore treatment. Its independent effect explained 2.8% of the observed variation ($f = 2.6$, $p = 0.0001$). The willow species identity explained 18.4% of the observed variation ($f = 9.5$, $p = 0.0001$). The herbivory damage did not explain a significant amount of the observed variation in VOCs ($f = 0.7$, $p = 0.5024$).

Some willow species formed groups and shared similar responses when comparing the emissions of individual VOCs (Fig. 2). For example, herbivory by the specialist leaf beetle *Chrysomela vigintipunctata* increased the production of monoterpenes such as β-ocimene and linalool and the majority of GLVs in *Salix cinerea* and *S. purpurea*. The leaf beetle also increased the emissions of the sesquiterpenes farnesene, α-farnesene, and (*Z*, *E*)-α-farnesene compared to control samples in most species (Fig. 2). Herbivory by the generalist caterpillar *Lymantria dispar* decreased the emissions of GLVs such as (*E*)-2-Pentenal, n-Hexyl acetate in *S. aurita*, *S. triandra*, *S. purpurea* and *S. viminalis*. Herbivory by the generalist caterpillar *Operophtera brumata* elicited an increase in emissions of the aldehyde nonanal in *S. aurita* and *S. cinerea* and the monoterpene limonene in *S. cinerea.*, *S. fragilis* and *S. viminalis*. The plants exposed to the sapsucker *Aphrophora salicina* generally showed weaker emission of VOCs than the other treatments, except for the sesquiterpene caryophyllene that increased in *S. fragilis* and *S. triandra*.

There was a significant positive correlation between the similarity in induced responses in VOCs upon feeding of the leaf beetle *C. vigintipunctata* and the phylogenetic distance between the studied willow species ($r = 0.733$, $p = 0.004$; Appendix S3 – Table S3). Other correlations were not significant.

2.2. Non-VOCs (lasso-selected metabolites)

We detected 13,414 non-VOCs, of which 8871 occurred in at least

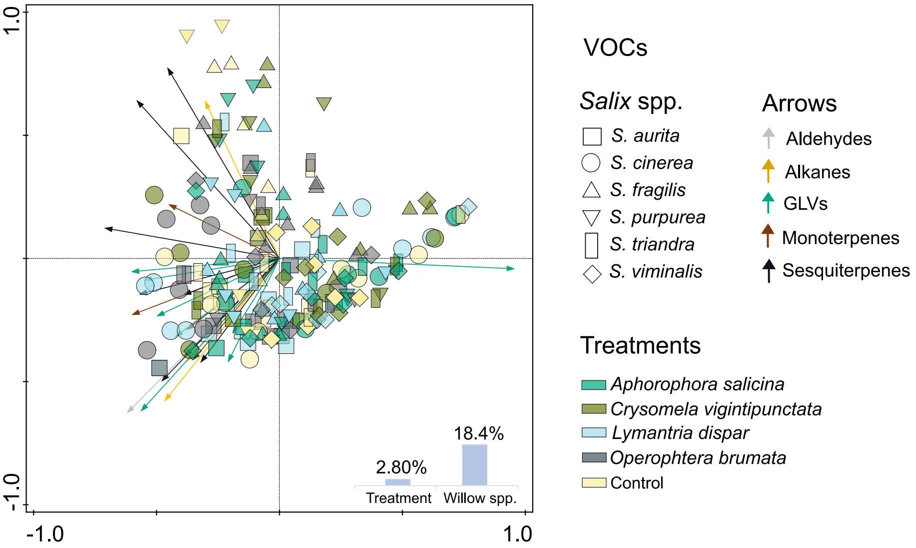

Fig. 1. Principal Component Analysis (PCA) diagram showing the variation in VOCs in the Hellinger standardised dataset. The first two axes of the PCA explained 64.8% of the variation. Symbols represent willow species and are colour-coded according to insect and control treatments. Arrows represent individual VOCs and are colour-coded according to the VOC classes (GLVs = Green-leaf volatiles). The significant results of RDA-based variation partitioning analysis are expressed in the column plot at the bottom. Willow species explained 18.4% of the observed variation (f = 2.6, p = 0.0001), and treatments explained 2.8% of the observed variation (f = 9.5, p = 0.0001) in VOCs.

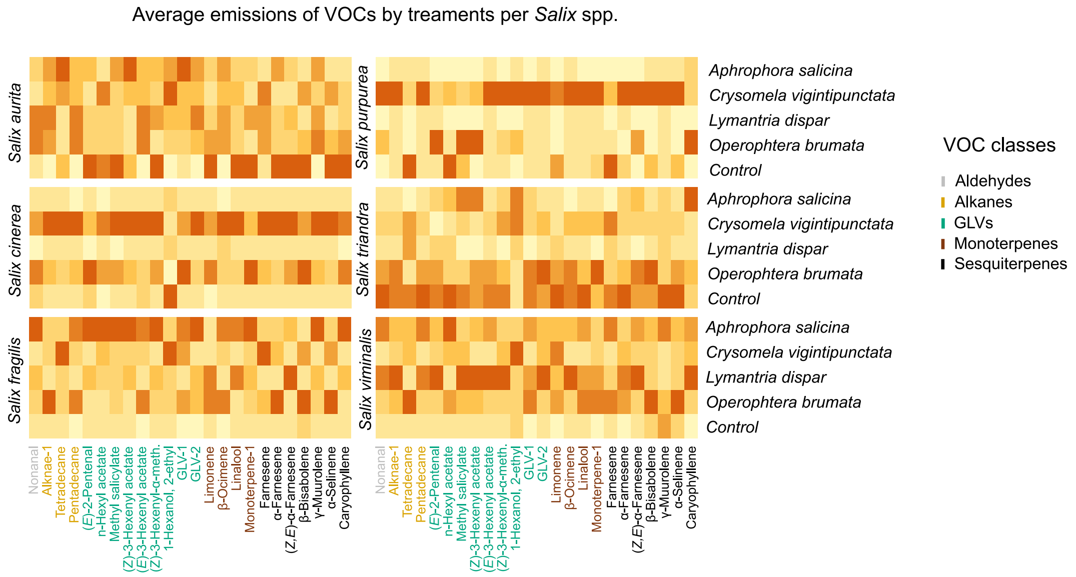

Fig. 2. Heatmap plots showing the average emissions (area under peaks) of VOCs as elicited by different treatments per willow species. VOCs are colour-coded according to VOC classes. The colour gradient shows the emissions of VOCs. Higher emissions are in darker colours, and lower emissions are in lighter colours. Grey columns represent metabolites no detected in that species.

10% of the samples. The metabolites were classified into 14 different classes, with most of the non-VOCs belonging to organic acids and derivatives (N = 2350), lipids (N = 2229), organooxygen compounds (N = 1288), phenylpropanoids and polyketides (N = 1105), and benzenoids (N = 851) (Appendix S2 – Table S2).

We selected 189 out of 8871 non-VOCs with non-zero coefficients using the LASSO regression, out of which secondary metabolites were selected. The LASSO-selected metabolites included 39 (20.63%) secondary metabolites. The unique effect of the treatment explained 0.5% of the observed variation (f = 1.4, p = 0.0358). The willow species identity explained 41.1% of the observed variation in secondary metabolites (f = 26.0, p = 0.0001). The amount of herbivory damage did not explain the observed variation in secondary non-VOCs (f = 1.0, p = 0.4341) (Fig. 3).

Individual willow species showed largely specific non-VOC responses (Fig. 4). Overall, there were few discernible patterns concerning the upregulation or downregulation of individual non-VOCs or their classes in response to the herbivore treatments. One of the visible patterns included feeding by the sapsucker *A. salicina,* which upregulated most of the non-VOCs in *S. viminalis*. At the same time, feeding by this species elicited the downregulation of two benzenoids, a few organooxygens and heterocyclic compounds compared to the controls.

There was no significant correlation between the similarity in induced responses in LASSO-selected secondary non-VOCs and the phylogenetic distance between the willow species for any treatments (Appendix S3 – Table S3).

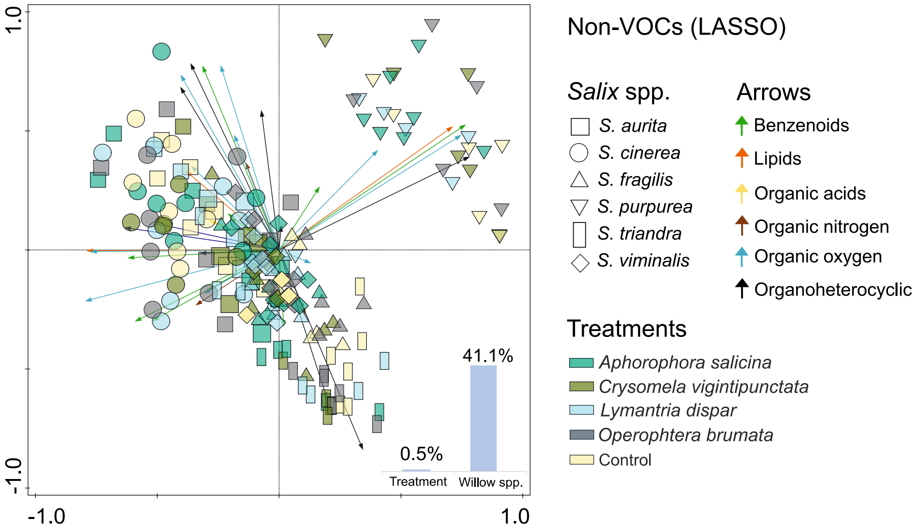

Fig. 3. Principal Component Analysis (PCA) showing the variation in non-VOCs in the Hellinger standardised dataset. The first two axes explained 46.74% of the. Symbols represent willow species and are colour-coded according to insect and control treatments. Arrows represent individual non-VOCs and are colour-coded as different classes of secondary non-VOCs. The significant results of RDA-based variation partitioning analysis are expressed in the column plot at the bottom. Willow species explained 41.1% of the observed variation (f = 26.0, p = 0.0001), and treatments explained 0.5% of the observed variation (f = 1.4, p = 0.0358) in non-VOCs.

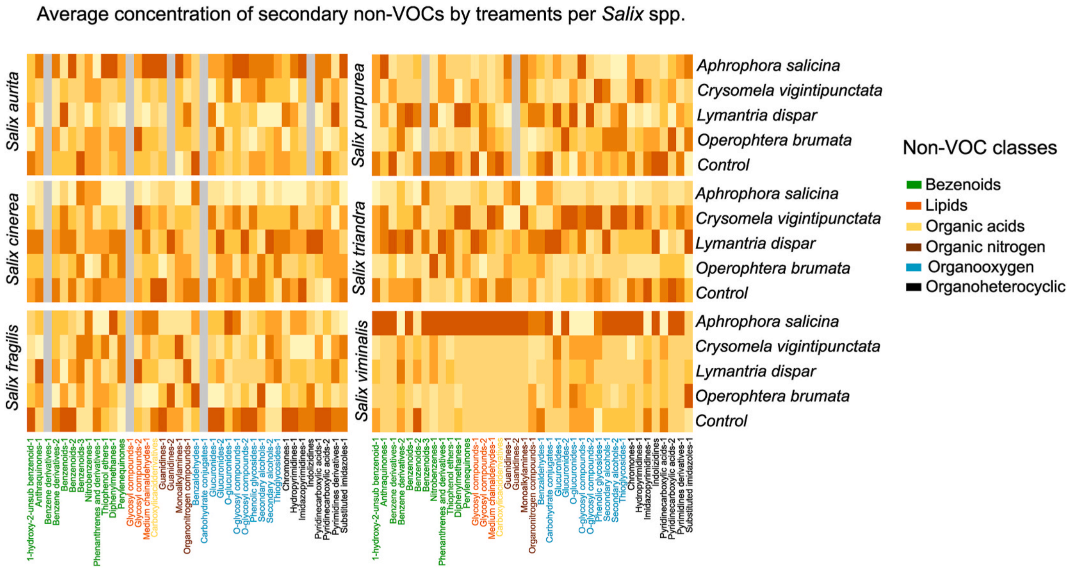

Fig. 4. Heatmap plots showing the average concentration (peak area per sample dry weight) of secondary non-VOCs selected by LASSO regression as elicited by different treatments per willow species. Non-VOCs are colour-coded according to non-VOC classes. The colour gradient in the panels shows the concentration of non-VOCs. Higher concentrations are in darker colours, and lower concentrations are in lighter colours. Grey columns represent metabolites no detected in that species.

2.3. Salicinoids

We detected 32 salicinoids and their derivatives among the six studied *Salix* species. The unique effect of the treatment explained 0.5% of the observed variation in salicinoids (f = 1.5, p = 0.0496). At the same time, the willow species identity explained 60.7% of the observed variation in salicinoids (f = 57.7, p = 0.0001). As before, the amount of herbivory damage did not explain the observed variation in salicinoids (f = 0.7, p = 0.6100) (Fig. 5).

Similarly to non-VOCs, salicinoids showed largely species-specific responses. Feeding by most insect herbivores did not produce any pattern in salicinoids that would appear uniformly across several of the studied willow species (Fig. 6). Again, the only treatment that seemed to elicit a more uniform response across the willow species included feeding by the sapsucker *A. salicina*. It caused the upregulation of most salicinoids and their derivatives in *S. aurita*, *S. cinerea*, *S. triandra* and *S. viminalis* compared to controls.

There was no significant correlation between the similarity in induced responses in salicinoids and the phylogenetic distance between the willow species for any treatments (Appendix S3 – Table S3).

2.4. Correlation of VOCS and non-VOCs (LASSO-selected metabolites)

Generally, there was no significant correlation between the similarity in responses in VOCs and secondary non-VOCs when measured among willow species or treatments (Appendix S3 – Tables S4 and S5). The only exception was *S. fragilis*, which showed a positive correlation between the similarity in VOCs and secondary non-VOCs among the treatments (r = 0.474, p = 0.037) (Appendix S3 – Table S5).

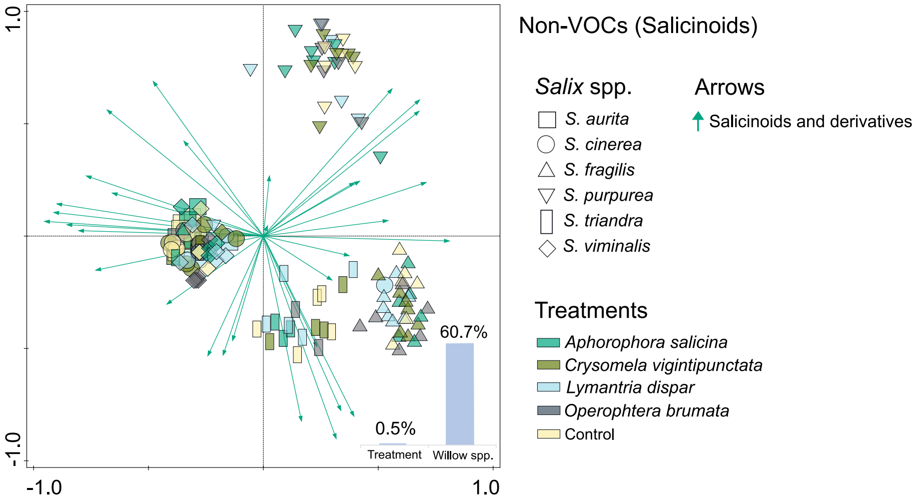

Fig. 5. Principal Component Analysis (PCA) showing the variation in salicinoids. The first two axes explained 56.40% of the variation. Symbols represent willow species and are colour-coded according to insect and control treatments. Arrows represent individual salicinoids and are colour-coded as different classes of salicinoids and their derivatives. The significant results of RDA-based variation partitioning analysis are expressed in the column plot at the bottom. Willow species explained 60.7% of the observed variation (f = 57.7, p = 0.0001), and treatments explained 0.5% of the observed variation (f = 1.5, p = 0.0496) in salicinoids.

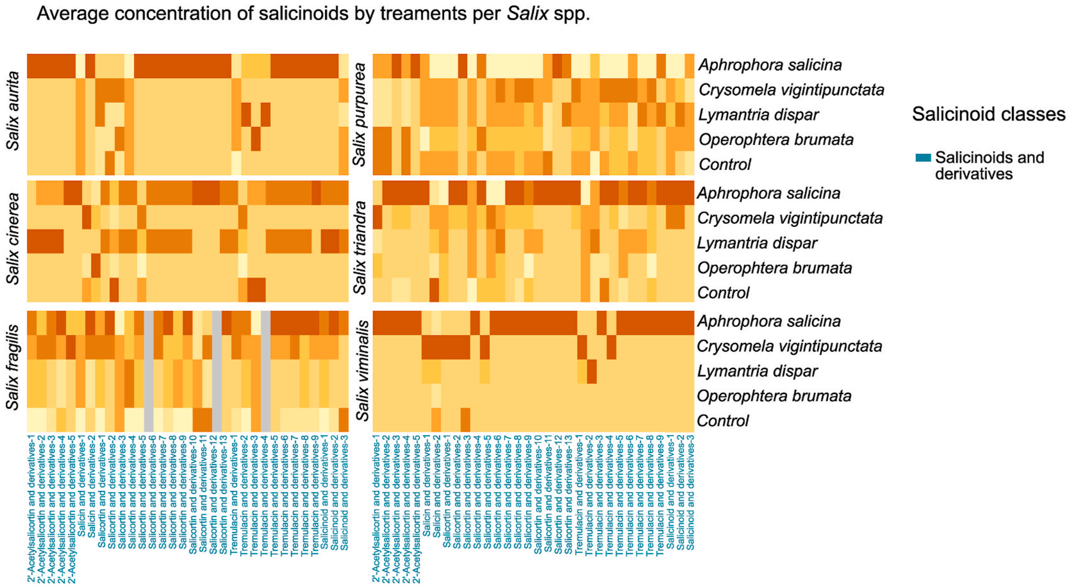

Fig. 6. Heatmap plots showing the average concentration (peak area per sample dry weight) of salicinoids as elicited by different treatments per willow species. The colour gradient in the panels shows the concentration of salicinoids and their derivatives. Higher concentrations are in darker colours, and lower concentrations are in lighter colours. Grey columns represent metabolites no detected in that species.

2.5. *Correlation of VOCs and salicinoids*

There was no significant correlation between the similarity in responses in VOCs and salicinoids when measured among willow species or treatments (Appendix S3 – Tables S4 and S5). There was a positive correlation between the responses in VOCs and salicinoids among willow species, but only when induced by the generalist caterpillar *Operophtera brumata* (r = 0.561, p = 0.004).

3. Discussion

Chemical variation is a key aspect affecting co-occurring plants' performance (Volf et al. 2019; Salazar et al. 2016). Here we explored the variation in volatile and nonvolatile induced responses elicited by different insect herbivores and compared it with the interspecific variation between the willow species. Our results suggest pronounced differences in the induced responses among the studied willow species, particularly in the case of non-VOCs. However, we also observed some similarity in induced responses in VOCs among different willow species when damaged by the same herbivore. Instead of finding universally shared responses among the willow species, our results imply that there may be groups of species, each with a somewhat specific VOC response to their herbivores.

The herbivory treatment had a stronger effect on VOCs than non-VOCs and salicinoids. Furthermore, some similarities in VOC responses among willow species were observed as a response to damage by the same herbivore species. The most notable similarity was a strong upregulation of VOCs in *S. cinerea* and *S. purpurea* when damaged by *Chrysomela vigintipunctata*. This specialised leaf beetle can sequester salicinoids and cause extensive damage to its host plants (Hespenheide 2001). Several studies showed that specialist leaf beetles elicit strong responses in VOC emissions in willows and poplars (Unsicker, Gershenzon, and Köllner 2015; Mezzomo et al., 2023). Here, the beetle triggered the most pronounced response in VOC emissions out of all the herbivores, upregulating mainly various terpenoids. We did not test the efficacy of the detected VOCs in attracting predators and parasitoids in this system. However, many detected terpenoids emitted upon herbivory are known for their roles in insect-plant interactions, with both direct and indirect effects on insect herbivores. For example, the monoterpene β-ocimene, which increased in plants exposed to *C. vigintipunctata*, is involved in attracting predators and parasitoids in various plants (De Moraes et al., 1998; De Moraes, Mescher, and Tumlinson 2001).

The similarity in VOC responses to *C. vigintipunctata* positively correlated with phylogenetic distance among the studied willow species, suggesting that closely related willows respond similarly to this herbivore. In contrast, other notable similarities in VOC responses seemed to occur among species that were not closely related. For example, *S. aurita* responded to herbivory by downregulation of many VOCs, which was more similar to distantly related *S. triandra* than to more closely related *S. cinerea*, *S. purpurea*, or *S. viminalis* (Volf et al., 2023). Likewise, we found a relatively strong response to *Aphrophora salicina* in *S. aurita*, *S. viminalis*, and *S. fragilis*, the last of which belongs to a different subgenus than the first two. These results show that although there was some similarity in VOC responses among the willow species, it was not universal, nor did it always correlate with their relatedness (Carrillo-Gavilán et al., 2015; Salazar et al., 2016). The results align with simulation models that predict co-occurring plant species to form clusters based on their trait similarity, disregarding their relatedness (D'Andrea et al. 2019). Plant species within a cluster should interact more strongly with each other than with plants from different clusters (D'Andrea et al. 2019; Sedio 2019). In the case of VOCs, forming such species clusters may theoretically allow for recruiting specialised natural enemies of herbivores by each willow cluster while sharing them among the species within. The similarity in VOC responses among willow species can also potentially reduce the information complexity and navigate natural enemies towards the plants more efficiently (Zu et al., 2020).

However, experimental tests would be required to explore if the patterns we detected translate to improved recruitment of predators and parasitoids of herbivores and reduced herbivory damage to the plants. Some of the willow VOCs have also been shown to mediate plant-plant communication and induction of defences with direct effects on herbivores (Hughes et al., 2015). Additionally, it is important to note that some of the similarities in VOC responses may not necessarily be adaptive and contribute towards the attraction of predators and parasitoids of herbivores. For example, feeding by the generalist leaf-chewer *Lymantria dispar* caused a decrease in GLVs, such as methyl salicylate, among most of the studied willow species. Methyl salicylate is a phytohormone derivative of salicylic acid and is often involved in plant-insect interactions and the recruitment of insect predators and parasitoids (James 2003; Salamanca et al., 2017; Silva et al. 2021). Its downregulation thus likely results from a negative crosstalk between the salicylic pathway and the jasmonic acid pathway, which produces other VOCs, such as various terpenoids we detected across the willow species (Barker et al., 2019; Schmelz et al., 2003).

The trends in LASSO-selected secondary non-VOCs and salicinoids largely differed from those we observed in VOCs. The willow species identity explained roughly twice the variation in non-VOCs and salicinoids than in VOCs. In contrast, the independent effect of treatment identity explained much less variation than in the case of VOCs for both LASSO-selected metabolites and salicinoids and their derivatives. This suggests that each willow species responded largely in a specific way, as they show completely different responses for most herbivores. This corresponds with the findings of Barker et al. (2019), that the variation in defence compounds such as salicinoids and the performance of insect herbivores seems to be highly genetically determined in Salicaceae. The high variability in induced responses we detected can further increase the effects of these constitutive differences in secondary chemistry among willow species. High variability in leaf secondary metabolites among co-occurring plant species has been shown to reduce herbivory damage (Massad et al., 2017; Wang et al., 2023). Theoretically, such patterns should translate into chemical divergence among co-occurring plant species and low phylogenetic signal in their chemical defences (Becerra 2007; Salazar et al., 2016; Endara et al., 2017; Volf et al., 2023). Here, we did not find any correlation between the phylogenetic distance and similarity in induced responses in non-VOCs and salicinoids, which seems to be in line with these findings. However, our taxonomic sampling is too small to make a more specific conclusion.

The only treatment that elicited a similar response across several willow species in both non-VOCs and salicinoids was the response to the sapsucker *Aphrophora salicina*. The pattern was particularly strong in the case of salicinoids, where *S. aurita*, *S. cinerea*, *S. triandra*, and *S. viminalis* responded to feeding by the sapsucker *A. salicina* by upregulating most of its salicinoids. Sapsucking herbivores typically activate the salicylic acid pathway, which regulates salicinoid production in plants (Walling, 2000; Erb et al., 2012). In contrast, leaf-chewing herbivores, like the other insect herbivore species studied here, commonly trigger the jasmonic acid (JA) pathway, which in turn can suppress the salicylic acid pathway (Walling, 2000; Erb et al., 2012). This could explain the differential response to the three chewing herbivores and the sapsucker we observed. However, since the concentration of salicinoids is typically high in leaves but not in the xylem that *A. salicina* feeds on (Thompson, 2022), it is difficult to assess if this upregulation can have any positive consequences for the plant.

Overall, there was no significant correlation between the similarity in responses in VOCs and the secondary non-VOCs and salicinoids. The only exception was *S. fragilis*, which responded to the treatment similarly in both VOCs and LASSO-selected non-VOCs. The general absence of either a positive or negative correlation suggests that VOCs and non-VOCs may display largely independent trends in willows (Mezzomo et al., 2023). Negative correlations among various chemical defences can arise between constitutive and induced defences in slow-growing vs. fast-growing plant species (Moreira et al., 2014) or among structurally related metabolites that share their precursors or result from competing metabolic pathways (Schmelz et al., 2003; Wei et al., 2014; Leong et al., 2024). In contrast, our results suggest that different types of induced responses may be largely independent among related plants with similar life history traits, contributing to their flexible responses to various environmental stimuli (Agrawal and Fishbein 2006).

4. Conclusions

Our results point to different trends in variation and specificity in induced responses in VOCs and non-VOCs in willows. Host-plant identity and herbivore species accounted for a different amount of variation in the induced responses in VOCs, non-VOCs, and salicinoids. This likely reflects the different roles of these metabolites in plant defence. Large interspecific variation in non-VOCs and salicinoids may help willows partition their chemical niches and avoid sharing herbivores (Volf et al., 2023). In contrast, similar VOC responses may improve communication between plants or the attraction of specialised enemies of herbivores (De Moraes et al., 1998; Mrazova and Sam, 2018; Li et al., 2022). Another level of variation is then also explained by the herbivore feeding mode and metabolic pathways it upregulates, as evidenced by the relatively uniform response by several willow species to the feeding by the sapsucker *Aphrophora salicina*. In turn, various induced responses seem to be largely independent in the studied willows. Overall, these patterns contribute to the diversity of willow defences and their ability to tailor the responses somewhat to cope with the different stressors they encounter. However, further experiments are needed to test whether these differences result from different functional roles of specialised metabolites in plant defence and to reveal if this shapes their variation in natural plant communities.

5. Experimental

5.1. Host plants

We focused on six willow species: *Salix aurita*, *S. cinerea*, *S. fragilis*, *S. purpurea*, *S. triandra*, and *S. viminalis*. These species occur in sympatry and often form mixed local assemblages. Three mother plants were selected per willow species from wild populations around Ceske Budejovice, Czech Republic, to obtain enough individuals for the experiment. In total, 20 cuttings (40 cm long) were sampled from each mother plant in February 2021. We grew the cuttings in a greenhouse at ambient temperature for three months. Further information on plant treatment and preparation for the experiment is available in Appendix S1. Initially, the mother genotype was included in the preliminary statistical analyses. The genotype information was excluded from the final analyses as it did not significantly affect the chemistry.

5.2. Model insects

Four insect herbivore species with different feeding strategies and host plant preferences were used: two Lepidoptera species (*Lymantria dispar* and *Operophtera brumata*), one Coleoptera species (*Chrysomela vigintipunctata*), and one Hemiptera species (*Aphrophora salicina*). All of them are naturally associated with the willow species used in this study. *L. dispar* (Erebidae) and *O. brumata* (Geometridae) caterpillars are generalist external and tying leaf-chewers, respectively, that feed on various broadleaf trees as larvae. The leaf-beetle *C. vigintipunctata* (Chrysomelidae) is a specialised external leaf-chewer that feeds on willows as both larvae and adults and can sequester salicinoids. *A. salicina*, is a specialist sapsucker that feeds on various willow species as nymphs and adults. We obtained *L. dispar* and *O. brumata* from overwintered eggs, whereas *A. salicina* and *C. vigintipunctata* were obtained from wild populations before the experiment. The second to third instars were used for the caterpillars. A combination of the last two instar larvae and adults was used for the leaf beetle. Nymphs were used for the sapsucker (Table 1).

The number of individuals introduced to the plants differed among insect species to ensure that the insects would cause comparable plant damage (Table 1). Herbivory damage (in cm^2) was accounted for in all the statistical analyses since a variation in the herbivory damage caused by the external feeders was observed. Although we observed feeding by the sap-sucker species, it was impossible to directly quantify the damage they caused to the plants, as they feed on the xylem. To account for this, the weight of the individuals introduced to each plant was included in the preliminary analyses. As sap-sucker weight did not affect the results, it was excluded from the final analyses.

5.3. Host plant exposure to insect herbivores

Plants were exposed to the insects in May 2021. The insects were introduced to the terminal part of the largest shoot on each plant. The insects were enclosed with ca. ten leaves in 26 × 35 cm fine mesh transparent tissue bags. Control plants received bags without herbivores. Five to seven replicates were used for each herbivore and control treatment per willow species, accounting for 192 plants in total. Plants were randomly distributed in the greenhouse. The insects were allowed to feed for 72 h and checked several times per day. Inactive or dead insects were immediately replaced with conspecific individuals of the same developmental stage. After 72 h, all herbivores and their frass were removed from the plants. Two PDMS (polydimethylsiloxane) tubes (2 cm cuttings, inner diameter 1.0 mm, outer diameter 1,8 mm, Carl Roth, Karlsruhe, Germany) were attached to each plant immediately after removing the herbivores. The PDMS tubes were attached to a cleaned stainless-steel wire (0,60 mm diameter, Stabilit, Gutenbergstr, Germany) to avoid contact with the plant surface. PDMS tubes were enclosed in 25 × 38 cm polyamide bags (Alufix Bohemia, Cerniky, Czech Republic). VOCs were passively sampled from headspace for 24 h following Kallenbach et al., 2015. This method is particularly suitable for sampling monoterpenoids and sesquiterpenoids, two groups of VOCs we were interested in due to their involvement in plant-herbivore interactions (Peacock et al., 2001; Danner et al., 2011).

Table 1
Herbivore species, life stage, feeding guild and the number of individuals (N) added to each plant.

Herbivore species	Life stage	Feeding guild	N
Chrysomela vigintipunctata (Linnaeus, 1758)	Larvae + Adults	External leaf-chewer	4 + 2
Lymantria dispar (Linnaeus, 1758)	Larvae	External leaf-chewer	1
Operophtera brumata (Linnaeus, 1758)	Larvae	Tying leaf-chewer	2
Aphropohora salicina (Goeze, 1778)	Nymphs	Sap-sucker	10

As a slow upregulation of non-VOCs has been documented in other woody plants (Volf et al., 2021), we chose to sample the leaves to measure the non-VOCs six days after the VOCs sampling. All the leaves enclosed in the mesh bags on the treated and control plants were removed from the plants. The leaves were flattened and photographed, and the images were processed in ImageJ (Abramoff et al., 2004) to measure each plant's leaf area and amount of herbivory damage (in cm^2). After calculating the herbivory damage, the first three fully developed leaves were also removed from each plant, freeze-dried, and homogenised for the untargeted metabolomic analyses.

5.4. Chemical analysis

5.4.1. VOCs quantification

The PDMS tubes were analyzed by thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) in a thermodesorption unit (MARKES, TD100-xr, Llantrisant, United Kingdom) with an integrated autosampler. TD-GC-MS used the following conditions: carrier gas helium (constant flow rate of 2 ml/min), flow path temperature 160 °C; processing method: dry purge 5 min at 20 ml/min, desorption 8 min at 200 °C with 20 ml/min, pre trap fire purge 1 min at 60 ml/min, trap heated to 300 °C and hold for 4 min, desorption split flow was 20 ml/min. VOCs were separated on a gas chromatograph Trace 1300 connected to an ISQ quadrupole mass spectrometer (both Thermo Fisher Scientific, San Jose, CA, USA) equipped with an LN-WAX column, 30 m × 0.25 mm i.d., 0.25 μm film thickness (Chromservis, CZ). The temperature program was set up at 60 °C (hold 2 min), 30 °C/min at 150 °C, 10 °C/min at 200 °C, and 30 °C/min at 230 °C (hold 5 min). MS conditions were set at 240 °C at the transfer line and 220 °C for the EI ion source (70 eV). The scan range was 33–500 m/z for a full scan (scan time 250 ms). The Xcalibur 2.0 software (Qual Browser, Quan Browser) was used for data processing, method calibration, and validation. VOC emissions were quantified as the area under the chromatographic peak. We standardised them by dividing the peak areas with the herbivory damage (cm^2) caused by the herbivores and log-transformed them for analysis.

5.4.2. Untargeted metabolomics for non-VOCs

Small organic non-VOCs were analyzed with untargeted metabolomics following Sedio et al., 2018b and Sedio et al. (2021). Metabolites were separated by UHPLC followed by heated electrospray ionisation (HESI) in positive mode using full scan MS1 and data-dependent acquisition of MS2 (dd-MS2) on a Thermo Fisher Scientific QExactive hybrid quadrupole-orbitrap mass spectrometer. As samples are freeze-dried for UHPLC/MS analysis, all compounds with a boiling point below 100 °C (i.e., most VOCs) sublimate. Furthermore, such compounds also show poor ionisation efficiency of the ESI ion source due to their structure's absence of a heteroatom. Together, this ensures that our UHPLC analysis does not detect VOCs emitted from the plants at average outdoor temperatures, as measured with TD-GC/MS. Details on sample processing and instrument setup are available in Appendix S1.

Raw data from the UHPLC-MS extraction were centroided and processed for peak detection, peak alignment, and peak filtering using Mzmine2 (Pluskal et al., 2010), which groups chromatographic features into putative compounds based on molecular mass and LC retention time. We used the same parameters as Sedio et al. (2021), except for setting the MS1 noise threshold to 15,000 ion count and the MS2 noise threshold to 1500 ion count. The MZmine output was used to calculate metabolite concentrations (peak area per sample dry weight) and putative identities of the metabolites detected. The molecular formulae were inferred using Sirius (Dührkop et al., 2019), predicted structures using CSI: finger ID (Dührkop et al., 2015), and the metabolites were classified using CANOPUS and ClassyFire compound class predictor (Djoumbou Feunang et al., 2016; Dührkop et al., 2021). The canonical SMILES and retention times were used to classify salicinoids and their derivatives. We excluded all metabolites in the blanks and those we could not classify. All individual metabolite concentrations were standardised as peak area divided by the dry weight of leaf tissue (in mg) and log-transformed for the analysis.

5.5. Statistical analysis

We analyzed three datasets: i) VOCs, ii) non-VOCs, and iii) salicinoids). All detected metabolites were used in the case of VOCs and salicinoids. In the case of non-VOCs, we first reduced the complexity of the dataset only to include secondary metabolites that best described the variation among samples without constricting the variation to treatments or willow species. First, all metabolites that occurred in less than 10% of the samples were removed. Then, the Least Absolute Shrinkage and Selection Operator (LASSO) regression was applied to the remaining dataset. The LASSO regression allowed us to select a reduced dataset and investigate overall changes in non-VOC composition at the compound level. A 100-fold cross-validation method was used with the package “glmnet” in R (Friedman et al. 2010). From this dataset, secondary metabolites were selected based on their previously identified classes from CANOPUS and ClassyFire, using their predicted canonical SMILES.

To test our first hypothesis that the plants would respond to treatments, an exploratory principal component analysis (PCA) was performed to visualise the induced variation among the willow individuals. A Hellinger standardisation was applied to the data, eliminating the differences in total emissions (VOCs) and total concentration (non-VOCs and salicinoids) and focusing on their relative importance. Combined heatmaps computed in R 4.1.1 (R Core Team, 2023) were used to show the changes in VOC emissions, non-VOC and salicinoid concentrations across the *Salix* species as elicited by different treatments.

To test our second and third hypotheses to determine whether the induced responses in VOCs, non-VOCs, and salicinoids would mainly reflect herbivore identity or willow species, three separate redundancy analyses (RDA) were performed. The variation in each dataset was partitioned as explained by the willow species, herbivore species, or amount of herbivory damage (in cm^2). The significance of individual variables and the best overall model was tested with the Monte-Carlo permutation test with 9999 permutations in CANOCO 5 (ter Braak and Smilauer, 2012).

To explore if related willow species respond similarly to the herbivory, we tested for the correlation between phylogenetic distance and similarity in either VOC, non-VOC or salicinoid responses among the willow species. The willow phylogeny was pruned from Volf et al. (2023) to include only our six focal species to obtain the phylogenetic distance matrix. Then, the phylogenetic distances were transformed into a patristic distance matrix. To obtain the distance matrices on VOCs, non-VOCs, and salicinoids, we first calculated the average emissions of individual VOCs or concentration of non-VOCs and salicinoids within individual willow species. The averages for each herbivore treatment and control were calculated separately, resulting in five datasets (four for the treatments and one for the controls). Then, these data were used to calculate five Bray-Curtis distance matrices showing the dissimilarity in VOCs, non-VOCs, and salicinoids among the willow species exposed to the same treatment or used as controls. The correlation between the phylogenetic and VOC/non-VOC/salicinoid matrices was tested with Mantel tests with 999 permutations in R.4.1.1. using the “vegan” package 2.6–4 (Oksanen et al., 2022).

To test our fourth hypothesis, Mantel tests were used to test the correlation between VOCs vs. non-VOCs and VOCs vs. salicinoids. First, we tested for the correlation between the dissimilarity in these metabolites among the willow species exposed to one of the treatments or used as controls. For that, the same Bray-Curtis distance matrices on VOCs, non-VOCs and salicinoids were used as in the case of Mantel tests testing for the correlation between metabolites and phylogenetic distance described above. Second, we tested for the correlation between the dissimilarity in these metabolites among the treatments within individual willow species. To obtain the distance matrices, the average emissions of individual VOCs and average concentrations of individual non-VOCs and salicinoids were calculated in plants exposed to one of the four treatments or used as controls. The averages for each willow species were calculated, resulting in six datasets representing treatment averages in different willow species. These data were then used to estimate the Bray-Curtis distance matrices showing VOC, non-VOC and salicinoid dissimilarity among the treatments in individual willow species. The correlation between the VOC, non-VOC and salicinoid matrices was tested with Mantel tests with 999 permutations in R.4.1.1. using the “vegan” package 2.6–4 (Oksanen et al., 2022).

CRediT authorship contribution statement

Priscila Mezzomo: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jing V. Leong:** Data curation. **Petr Vodrážka:** Investigation, Data curation. **Martin Moos:** Data curation. **Leonardo R. Jorge:** Formal analysis, Conceptualization. **Tereza Volfová:** Investigation, Data curation. **Jan Michálek:** Data curation. **Paola de L. Ferreira:** Data curation. **Petr Kozel:** Data curation. **Brian E. Sedio:** Investigation, Formal analysis, Data curation. **Martin Volf:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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