

Article

Volatile Profiling of Fifteen Willow Species and Hybrids and Their Responses to Giant Willow Aphid Infestation

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Abstract: The giant willow aphid (*Tuberolachnus salignus*) is a large stem-feeding insect which forms dense colonies on infested plants. Since *T. salignus* is a new invasive species in New Zealand, we have a poor understanding of the plant chemical responses to aphid infestation. This study aimed to characterize the volatile organic compounds (VOCs) emissions of fifteen different willow species and hybrids growing in New Zealand, and to evaluate changes in response to *T. salignus* attack in a field trial. Volatiles were collected using a headspace sampling technique and analysed using gas chromatography-mass spectrometry (GC-MS). We found high variability in the volatile profiles of different species and hybrids, with (*Z*)-3-hexenyl acetate and (*E*)- β -ocimene being the only common components to all blends. Taxonomically related plants showed an overlapping pattern of VOC emission, and there seemed to be a clear separation between shrub and tree willows. Responses to aphid infestation were variable, with only four species/hybrids showing changes in their total VOC emission, or that of at least one class of VOCs. A weak positive correlation between aphid population estimates and VOC emissions suggests that responses are species-specific and not infestation-dependent. These results reveal useful information about the interaction between *T. salignus* and its potential host plants for biological control and pest management purposes.

Keywords: *Salix*; *Tuberolachnus salignus*; volatile organic compounds (VOCs); plant-insect interactions; herbivore-induced plant volatiles (HIPVs)

1. Introduction

Plants naturally release a wide array of volatile organic compounds (VOCs) into the environment to perform various ecological and physiological processes [1]. Chemically, VOCs are low molecular weight lipophilic molecules, consisting of terpenoids, benzenoids, green leaf volatiles (GLVs), fatty acid and amino acid derivatives [2]. The production and release of these VOCs are highly responsive to biotic and abiotic factors, making them an excellent source of information for surrounding organisms [3,4]. VOCs mediate multiple ecological interactions: they can repel herbivores [5,6], attract the natural enemies of herbivores (predators and parasitoids), and lure pollinators and seed dispersers, which are key elements to the plant's defense and reproduction [7–9]. However, VOC can also enhance the attractiveness of plants to some herbivores, harming the emitting plants [10]. Plants also release VOCs to protect themselves from environmental stress, such as heat or UV-radiation [11,12].

A variety of factors are known to influence both the quality and quantity of VOCs emitted by plants [13]. Among them, the plant species is a determining factor, since some biosynthetic pathways

are taxon-specific [14,15]. As VOC emission quantitatively and qualitatively differs from one species to another, volatile blends (scents) are good indicators of a plant's identity and of evolutionary relationships among plant groups [4]. Some VOCs are released only from specific plant species, or plant groups, while others are ubiquitous to all plant species [16]. Within the same plant species, VOCs emission can vary within species, and with the phenological state, age and sex of the plant [17–20].

Although plants constitutively emit VOCs, both quantitative and qualitative changes in VOCs blends are detected when plants are attacked by insect herbivores [21]. These herbivore-induced plant volatiles (HIPVs) mainly consist of GLVs, aromatics and terpenoids [22]. The HIPV emission is a dynamic process between the host plant and herbivorous insects [23], where the abundance and identity of the attacker influences the responses to herbivore attack [24]. The feeding mode of the herbivore appears to play a key role in regulating the plant's responses, activating different signaling pathways [25]. Previous studies suggest that phloem feeders induce less pronounced changes in the volatile emission of their host plants than chewing insects [26,27].

Willow plants are known to release various VOCs, consisting of acetaldehydes, acetones, acetic acids, isoprenes, methanols, methyl ethyl ketones, methyl vinyl ketones and monoterpenes [28,29]. These emissions are species-specific [30], have been related to both direct and indirect plant defenses [31], and play an important role in host selection by aphids [32] and other insects [33]. As willows possess different growth forms [34], VOC blends and response to herbivore attack can differ between tree and shrub types.

Willows (*Salix* spp.) are known for their high genetic diversity encompassing more than 400 wild and cultivated species and hybrids all over the world [35]. New Zealand (NZ) currently has 59 species and hybrids [36]. Some of them are widely planted in NZ for biomass production, soil conservation on pastoral hill country and river banks, and as sources of spring pollen and nectar for honey bees [37–39]. Willows are now being infested by the giant willow aphid, *Tuberolachnus salignus* Gmelin (Hemiptera: Aphididae), a stem-feeding insect that was first reported in NZ in December 2013 [40]. Aphid infestation reduces the amount of photosynthetic storage in willow roots and stems, leading to changes in plant performance and growth [41]. Long-term sustainable management solutions are needed to reduce the impact of aphid infestation, such as the selection of resistant willow species or hybrids for planting [42]. A key aspect for selection is a better understanding of the aphid interaction with its host plant, including the plant's production and emission of volatile organic compounds (VOCs) in response to herbivore attack.

Some studies have explored the role of willow VOCs in host selection by herbivores, such as the willow sawfly (*Nematus oligospilus*) and willow leaf beetles (*Phratora* spp. and *Plagioderia* spp.) [30,43–45]. However, there is scarce information about willow responses to attacks by phloem-feeding herbivores [46]. Therefore, in this study, we explored the intra-genus (*Salix*) variation in VOCs from fifteen willow species and hybrids, and changes in VOCs emissions in response to infestation by *T. salignus*. We expect these results will shed some light on the aphid–plant interaction, and inform pest management decisions for successful willow growing.

2. Materials and Methods

2.1. Study Site and Plant Material

This study was conducted in a willow field trial at the Orchard Block, Plant Growth Unit, Massey University, NZ (40°22'41.70" S, 175°36'30.67" E). The field trial was set up to investigate the interactions of the giant willow aphid with its host plants and the environment. Fifteen willow species and hybrids, from different geographical origins [36], were grown in three blocks of paired rows, with the position of each species being random within each paired row. Twelve ramets (an individual of plant species, vegetatively reproduced from a single parent plant) of each species or hybrid were planted within each row. Within each block, one row was randomly selected as a control (aphid exclusion), while the

adjacent row was aphid-infested. Information on the willow species and hybrids, and the field trial layout are provided in Table 1 and Table S1.

The willow field trial was planted using stem cuttings in June 2017, with 0.4 m spacing between cuttings within the rows, and 4 m spacing between rows. The willow plants in the control rows were inspected for colonising aphids on a weekly basis, and any aphids found were removed manually. Mavrik[®] insecticide (Nelson, NZ) was applied on 28 February 2018 and 17 January 2019, when manual control was impractical due to high population densities of *T. salignus*.

Table 1. Willow species and hybrids used in this study.

Species/Hybrid	Code	Type	Sex	Geographical Origin
<i>S. candida</i>	PN 385	Shrub	Male	North America
<i>S. eriocephala</i>	PN 376	Shrub	Male	North America
<i>S. lasiolepis</i>	PN 751	Shrub	Male	North America
<i>S. lasiolepis</i> × <i>S. viminalis</i>	NZ 04-106-073	Shrub	Male	Hybridized in New Zealand
<i>S. purpurea</i>	PN 249	Shrub	Female	Europe, North Africa
<i>S. schwerinii</i>	PN 386	Shrub	Male	Eastern Asia
<i>S. viminalis</i>	PN 220	Shrub	Male	Europe, Western Asia
<i>S. × reichardtii</i>	PN 714	Shrub	Male	Europe
<i>S. alba</i>	PN 357	Tree	Male	Europe, Western and Central Asia
<i>S. lasiandra</i>	PN 747	Tree	Male	North America
<i>S. matsudana</i>	PN 227	Tree	Female	Eastern Asia
<i>S. matsudana</i> × <i>S. alba</i> (1)	NZ 1040	Tree	Female	Hybridized in New Zealand
<i>S. matsudana</i> × <i>S. alba</i> (2)	NZ 1184	Tree	Male/female	Hybridized in New Zealand
<i>S. matsudana</i> × <i>S. lasiandra</i>	NZ 03-003-073	Tree	Male	Hybridized in New Zealand
<i>S. × fragilis</i>	PN 218	Tree	Female	Europe and Western Asia

Numbers (1) and (2) represent two different cultivars of *S. matsudana* × *S. alba* (NZ 1040) and (NZ 1184), and will be used in subsequent tables and figures. Code corresponds to the Plant & Food Research (Palmerston North, NZ) willow collection numbers.

2.2. Aphid Inoculation

Willow plants in the aphid-infested rows were inoculated with five adult aphids per plant on 25–27 January 2018 and 6–7 December 2019. Additional inoculations with ten adult aphids per plant were done on 13–14 February 2018 and 30 January 2019. The aphid population per ramet of each sampled plant was quantified immediately before VOC collection using a visual scale from zero to six, with 0 = less than five aphids, 1 = 2 to 20 aphids, 2 = 20 to 50 aphids, 3 = 50 to 100 aphids, 4 = 100 to 300 aphids, 5 = 300 to 600 aphids, and 6 = 600 aphids or more per plant, as described by Collins [47] with slight modification.

2.3. VOCs Sampling

VOCs from willow branches were collected using the push-pull headspace sampling method as described in Effah, et al. [48]. Among the twelve plants in the row plots of each species or hybrid, one of the middle ramets (plants 5, 6 or 7) per plot was chosen to ensure that the VOCs collected were released from that treatment, without receiving VOCs from different neighboring plants, for a total of six plants per species. Willow branches of a suitable size, without visible sign of damage by insects and pathogens, were selected and enclosed in oven cooking bags (Glad[®], Melbourne, Australia). One inlet and one outlet tube were fastened with cable binders at each end of the bag. The portable volatile assay system (PVAS22 pump, VAS Rensselaer NY) was used to circulate carbon-filtered air through the bag (Figure 1). Incoming air was pumped at 1.70 L/min and outgoing air was pulled at 1.20 L/min creating a slight overpressure to avoid contaminants from entering the bag. The VOCs emitted from the willow foliage were trapped in Haysep-Q filters attached to the outlet (pull) tubes. The pump ran for two hours, and then the filters were removed and individually wrapped with labelled pieces of aluminum foil, and stored in a cooler box to prevent contamination and evaporation of the collected volatiles. The willow branches were cut just below the bags, and the oven-dried weight of the branches was

measured after drying at 60 °C for 72 h; therefore, volatiles measured are presented in nanograms per dry weight (g) per hour ($\text{ng}\cdot\text{g}\cdot\text{DW}^{-1}\cdot\text{h}^{-1}$). Negative controls were also included by taking air samples from empty bags to exclude potential contaminants.

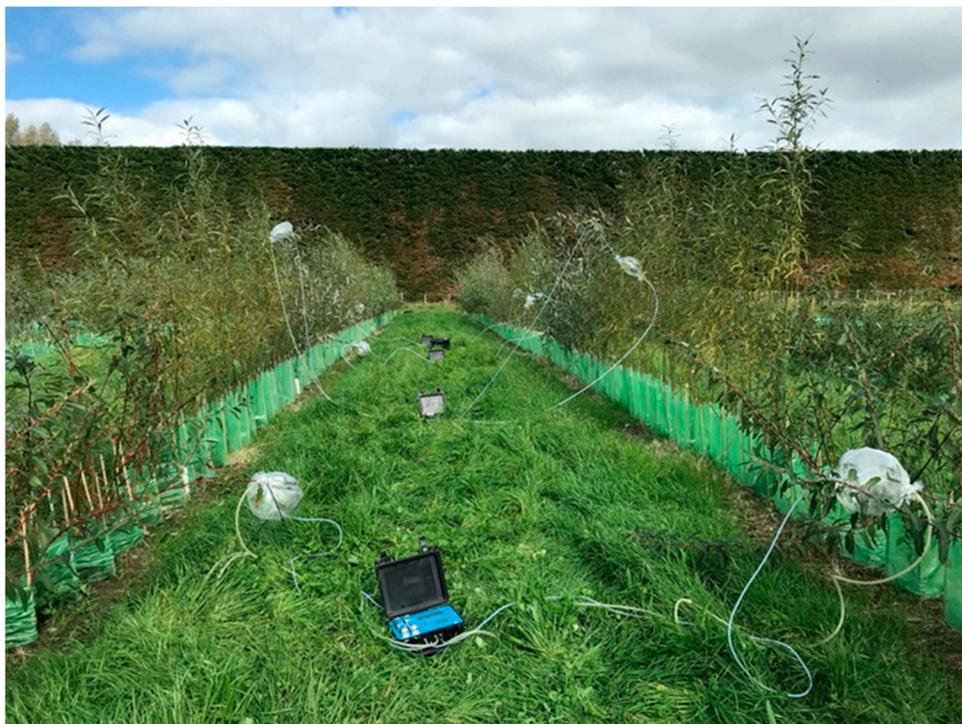


Figure 1. Volatile organic compound (VOC) sampling from willow foliage using a portable volatile assay system. Branches were enclosed in oven cooking bags, into which carbon-filtered air was circulated through a push-pull system.

The first VOC sampling was performed on 17–23 January 2018, to characterize the willow VOCs ($n = 6$ branches/species or hybrid, 90 in total) before giant willow aphid inoculation. To estimate the effect of *T. salignus* on the VOC emissions of the willow plants, second VOC sampling was done shortly after aphid inoculation from both the control and aphid-infested plants on 15–17 March 2019.

2.4. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

The volatile compounds in the filters were eluted using a solvent solution with an internal standard (200 μL hexane with 10 ng/mL of nonyl acetate) into gas chromatography-mass spectrometry (GC-MS) vials and then stored in a -80 °C freezer before analysis. The willow volatiles were separated and identified using the GC-2010 Plus Gas Chromatograph (Shimadzu, Japan) coupled to the AOC-20 I Auto-injector, QP2010 SE- gas chromatograph-mass spectrophotometer, and TG-5MS column (30 $\text{m} \times 250$ $\mu\text{m} \times 0.25$ μm). Helium (He) was used as a carrier gas with the flow rate of 0.5 mL/min into split mode (10:1). The injector port and detector were set up at 250 and 230 °C, respectively. The oven temperature was initially held at 50 °C for 3 min, then increased by 5°C/min to 95 °C, and then ramped to 240 °C at 15°C/min, where it was maintained for three minutes. VOCs were tentatively identified using the NIST (National Institute of Standards and Technology) Mass Spectral Library and confirmed by comparing their retention times with those of commercial standards whenever available. Post-run analyses were carried out using the Shimadzu Lab Solutions software (version 2.50). VOCs were quantified by dividing their peak area by that of the internal standard and expressed as nanogram per microliter per gram of dry weight of foliage per hour ($\text{ng}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$). Contaminants (toluene, p-xylene, o-xylene, diethyl phthalate, etc.) that were consistently identified in negative controls (empty oven cooking bags) were excluded from further analyses.

2.5. Statistical Analysis

The R statistical software (Version 3.6.1) [49] was used for all analyses. For the VOC profiling of the fifteen willow species and hybrids, 19 VOCs were chosen, based on their consistent occurrence in the samples of at least one species or hybrid. One replicate of *S. × fragilis* that did not emit the selected compounds was dropped from analyses. The VOC data were square-root transformed to achieve normality [50], and to allow rare VOCs to have equal weight by reducing the overestimation of highly-occurring VOCs in the headspace samples [51]. We performed a permutational multivariate analysis of variance (PERMANOVA) with Bonferroni adjustment to differentiate the VOC blends of the fifteen willow species and hybrids. The analysis was done using the Adonis function with Bray–Curtis distance matrix and 999 permutations [52]. Non-metric Multi-Dimensional Scaling (NMDS) was performed to depict differences in the VOC profiles of the willow species and hybrids. The PERMANOVA and NMDS were performed using the *vegan* [53] package.

The relative proportion of each major VOC class (aldehydes, GLVs, monoterpenes and sesquiterpenes) was calculated by summing up the specific VOC concentrations for each group, and then dividing by the whole blend as described by Digilio, et al. [54]. We constructed linear mixed model (LMM) ANOVAs on the square root transformed relative VOC proportions using the *lme4* package, to further differentiate the emission of VOC class within each species or hybrid. The fifteen willow species and hybrids were treated as a fixed factor, while the row number of the VOC sampling was considered as a random variable. The linear mixed model was fitted on a proxy-log scale and a multiple comparison was then performed using Tukey's HSD test in *multcomp* and *lsmeans* packages.

To test the response of the willows to aphid infestation, a Tweedie generalized linear model with gamma distribution and log-link function was used to compare the total VOC emissions, and that of each of the four major VOC groups (aldehydes, GLVs, monoterpenes and sesquiterpenes). The concentrations of specific VOCs were summed up to become the total concentrations of the major VOC classes. A one-way ANOVA, followed by Tukey's HSD test was used to compare aphid population levels on willow species, monitored just before VOC sampling. Finally, a Spearman's rank correlation was performed to correlate the aphid population level (proxy-log scale) and the total VOC emissions for the aphid-infested willows; the relationship was visualized using the *ggpubr* package.

3. Results

3.1. Characterisation of Willow VOCs

The VOCs in the headspace samples from the willow plants, before inoculation with aphids, included: one aldehyde (nonanal), four GLVs ((*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl benzoate and (*Z*)-3-hexenyl- α -methylbutyrate), four monoterpenes ((*E*)- β -ocimene, (*Z*)- β -ocimene, α -ocimene and β -myrcene) and ten sesquiterpenes (α -cubebene, (*E,E*)- α -farnesene, (*E*)- β -farnesene, germacrene D, δ -cadinene, (*E*)- α -bergamotene, copaene, (*Z,E*)- α -farnesene, β -caryophyllene and cedrene) (Table 2). *Salix candida* and *S. schwerinii* released the largest number of VOCs (15), whereas *S. matsudana*, *S. matsudana* \times *S. alba* (NZ 1040), *S. purpurea*, *S. lasiandra*, *S. lasiolepis* \times *S. viminalis* and *S. matsudana* \times *S. alba* (NZ 1184) emitted less than six out of the 19 selected VOCs. The remaining species and hybrids produced 7 to 11 VOCs in their headspace samples. The two VOCs released by all willows in this study were (*Z*)-3-hexenyl acetate and (*E*)- β -ocimene. More than 50% of the species and hybrids released α -farnesene, (*Z,E*)- α -farnesene and α -ocimene. The VOCs (*E*)- β -farnesene, germacrene D, δ -cadinene, β -myrcene, cedrene, (*Z*)-3-hexenyl- α -methylbutyrate, α -cubebene, copaene and (*E*)- α -bergamotene were released from 25% of the willow species and hybrids. *Salix candida* was the only species that emitted (*Z*)-3-hexenyl benzoate (Table 2).

Table 2. Gas chromatography-mass spectrometry (GC-MS) analysis of VOCs released from the foliage of fifteen willow species and hybrids.

Species and Hybrids	Major VOC Groups																		Number of VOCs Emitted	
	Aldehyde		GLVs			Monoterpenes					Sesquiterpenes									
	Nonanal	(Z)-3-hexenol	(Z)-3-hexenyl acetate	(Z)-3-hexenyl benzoate	(Z)-3-hexenyl- α -methylbutyrate	(Z)- β -ocimene	(E)- β -ocimene	α -ocimene	β -myrcene	α -cubebene	(E,E)- α -farnesene	(Z,E)- α -farnesene	(E)- β -farnesene	Germacrene D	β -caryophyllene	Cedrene	Copaene	δ -cadinene	(E)- α -bergamotene	
<i>S. candida</i>	+	+	+	+	+	o	+	+	+	+	+	+	o	+	+	+	+	o	o	15
<i>S. eriocephala</i>	o	+	+	o	+	o	+	o	o	+	+	+	o	o	+	o	+	o	o	9
<i>S. lasiolepis</i>	o	o	+	o	o	o	+	+	o	+	+	+	o	+	+	+	+	+	o	11
<i>S. lasiolepis</i> \times <i>S. viminalis</i>	+	+	+	o	o	o	+	o	o	o	+	+	o	o	o	o	o	o	o	6
<i>S. purpurea</i>	o	+	+	o	o	o	+	+	o	o	+	o	o	o	o	o	o	o	o	5
<i>S. schwerinii</i>	+	o	+	o	+	+	+	+	+	+	+	+	o	o	+	+	+	+	+	15
<i>S. viminalis</i>	o	+	+	o	o	+	+	+	+	o	+	+	+	o	o	o	o	o	+	10
<i>S. \times reichardtii</i>	+	+	+	o	o	o	+	o	o	o	+	+	o	o	+	o	o	o	o	7
<i>S. alba</i>	o	+	+	o	+	o	+	o	o	o	+	+	o	o	+	o	o	o	+	8
<i>S. lasiandra</i>	+	o	+	o	o	o	+	o	o	o	+	+	o	o	o	o	o	o	o	5
<i>S. matsudana</i>	o	o	+	o	o	+	+	+	o	o	o	o	o	o	o	o	o	o	o	4
<i>S. matsudana</i> \times <i>S. alba</i> (1)	o	+	+	o	o	+	+	+	o	o	o	o	o	o	o	o	o	o	o	5
<i>S. matsudana</i> \times <i>S. alba</i> (2)	+	o	+	o	o	+	+	+	o	o	+	o	o	o	o	o	o	o	o	6
<i>S. matsudana</i> \times <i>S. lasiandra</i>	+	o	+	o	o	+	+	+	o	o	+	+	o	o	o	o	o	o	+	8
<i>S. \times fragilis</i>	+	+	+	o	o	o	+	+	o	o	+	o	+	o	o	o	o	o	o	7
Number of species and hybrids	8	9	15	1	4	6	15	10	3	4	13	10	2	2	6	3	4	2	4	

The “+” and “o” symbols indicate the presence and absence of the compounds in the headspace samples, respectively. Compounds found in all replicates were used to show their presence or absence in VOC blend of each willow species and hybrid.

The VOC profiles of the tested willow species and hybrids differed significantly (PERMANOVA; Pseudo- $F_{14,88} = 5.83$, $p < 0.001$). Due to the high overlap of the VOC profiles, the NMDS algorithm yielded a low stress value (badness-of-fit; 0.20). However, some of the willow species and hybrids were distinguishable from others. For instance, there was clear differentiation between *S. lasiandra* and *S. lasiolepis*, *S. schwerinii*, *S. matsudana* and *S. matsudana* × *S. alba* (NZ 1040). *Salix schwerinii* had no overlap with *S. eriocephala*, *S. × reichardtii*, *S. lasiolepis* × *S. viminalis*, *S. lasiandra* and *S. matsudana* × *S. alba* (NZ 1040) (Figure 2). As expected, the closely related species and hybrids had more similar VOC profiles, as observed for *S. matsudana* and its hybrids. Tree willows were clustered separately from the shrub willows (Figure 2).

The fifteen willow species and hybrids emitted different proportions of the four classes of VOCs. The relative proportions of long-chain aldehydes in the whole blends differed significantly among the willow species and hybrids ($F_{14,88} = 3.78$, $p < 0.001$) (Figure 3a). The (LMM) ANOVA results also showed a significant fixed effect in GLVs proportion ($F_{14,88} = 8.49$, $p < 0.001$). Three species (*S. lasiolepis* × *S. viminalis*, *S. × reichardtii* and *S. eriocephala*) released more GLVs than five other species or hybrids (*S. schwerinii*, *S. matsudana*, *S. matsudana* × *S. lasiandra*, *S. viminalis* and *S. × fragilis*) (Figure 3b). Monoterpene production varied greatly among the willow species and hybrids ($F_{14,88} = 9.24$, $p < 0.001$). *Salix matsudana*, *S. matsudana* × *S. alba* (NZ 1184), *S. purpurea*, *S. viminalis* and *S. matsudana* × *S. alba* (NZ 1040) had the largest monoterpene emissions, while *S. lasiandra* and *S. lasiolepis* × *S. viminalis* released the lowest amount (Figure 3c). There were significant differences in the relative proportion of sesquiterpenes too ($F_{14,88} = 12.03$, $p < 0.001$). *Salix schwerinii*, *S. lasiolepis*, *S. lasiandra*, *S. matsudana* × *S. lasiandra*, *S. candida*, and *S. alba* were all high sesquiterpene-emitters, while *S. matsudana* × *S. alba* (NZ 1040) and *S. matsudana* had zero emissions (Figure 3d).

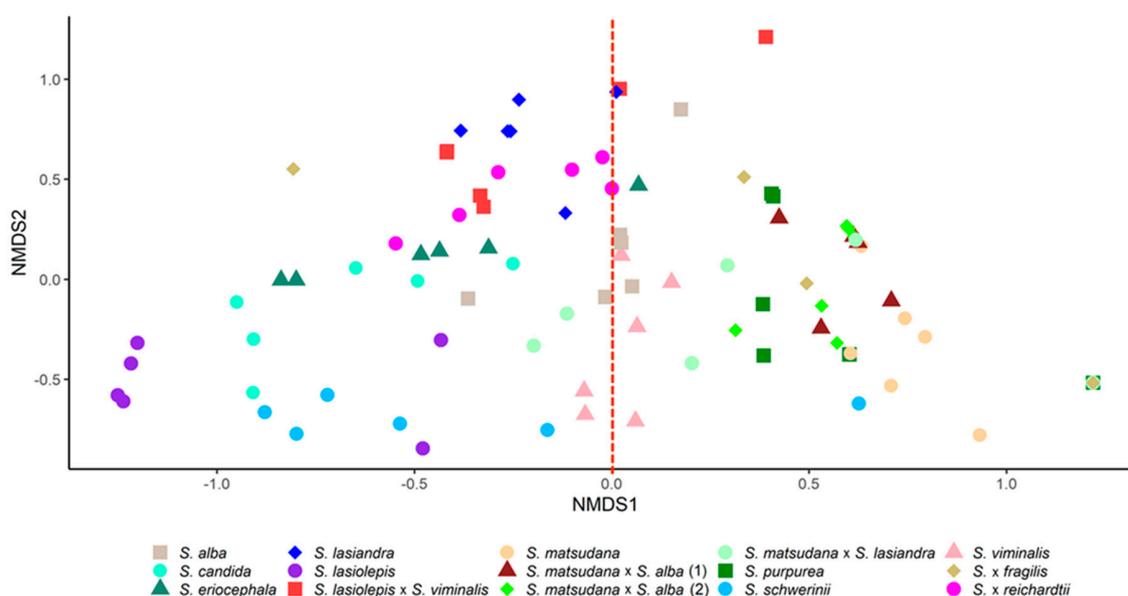


Figure 2. Non-metric Multi-Dimensional Scaling (NMDS) plot of similarities in VOC profiles released by the fifteen willow species and hybrids. Bray–Curtis dissimilarities were calculated on the square-root transformed VOC profiles containing 19 compounds. Each point represents a headspace sample (replicate). Most samples at the left side of the vertical dotted line correspond to shrub willows, whereas most samples at the right of the dotted line are tree willows.

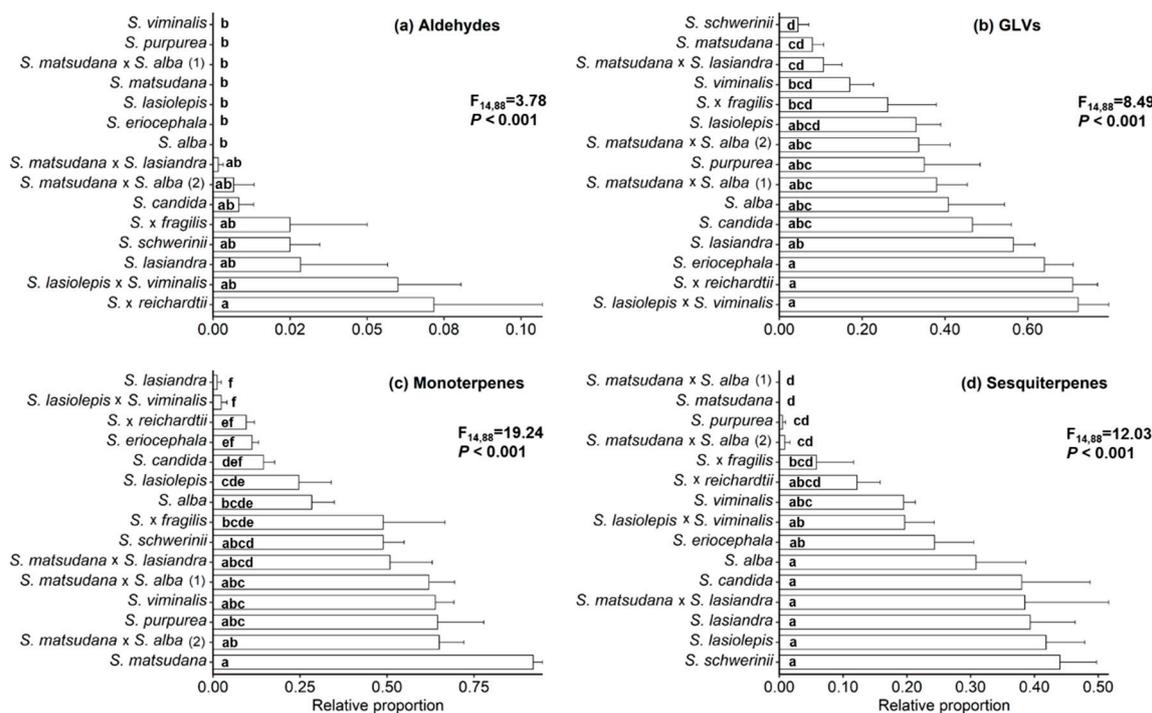


Figure 3. Relative proportions of each major VOC class: (a) aldehydes, (b) GLVs, (c) monoterpenes and (d) sesquiterpenes, emitted by the fifteen willow species and hybrids. The values and error bars are means \pm SE. Different letters represent statistically significant differences ($\alpha = 0.05$) after post-hoc comparisons using Tukey's HSD test.

3.2. VOC Response of Willow Species and Hybrids to *T. salignus* Infestation

Eighteen VOCs were identified in the headspace samples from aphid-infested plants (Table S2) and different willow species responded differently to *T. salignus* infestation. In most cases, the VOC profiles of aphid-infested willow plants did not differ significantly from those of control plants (Figure 4). However, upon closer inspection, aphid infestation was found to significantly decrease total VOC emission in *S. x reichardtii* (Figure 4), GLV emission in *S. matsudana* x *S. lasiandra*, monoterpene emission in *S. x reichardtii* and *S. candida*, and sesquiterpene emission in *S. x reichardtii*, *S. matsudana* x *S. alba* (NZ 1184) and *S. candida* (Figure 5).

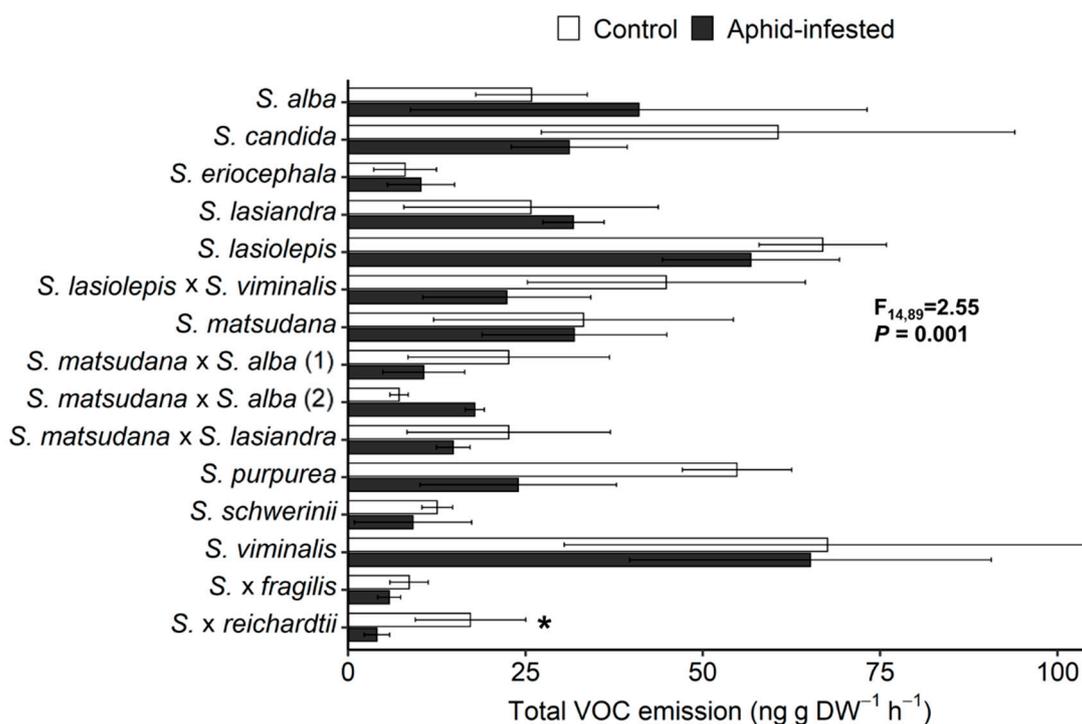


Figure 4. Total VOCs emitted by fifteen willow species and hybrids in the control and aphid-infested treatments. The values and error bars indicate means \pm SE. Asterisks indicate significant differences between the treatments within a species/hybrid, Tukey’s HSD test, $\alpha = 0.05$.

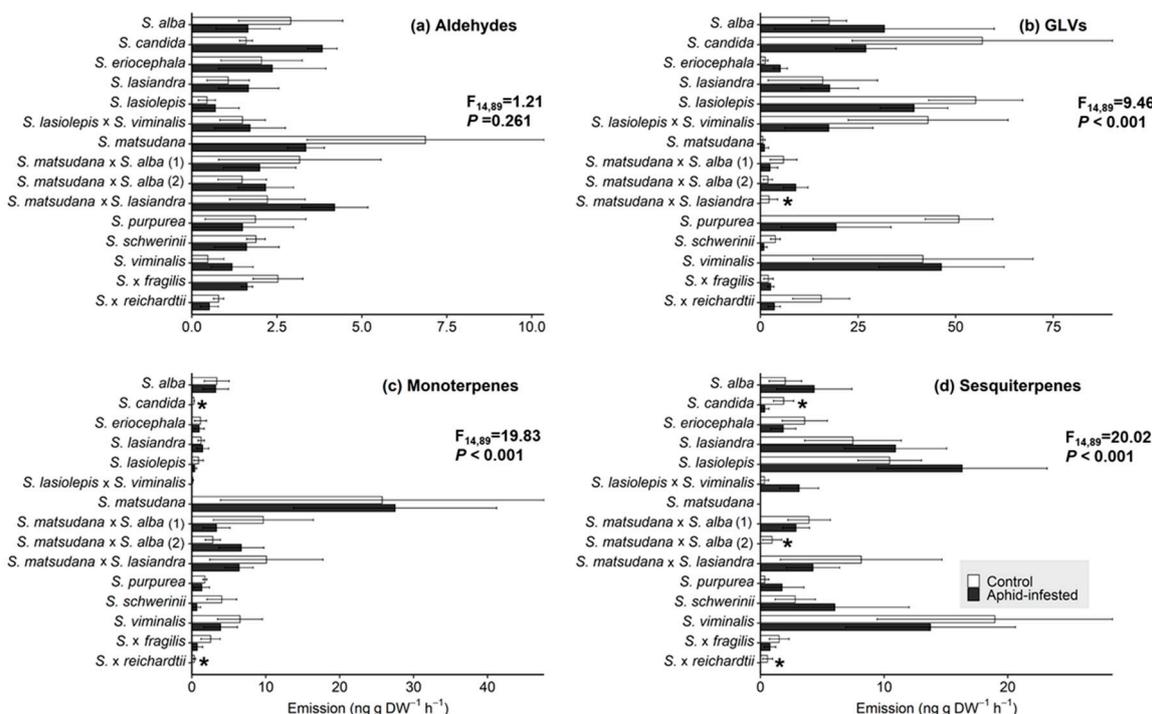


Figure 5. Total emissions of (a) aldehydes, (b) GLVs, (c) monoterpenes and (d) sesquiterpenes by the fifteen willow species and hybrids in the control and aphid-infested treatments. Values and error bars indicate means \pm SE. Asterisks indicate significant differences between the treatments within each species/hybrid, Tukey’s HSD test, $\alpha = 0.05$. Detailed multiple comparisons can be seen in Table S3.

3.3. Correlation between Aphid Infestation and VOC Emissions

Our observation of aphid populations before VOC collection revealed different degrees of infestation among willow species (Figure 6), with *S. eriocephala*, *S. matsudana*, and *S. lasiolepis* × *S. viminalis* having very low infestation rates (most plants having 20 aphids or less) and *S. viminalis* having the highest infestation rates (most plants having 300 aphids or more), followed by *S. candida* (most plants having over a 100 aphids). Correlation analysis revealed only a weak relationship between aphid infestation and VOC emissions ($n = 45$, $\rho = 0.34$, $p = 0.02$).

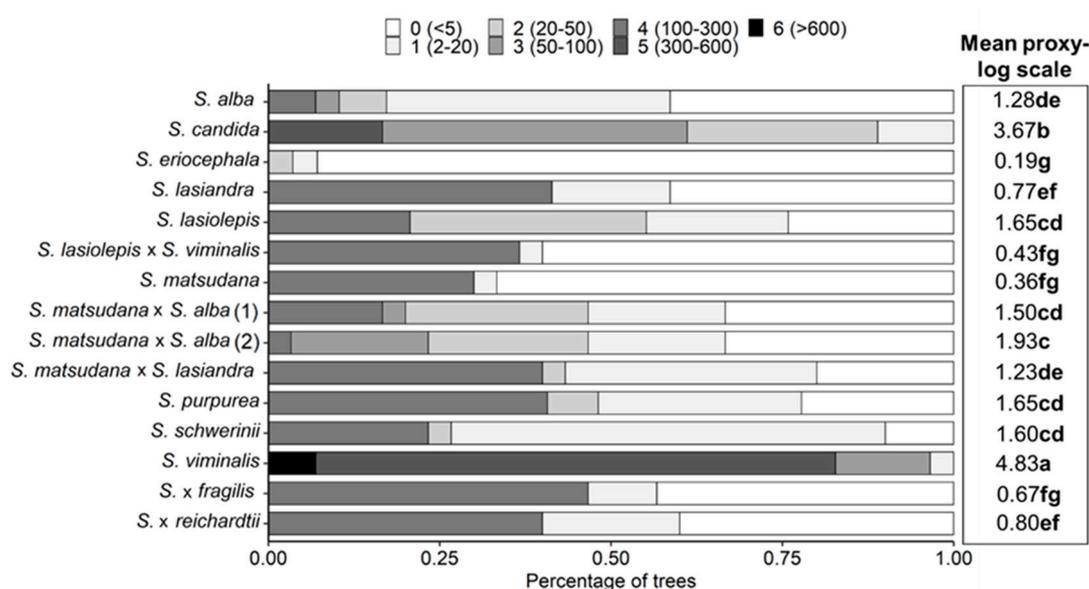


Figure 6. Proportion of willow plants hosting different population levels of *T. salignus* as monitored on 15 March 2019. The numbers on the right side represent mean proxy-log scales, with different letters indicating statistically significant differences among species; Tukey's HSD test, $\alpha = 0.05$.

4. Discussion

Our results showed variation in the VOC profiles of the different willow species and hybrids, attributed to the diversity of *Salix* spp. used in current study (Table 1). The VOC emissions were found to be more similar between closely related plants, as shown by close clustering between *S. matsudana* and its hybrids (*S. matsudana* × *S. alba* (NZ 1040), *S. matsudana* × *S. alba* (NZ 1184) and *S. matsudana* × *S. lasiandra*). VOC composition was distinct between shrub and tree willows, supporting the genetic clustering between these two growth forms as recently reported by Ngantcha [55].

Although species and hybrids varied in the number of compounds emitted, two compounds were common to all blends: GLVs (Z)-3-hexenyl acetate and monoterpene (E)- β -ocimene. Both compounds have been reported for *S. eriocarpa* [45], *S. viminalis* [43] and in related poplar trees (*Populus nigra* and *P. trichocarpa*) [9,56], suggesting that they are ubiquitous to plants species in the family Salicaceae. Studies suggest that these two compounds play important ecological roles. For example, (Z)-3-hexenyl acetate has been identified as a key compound associated with herbivore damage in different willow varieties and in poplar species, and is known to attract natural enemies [9,30,57]. In other willow and poplar species (E)- β -ocimene is involved in within-plant communication as a signal emitted by damaged plant parts to alert nearby undamaged parts of potential attack [58]. In our study, some species and hybrids reduced their emissions of these two compounds in response to *T. salignus* herbivory (Figure S1, Table S4). The reasons behind this reduction are not yet known and deserve further investigation.

Few studies have explored willow VOC responses to herbivore attack. HIPV emission by willows can be highly specific, varying with the life stage of their attacker (larvae vs. adult), and informing natural enemies about the suitable stage of their prey [59]. The predatory ladybird (*Aiolocaria*

hexaspilota) was more attracted to VOC blends induced by willow beetle (*Plagioderia versicolora*) larvae, containing higher amounts of the GLVs (Z)-3-hexenol and (Z)-3-hexenyl acetate, the monoterpenes (E)- β -ocimene, (Z)- β -ocimene, allo-ocimene and linalool, the sesquiterpene (E)- α -farnesene, and two oximes (nitrogenous compounds) [59]. These results show that HIPVs play a role in host selection by herbivores, and in indirect defense in willows.

In the present study, we observed that not all willow species and hybrids reacted to aphid damage in the same way. While some willows (*S. × reichardtii*, *S. matsudana × S. lasiandra*, *S. matsudana × S. alba*, and *S. candida*) responded by decreasing their VOC emissions, the majority of species and hybrids did not show a significant change. Other studies on phloem feeders show contrasting results, with some reporting increases in VOC emissions after attack [26,60–63]. For instance, infestation by spiral gall aphid *Pemphigus spyrothecae* (Hemiptera: Aphididae) on leaf tissue can alter leaf's photosynthetic activity that in turn triggers jasmonate transportation from petiole to lamina and finally modifies VOC emission in poplar (*Populus × petrovskiana*) [63]. However, there are also reports showing reductions in VOC emissions or no response at all [27,61,64]. Furthermore, studies comparing chewers and phloem feeders typically indicate that the latter have a less pronounced effect on VOC emissions than chewing herbivores [27,64].

The lack of response in most species and hybrids may be due to the fact that the giant willow aphid does not directly damage the photosynthetically active tissue (leaves), nor causes severe mechanical damage (as chewing herbivores do), and thus may not trigger strong changes in VOC emissions [64]. However, it has been suggested that aphids actively suppress plant responses to escape their natural enemies [61]. This manipulation of plant responses is possibly mediated by microbial endosymbionts of aphids in order to protect their hosts [65]. Further studies are required to clarify the mechanism behind the observed responses (or lack of them).

The VOC emission reduction observed in some species and hybrids may also be due to a trade-off between indirect and direct defense (i.e., production of VOCs vs. non-volatile secondary metabolites) [66–69]. Both direct and indirect defenses have a metabolic cost to the plant, and plants typically favor one type of defense over another. For instance, a study investigating wild and cultivated accessions of lima bean (*Phaseolus lunatus*) found that plants producing high levels of cyanogenic compounds (direct defenses) released low amounts of VOCs (indirect defenses) and vice versa [69]. In this study, some species and hybrids hosted lower aphid populations and appear to be more naturally resistant to aphid attack than others (Figure 6). This resistance is possibly associated with the presence of physical defense mechanisms (e.g., rough bark of resistant species), suggesting that defense trade-offs could exist in different types of willows.

Plant volatiles are known to play a role in deterring herbivores and attracting natural enemies in related tree species [6,9,59], but considering the costs involved in VOC production and emission [70,71], it would be disadvantageous for a plant to increase its emissions if there was an elevated fitness cost (e.g., higher appetency to generalist herbivores) with no net benefit (e.g., no attraction of natural enemies); such as in our case, where the invasive aphid lacks specialist natural enemies.

The degree of infestation (Figure 6) could also have contributed to different outcomes. The emission of HIPVs can qualitatively and quantitatively vary depending on the population density of the insect feeding on host plants [72,73], with studies typically reporting a positive correlation between herbivore population density and VOC emission [74–76]. However, we only found a weak correlation between aphid infestation and VOC emission, and responding species and hybrids (except *S. candida*) were not heavily infested. This shows that responses are host-specific and less dependent on the degree of infestation, although within the responding species and hybrids, changes in aphid density may affect VOC emissions. Further studies are required to test this hypothesis.

A study by Aradottir et al. [46] found that *T. salignus* was significantly attracted to certain willow varieties but not to others in laboratory olfactometry tests. Although the compounds involved were not identified, this evidence shows that the giant willow aphid uses plant volatiles to choose their host plants. Therefore, future research should explore the role of VOCs in *T. salignus* host selection

and colonization, and the behavioral responses of potential natural enemies of *T. salignus*, such as the harlequin ladybird *Harmonia axyridis* [77] to willow VOCs. Our results suggest that some naturally resistant species and hybrids (*S. lasiolepis* × *S. viminalis* and *S. eriocephala*) are rich GLV emitters, which are known to repel herbivores and attract natural enemies in other systems [78]. Representative compounds from this group, such as (Z)-3-hexenyl acetate, are good candidates for further testing.

In this study, plants belonging to the same species or hybrid had the same sex, so we did not explore the influence of plant sex on VOC emissions or responses to herbivory; this is an aspect that requires further investigation. Being an exploratory study, our results were limited to a low number of replicates, and therefore, we encourage additional studies with higher replication to confirm these findings. Aphid infestation was unequal between plants, as we wanted to explore aphid behavior in nature, and in doing so, were able to identify some species and hybrids which appear to be naturally more resistant than others to aphid attack. We are following this lead towards the selection of resistant species and hybrids for sustainable willow growing.

5. Conclusions

To summarize, there was a high variation in VOC emissions by different willow species and hybrids, with clear clustering between tree and shrub species. Most species and hybrids did not show significant changes in their VOC emissions in response to *T. salignus* infestation, but in those that did, this response was typically a reduction in VOC emissions. Whether this occurs due to the lack of response by the plant, trade-offs between direct and indirect defenses, or the active suppression of plant defenses by the aphid, requires further testing. Our study provides the foundation to further explore the role of willow VOCs in host selection by *T. salignus*. This information will contribute to the selection of willow species and hybrids for future planting, to reduce the ecological and economic impacts of this emerging pest.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/9/1404/s1>. Table S1: Split-plot experimental layout of the willow field trial, Table S2: GC-MS analysis of VOCs released from the foliage of 15 willow species and hybrids, for the control and aphid-infested treatments, Table S3: Mean emissions of total and major VOC classes as influenced by willow species/hybrid and aphid infestation, Table S4: Mean emissions of selected VOCs as influenced by willow species/hybrid and aphid infestation, Figure S1: Total emissions of the terpenoid (*E*)- β -ocimene and the GLV (Z)-3-hexenyl acetate. Asterisks indicate significant differences between the treatments within each species or hybrid, Tukey's HSD test, $\alpha = 0.05$. Details can be seen in Table S4.

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