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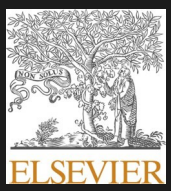

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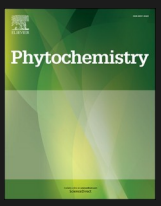

Neighbourhood effects on herbivory damage and chemical profiles in short-rotation coppice willows and their hybrids

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ABSTRACT

Short rotation coppices (SRCs) represent an important source of biomass. Since they are grown in various mixtures, SRCs represent an excellent opportunity for assessing the effects of local plant neighbourhoods on their performance. We used a common garden experiment consisting of plots that varied in genotype diversity of SRC willows to test for the effects of chemical traits of individual plants and chemical variation in the plots where they grew on insect herbivory. We also explored whether the composition of willows planted in a plot affected their chemistry. To do this, we performed untargeted metabolomics and quantified various chemical traits related to the total set of metabolites we detected, flavonoids, and salicinoids in four willow genotypes. We measured the leaf herbivory that the plants suffered. The genotypes differed in most chemical traits, yet we found only limited effects of individual traits on herbivory damage. Instead, herbivory damage was positively correlated with structural variation in salicinoids in a plot. When analysing the effects of plot chemical variation on herbivory damage separately for each genotype, we found both positive and negative correlations between the two, suggesting both associational resistance and susceptibility. Finally, we also observed a significant effect of the interaction between genotype and plot composition on structural variation in plant chemistry. Overall, our results suggest that high chemical variation in mixed willow SRCs does not necessarily lower the herbivory damage, possibly due to spillover effects of insect herbivores among genotypes. Our results also show that different genotypes respond differently to plot composition in terms of herbivory damage and chemical composition, which may affect their suitability for growing in mixed stands.

1. Introduction

Short rotation coppices (SRCs) are a cropping system with perennial trees or shrubs from which wood can be harvested annually within a few years of planting (Dillen et al., 2016 Eufrade et al., 2016). Willows, poplars, or eucalypts are favoured for their rapid growth, high biomass yield, and ability to be harvested on a short rotation cycle, making them valuable feedstocks for bioenergy and bioproducts. Biomass produced by SRC can serve as a viable alternative to fossil fuels and this practice has received significant economic attention and governmental subsidies (Mola-Yudego and Pelkonen, 2008). Plantations of SRCs have also been suggested to improve soil carbon sequestration and support the biodiversity of arthropods in agricultural landscapes (Grogan and Matthews, 2002 Müller et al., 2018). However, SRCs are often grown in monoculture despite the apparent benefits of mixed plantations (Dillen et al., 2016). This not only reduces the beneficial secondary effects, such as biodiversity maintenance (Müller et al., 2018), but can also support outbreaks of pest insect herbivores or pathogens (Stewart and Cromey, 2011). SRCs thus represent an excellent opportunity for testing the neighbourhood effects on plant chemistry and herbivory damage.

Willow hybrids are important for their economic role in biomass production. This is because of their rapid growth rates, adaptability to diverse environmental conditions, and ability to thrive on marginal lands unsuitable for most other crops (Verwijst et al., 2013). Willow hybrids grown in mixed stands have been observed to vary in growth characteristics and susceptibility to herbivory damage (Fritz et al., 2001;

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Orians et al., 1997). Some genotypes may exhibit faster growth rates, leading to higher biomass production, while others may be more resistant to damage by insect herbivores (Hoeber et al., 2017; Müller et al., 2018). Understanding these differences is essential for optimizing biomass production strategies and mitigating potential losses caused by pests.

Willows invest in various chemical defences against insect herbivores and pathogens. Willow defensive chemistry is based primarily on phenolic compounds, such as flavonoids (Boeckler et al., 2011; Volf et al., 2023). Flavonoids have been shown to negatively affect insect herbivores, while they also protect plants from oxidative stress (Nybakken et al., 2012; Simmonds, 2003). They play important roles in protection against UV and as plant pigments, differing between plant individuals exposed to different light regimes (Nybakken et al., 2012). Other phenolics produced by willows include salicinoids, which are phenolic glycosides and derivatives of salicin (Boeckler et al., 2011). Salicinoids can deter generalist insect herbivores from feeding, retard their growth, or increase their mortality (Orians et al., 1997). They probably also have negative effects on fungal pathogens (Hjalten et al., 2007). In contrast, some groups of insect herbivores have adapted to salicinoids and can use them as feeding or oviposition cues (Kolehmainen et al., 1994, 1995). Several species of highly specialized leaf beetles can also sequester salicinoids to produce salicylaldehyde to deter their natural enemies (Pasteels et al., 1983). In turn, these specialists may prefer to feed on willow genotypes with high salicinoid content and cause a large biomass loss to such plants (Orians et al., 1997; Volf et al., 2015b). Some species of specialized leaf beetles are thus considered important pests in willow plantations (Stenberg et al., 2010).

In addition to the effects of the chemical composition of individual plants on the damage they suffer, herbivory is also influenced by intra- and interspecific chemical variation between neighbouring plants (Bustos-Segura et al., 2017; Wang et al., 2023). Most insect herbivores tend to feed on closely related and chemically similar hosts (Forister et al., 2015). For example, many leaf-chewing beetles associated with Salicaceae feed solely on the family but can utilize multiple willow species as their hosts (Leong et al., 2022; Topp et al., 2002). Stands consisting of plants showing pronounced chemical differences thus should suffer less herbivory and show a lower likelihood of herbivore outbreaks (Wang et al., 2023; Ziaja and Müller, 2023). The practice of mixing crops is now widely used in agriculture and forestry to reduce herbivore and pathogen damage (Kelty, 2006; Malézieux et al., 2009). This effect has also been observed in SRCs, including willows and poplars (Stewart and Cromey, 2011). However, it is unclear what aspects of trait variation play a primary role – if it is the presence of individual key metabolites, the concentration or structural diversity of broader metabolite classes, or a completely different set of traits expressed by willows in the mixture.

In addition to affecting herbivory through variation in chemistry among plants, community or mixture composition can also affect plant chemistry itself (Molleman et al., 2024; Mraja et al., 2011). Changes in plant chemical profiles can be mediated by allelopathic effects of surrounding vegetation that affect not only the growth or germination of the given plant (Mudrák and Frouz, 2012) but also its investment in specialized metabolites (Wäschke et al., 2015). Other factors can be linked to vegetation structure that influences abiotic conditions, such as light availability, which affects investment in metabolites like flavonoids (Molleman et al., 2024). Lastly, the changes in plant chemistry can be related to herbivore spillover effects from surrounding plants, which may affect the induction of defensive metabolites (Molleman et al., 2024; Mraja et al., 2011).

Here, we explore herbivory damage and variation in specialized metabolites in a common garden experiment consisting of plots that vary in the diversity of SRC willows (Müller et al., 2018). We quantify willow chemistry using the concentration of individual metabolites along with indices that account for their structural diversity and variation between plants (Petrén et al., 2023; Sedio et al., 2017). With this, we aim to test the effects of individual metabolites versus the variability in metabolomes among the studied plants on the observed patterns in SRC willow genotypes. First, we expect that the insect herbivory damage to individual plants will be correlated with their chemical profiles, particularly with the concentration of salicinoids. Second, we expect that the chemical variation between plants in a plot will negatively correlate with the herbivory damage to the plants growing within. Lastly, we expect that the genotype composition in a plot will affect the chemical profiles of the studied willows, particularly in the case of metabolites such as flavonoids that respond to shading by surrounding plants.

2. Results

In total, we detected 9810 metabolites with untargeted metabolomics. The genotypes differed in all the measured chemical variables except for the structural diversity of the total set of metabolites we detected (Table 2, Table S1). Generally, Loden was the most chemically distinct genotype, showing low investment in flavonoids and high investment in salicinoids (Fig. 1). The average herbivory damage per genotype ranged between 1.2 % and 2.2 %. It significantly differed between the genotypes, with Björn showing the lowest and Jorr the highest damage ($\chi^2(3) = 8.18$, $p = 0.042$). However, none of the individual chemical variables correlated with herbivory damage once we accounted for the effect of genotype (Table 3).

Using the Least Absolute Shrinkage and Selection Operator (LASSO) regression, we selected 55 metabolites with non-zero coefficients that best described the variation among the plants (Table S2). These metabolites included mainly fatty acids (20%) and terpenoids (15%), while 47% of the metabolites remained unclassified when applying an 80% classification probability threshold. The metabolites selected by LASSO included two flavonoids and no salicinoids. When correlating the LASSO-selected metabolites with herbivory damage, the best model included fixed effects of two unclassified metabolites ($\chi^2(2) = 12.66$, $p = 0.002$).

The plot genotype diversity (i.e., the number of genotypes in the plot) and genotype composition did not show a significant correlation with herbivory damage ($\chi^2(1) = 0.05$, $p = 0.822$ and $\chi^2(14) = 18.50$, $p = 0.185$, respectively). In terms of structural chemical variation, we observed that Loden was distinct in all types of analyses when visualized with Principal Component Analysis (PCA). Björn, Jorr, and Tora were intermixed and showed large variation in all metabolites we detected, while Jorr formed a largely separated cluster when considering flavonoids (Fig. 2). When we tested for the correlation between chemical variation in the plot and herbivory damage to the plants within, we found a positive correlation between the herbivory damage and the standard deviation in axis 2 coordinates for salicinoids ($\chi^2(1) = 4.65$, $p = 0.031$). This axis corresponded mainly to the differences in salicinoids within genotypes (Fig. 2). Other measures of chemical variation did not show significant effects (Table 3). When performing the models for individual genotypes, we found positive correlations between herbivory damage and the standard deviation in axis 2 coordinates for the total set of metabolites we detected ($\chi^2(1) = 5.40$, $p = 0.020$), salicinoids ($\chi^2(1) = 4.70$, $p = 0.030$), and flavonoids ($\chi^2(1) = 4.20$, $p = 0.041$) in Jorr. In contrast, we found a negative correlation between herbivory damage and the standard deviation in axis 2 coordinates for the total set of metabolites we detected ($\chi^2(1) = 9.24$, $p = 0.002$) in Loden.

The best Redundancy Analysis (RDA) model explaining the structural variation in the total set of metabolites included the effect of genotype and its interaction with the plot genotype composition. Together, these variables explained 28.80% of the variation (pseudo-F = 1.8, $p < 0.001$). The best model explaining the structural variation in flavonoids included solely the effect of genotype, which explained 18.00% of the variation (pseudo-F = 7.5, $p < 0.001$). Similarly, the best model explaining the structural variation in salicinoids included only the effect of genotype that explained 18.02% of the variation (pseudo-F = 10.5, $p < 0.001$). When we considered the concentration, richness, and

Table 1
Sampling design showing the plot composition in terms of genotype diversity a genotype composition (Bj ̈orn – B, Jorr – J, Loden – L, Tora – T).

Genotype diversity	Genotype composition	Plots sampled	Number of plots per genotype composition	Individuals sampled	Number of individuals per genotype composition
monoculture	B, J, L, T	12	3	20	5
two genotypes	BJ, BL, BT, JL, JT, LT	18	2–3	60	10
three genotypes	BJL, BJT, BLT, JLT	12	3	60	15
four genotypes	BJLT	3	3	20	20

The columns show the total number of plots sampled per diversity level, number of plots sampled per each genotype composition, total plant individuals sampled per diversity level, and number of plant individuals sampled per each genotype composition. In total, we sampled 44 plots (one JL plot was excluded due to planting error) and 160 plant individuals. Metabolomics analyses failed for 11 individuals, leaving us with 149 samples entering the statistical analyses.

Table 2
Results of LMEs testing the chemical differences between the studied genotypes.

Explained Variable	$\chi^2(3)$	p	ΔAIC
Concentration of flavonoids	26.60	<0.001*	−20.60
Concentration of salicinoids	202.97	<0.001*	−196.97
Richness of the total set of metabolites	19.916	<0.001*	−13.92
Richness of flavonoids	98.02	<0.001*	−92.021
Richness of salicinoids	174.26	<0.001*	−168.26
Structural diversity of the total set of metabolites	3.28	0.350	2.71
Structural diversity of flavonoids	183.65	<0.001*	−177.65
Structural diversity of salicinoids	98.79	<0.001*	−92.78

The table gives test statistics and differences in AIC between the null model and the model including genotype as a fixed effect. Significant results are marked with asterisks.

structural diversity of the total set of metabolites we detected, flavonoids and salicinoids as response variables, the best model explaining the variation included the genotype and its interaction with plot genotype composition. Together, they explained 71.13% of the variation (pseudo-F = 12.9, p < 0.001). Most of the variation among the plants of the same genotype growing in plots with different genotype compositions was related to the investment in the richness and structural diversity of the total set of metabolites we detected, particularly in Jorr and Tora (Fig. 3).

3. Discussion

We explored herbivory damage and variation in specialized metabolites in a common garden experiment consisting of plots with various diversity of SRC willows. Our results show a limited effect of the chemical composition of individual plants on the herbivory damage they suffered. Instead, we found a positive correlation between plot variation in salicinoids and herbivory damage to the plants within. When analysing the effects of plot chemical variation on herbivory damage separately for each genotype, we found both positive and negative correlations between the two, showing that different genotypes show different associational responses to the chemical variation in their neighbourhood (Plath et al., 2012; Ziaja and Müller, 2023). This may affect their suitability for growing in mixed stands. Our results also show that the plot composition affected the investment in composition, richness, and structural diversity of metabolites in the plants. Overall, our results provide further understanding of chemically mediated interactions in SRC plantations and in plant communities in general.

The four genotypes we worked with included *Salix viminalis* (Jorr), two hybrids of *S. schwerinii* and *S. viminalis* (Björn and Tora), and *S. dasyclados* (Loden). All these species are from a single section of the

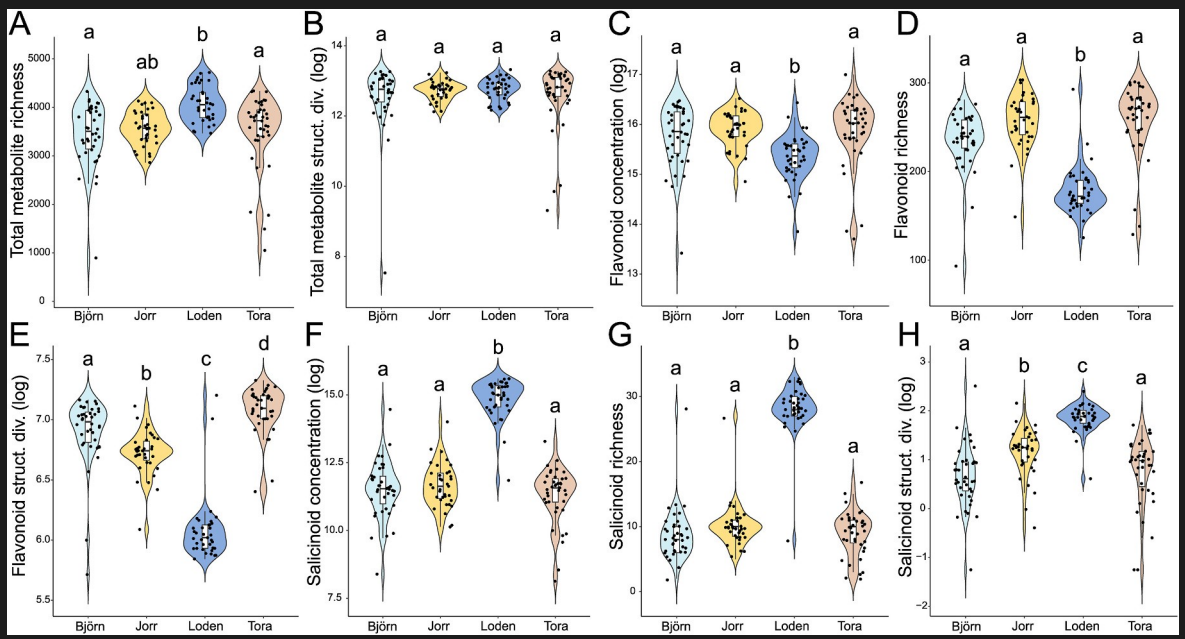

Fig. 1. Chemical traits measured in the studied genotypes. We analysed the richness of the total set of metabolites we detected (A), structural diversity of the total set of metabolites we detected (B), concentration of flavonoids (C), richness of flavonoids (D), structural diversity of flavonoids (E), concentration of salicinoids (F), richness of salicinoids (G), and structural diversity of salicinoids (H). The differences were analysed with LMEs and the letters above the plots indicate significant differences as recovered by Tukey post-hoc tests. The violins depict the data distribution, with the width showing the approximate frequency of data points. The boxes display the first to third quartile with the medians as horizontal lines. The whiskers indicate range. Differences in metabolite concentration and structural diversity are shown on a logarithmic scale.

Table 3
Results of GLMMs testing the correlation between herbivory damage and the standard deviation in PCA coordinates on axis 1 and 2 for the total set of metabolites we detected, flavonoids, and salicinoids.

Explanatory Variable	$\chi^2(1)$	p	ΔAIC
The total set of metabolites (axis 1)	1.73	0.188	0.27
The total set of metabolites (axis 2)	0.00	0.954	2.03
Flavonoids (axis 1)	2.12	0.145	−0.12
Flavonoids (axis 2)	2.92	0.088	−0.92
Salicinoids (axis 1)	1.58	0.208	0.42
Salicinoids (axis 2)	4.65	0.031*	−2.65

The table gives test statistics and differences in AIC between the null model including the fixed effect of genotype and the model including the fixed effects of genotype and the given chemical variable. Significant results are marked with asterisks.

genus *Salix* and are closely related (Skvortsov, 1999; Wagner et al., 2020). Despite that, our results show pronounced chemical differences between the four genotypes in almost all traits concerning their investment in concentration, richness, and structural diversity of metabolites. This matches the results from natural willow populations where chemical profiles typically show a low correlation to the relatedness among willow species (Volf et al., 2015b, 2023). Pronounced differences in functional traits, including various physical or chemical traits, can promote the co-occurrence of closely related plant species (Cavender-Bares et al., 2004; Sedio et al., 2017). Similarly, different growth characteristics and chemical profiles among related willow species and their hybrids also improve their suitability as SRCs grown in mixed cultures, probably contributing to their success in SCR mixed stands (Hoeber et al., 2018).

Despite the pronounced chemical differences we detected, we found that out of all the chemical traits we measured, only two unclassified metabolites correlated with herbivory damage. This contrasts with our original predictions, and it also suggests that the differences in plant chemistry we observed did not occur due to induction following herbivory. We originally supposed that damage by leaf-chewing herbivores would correlate with the concentration, richness or structural diversity of salicinoids and flavonoids, two groups of metabolites with known defensive roles in willows (Boeckler et al., 2011; Volf et al., 2015a). A large part of leaf-chewing herbivore fauna on willows is represented by adult beetles (Topp et al., 2002; Volf et al., 2015a). High numbers of adult beetles were also found by Müller et al. (2018) who studied the same experimental site before us. Adult beetles can efficiently move between plants and mix their diets. This may help them to avoid the negative effects of defensive metabolites, and it can potentially explain the relatively low correlation between their communities and willow chemistry found by some previous studies (Leong et al., 2022; Topp et al., 2002). The feeding preferences of adult beetles, thus may mainly follow traits that relate to nutrient variability between the studied genotypes, such as the differences in their specific leaf area or nitrogen economy (Hoeber et al., 2017). This could explain why we detected differences in the herbivory damage between the genotypes but only a weak correlation to their defensive chemistry.

Chemical dissimilarity between plants promotes the differences in their herbivore communities (Becerra, 2007; Volf et al., 2015b). Increased β-diversity in herbivore communities between chemically distinct genotypes could be one of the mechanisms explaining the previously recorded higher diversity of arthropods in mixed plots consisting of the four willow genotypes we studied (Müller et al., 2018). Higher chemical variation and the lower number of shared herbivores should also reduce the herbivory damage plants suffer (Salazar et al., 2016; Wang et al., 2023). Here, we observed the negative correlation between herbivory damage and chemical variation in the plot in the case of Loden when considering the variation in the total set of metabolites we detected. Loden was the genotype with the highest concentration, richness, and structural diversity of salicinoids that serve as feeding and oviposition cues to specialized herbivores feeding on willows, such as various leaf beetles or sawflies (Kolehmainen et al., 1994, 1995; Orians et al., 1997). These specialized herbivores can, in turn, prefer to feed on plant individuals with higher salicinoid concentration or diversity (Orians et al., 1997). Our results suggest that, in terms of herbivory damage, this genotype benefited from growing in mixtures of plants with structurally different metabolomes that are less likely to share such specialized herbivores (Becerra, 2007). These results are thus similar to other plot-based studies that reported associational resistance to herbivores mediated by increased chemical dissimilarity between neighbouring plants (Ziaja and Müller, 2023).

In contrast, when considering all genotypes in one model, we observed higher herbivory damage on plants that grew in plots with higher chemical variation in salicinoids. It was driven mainly by Jorr,

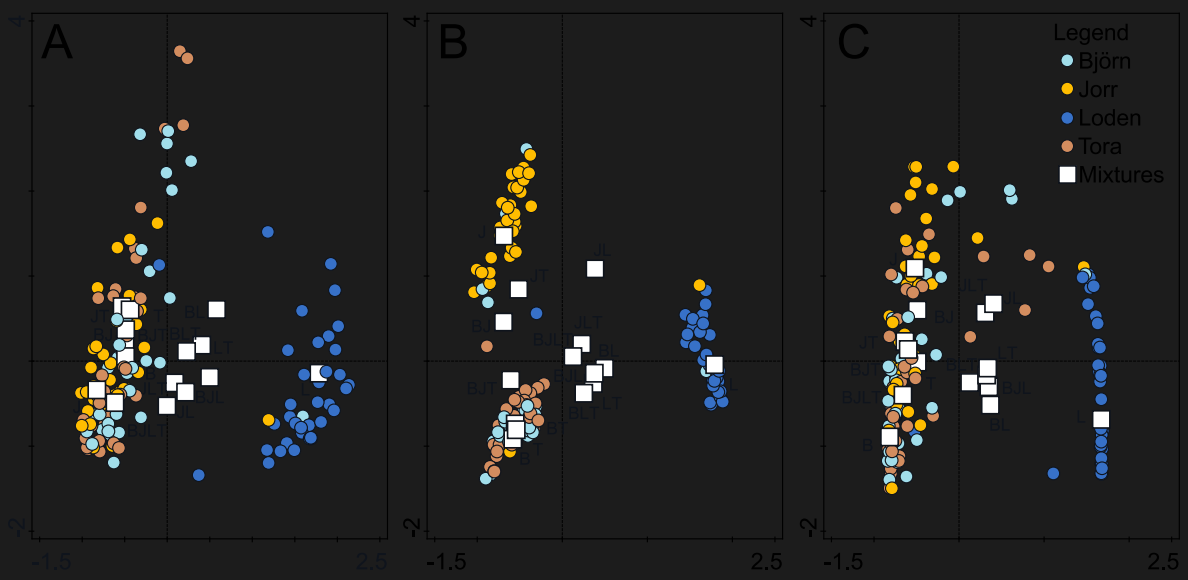

Fig. 2. Structural chemical variation between the studied plants as visualized with PCAs based on axes derived from CSCS matrices for the total set of metabolites we detected (A), flavonoids (B), and salicinoids (C). The first two unconstrained axes explained 13.11% of variation in the total set of metabolites we detected, 13.80 % of variation in flavonoids, and 13.50 % of variation in salicinoids. Plant individuals appear as circles. We used genotype composition of the plots (Bj örn – B, Jorr – J, Loden – L, Tora – T) as a supplementary variable not constraining the ordination space (indicated by squares).

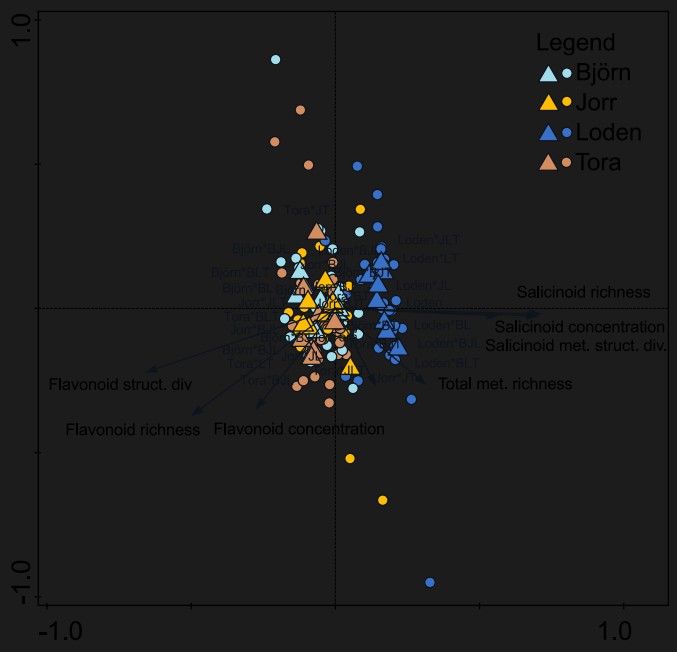

Fig. 3. Variation in the concentration, richness and structural diversity of metabolites as explained by an RDA with forward selection. The best model explaining the variation included the genotype and its interaction with plot genotype composition. Together they explained 71.13% of the variation (pseudo-$F = 12.9$, $p < 0.001$). Chemical traits used as response variables appear as black arrows, plant individuals used as samples appear as circles, and explanatory variables appear as triangles. Samples and explanatory variables are coloured based on the genotype identity of the sample or the identity of genotype interacting with the genotype composition of the plot. Combinations of genotypes and plot composition that were linearly dependent on the other combinations are not shown.

which seemed to be most susceptible genotype of the four we studied and showed the highest herbivory damage. In Jorr, the herbivory damage increased with all three measures of chemical variation we quantified. Previous studies have shown that different herbivores can respond differently to plot diversity, which can result in some plants experiencing associational susceptibility in mixed plots (Plath et al., 2012). Although chemical diversity can reduce damage by insect herbivores (Salazar et al., 2016; Wang et al., 2023; Ziaja and Müller, 2023), it can also increase herbivore abundance (Bustos-Segura et al., 2017). Generalist herbivores may prefer to feed in chemically diverse stands as it allows them to mix their diet and avoid some of the negative effects of host-plant defences (Unsicker et al., 2008). Potentially, the observed effects thus could occur due to a spillover of generalist herbivores to Jorr from surrounding plants. Although insect community data would be required to explore the exact mechanism, our results thus indicate that the genotype Jorr may be less suitable for growing in chemically diverse stands.

We observed a significant effect of the interaction between genotype and plot composition on structural variation in the chemistry of individual plants. Neighbourhood effects on plant chemistry can be mediated by various factors, including shading or spill-over of herbivores from surrounding vegetation (Molleman et al., 2024; Mraja et al., 2011). Here, we observed a significant interaction between genotype and plot composition in the case of the total set of metabolites we detected. No such interaction was observed for salicinoids or flavonoids that serve as defences against herbivores or, in the case of flavonoids, change in response to light conditions (Boeckler et al., 2011; Nybakken et al., 2012; Simmonds, 2003). This potentially rules out the herbivores or shading as the main drivers of the observed pattern. Previous studies on the focal four genotypes showed that increased diversity does not reduce their growth compared to monocultures (Hoeber et al., 2018). The observed changes in chemistry thus may be unrelated to direct or indirect heterospecific competition between the studied plants and they may rather relate to changes in microclimate conditions. Microclimate likely varies between plots with different genotype compositions since the four genotypes show different growth and leaf architecture (Müller et al., 2018). This would correspond to the fact that we observed the interaction between the genotype and the specific genotype composition of the plots rather than between genotype and plot diversity. Additionally, the significant interactions suggest that the genotypes reacted to plot composition with different chemical responses, particularly with their investment into its richness and structural diversity. Such genotype-specific trends may allow for more flexible compensatory responses to changing microclimatic conditions, which may further explain why previous studies did not find mixture-dependent growth differences in the studied genotypes (Hoeber et al., 2018).

4. Conclusions

Our results showed neighbourhood effects on herbivory damage and chemical profiles in SRC willows and their hybrids. The genotype-specific responses in the chemical composition of individual plants to the plot composition may be one factor that contributes to the successful growth of these genotypes in the mixed plots, similarly as chemical variation among plant species does in rich natural communities (Sedio et al., 2017). Yet, our results also illustrate that the effects of chemical variation on herbivory damage can vary between plants and the groups of metabolites considered (Hauri et al., 2021; Salazar et al., 2016). Some genotypes can benefit from growing in chemically diverse stands, while others may show higher herbivory damage under such conditions. This shows that both associational resistance and susceptibility can play an important role among willows and their hybrids. Overall, our results thus do not suggest that high chemical variation in mixed SRCs would universally lower the herbivory damage the plants suffer (Müller et al., 2018; Stolarski et al., 2011). The results likely depend on the particular chemical composition of the genotype and its susceptibility to specialist versus generalist herbivores (Plath et al., 2012; Salazar et al., 2016).

5. Experimental

5.1. Experimental design and sampling

The study was conducted at the Freiburg Experimental Ecology field research area (48°01′N, 7°49′E, elevation: 240 m). It was conducted within the ECOLINK-*Salix* experiment (Verheyen et al., 2016; Müller et al., 2018), which is part of the global network of tree diversity experiments (TreeDivNet). The area has an oceanic climate with a mean annual temperature of 11.8 °C and 858 mm of annual precipitation. See Wein et al. (2016) and Müller et al. (2018) for further details on the site.

The experiment employs a randomized block design with three replicates of 15 plots measuring 9.6 × 9.6 m each. Each plot consists of 12 rows with 12 individuals each, with 0.8 m between individuals. The experimental plots have an artificial willow diversity gradient comprised of clones of four genotypes: Björn (B; *Salix schwerinii* × *viminalis* hybrid), Jorr (J; *S. viminalis*), Loden (L; *S. dasyclados*), and Tora (T; another *Salix schwerinii* × *viminalis* hybrid). The tree diversity gradient is composed of monocultures (B, J, L, T), mixtures of two genotypes (BJ, BL, BT, JL, JT, LT), mixtures of three genotypes (BJL, BJT, BLT, JLT), and finally mixtures of all four genotypes (BJLT). The plants were planted in March 2014. The short rotation coppice was cut in February 2017. Hence, the aboveground parts of the plants were at maximum of 3 years old during our sampling.

Leaf samples were collected from 160 individual trees from 44 plots (one plot was excluded due to an error in planting), including 40 individuals per genotype and representing all genotype combinations and diversity levels (Table 1) on June 13–14, 2020. The collected leaf samples consisted of 20 freshly matured, undamaged leaves from each

individual and were stored in a portable cooler at 4 °C in the field. After returning from the field, the leaves were frozen at −20 °C and freeze-dried for metabolomics analyses. Additionally, another set of 20 leaves was haphazardly collected from each plant and visually assessed for damage by leaf-chewing insects, which accounted for over 99% of all recorded herbivory. Galling and mining damage on leaves was extremely rare and not scored. Each leaf was individually scored for damage by leaf-chewers following Johnson et al. (2016). Damage values between 1% and 5% were scored in steps of 1% (i.e. 0.01, 0.02 …), and values larger than 5% were scored in steps of 5% (i.e. 0.05, 0.10, 0.15 …). The damage values were then averaged across the 20 leaves sampled from the given individual. The obtained mean herbivory damage for each plant (on a scale from 0.00 to 1.00) was used in the subsequent statistical analyses.

5.2. Metabolomics analyses

We performed untargeted metabolomics to analyse the chemical profiles of the studied plants. Small organic molecules were extracted from ca. 10 mg (in 0.01 mg accuracy) of homogenized material using 1.8 ml methanol/water (90:10, v/v) solvent. The samples were extracted overnight at 4 °C and 300 rpm, centrifuged at 14,000 rpm for 30 min, and the supernatant was removed and filtered for analysis using LC-MS (Sedio et al., 2021). We optimized UHPLC-MS parameters to detect and fragment metabolites representing a wide range of polarity and mass (Sedio et al., 2018). Metabolomic extracts were separated using a Thermo Fisher Scientific (Waltham, MA, United States) Vanquish Horizon Duo ultra-high performance liquid chromatography (UHPLC) system with an Accucore C18 column with 150 mm length, 2.1 mm internal diameter, and 2.6- μm particle size. UHPLC buffer A (0.1% v/v formic acid in water) and buffer B (0.1% v/v formic acid in methanol) were employed in a solvent gradient from 5 to 100% buffer B over 18 min. Separation of metabolites by UHPLC was followed by heated electrospray ionization (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. An MS1 full scan (115–1725 *m/z*) was collected at a resolution of 140,000. Automatic gain control target values were 1e6 for full scan MS1 and 1e5 for dd-MS2. Maximum ion injection times were 200 ms for full scan MS1, 100 ms for QC MS1, and 50 ms for MS2. For dd-MS2, the isolation window was set to 1.5 *m/z* and stepped collision energy at 20, 40, and 60.

The adjusted data were processed for peak detection, peak alignment, and peak filtering using MZmine 3 (Schmid et al., 2023). Using the MZmine 3 output, molecular formulae were inferred with Sirius (Dührkop et al., 2019), structures were predicted with CSI: finger ID (Dührkop et al., 2015) and the metabolites were classified with CANOPUS (Dührkop et al., 2021). Using this information, we created three datasets of metabolites, including i) the total set of metabolites we detected, ii) flavonoids, and iii) salicinoids plus their derivatives. We manually checked the classification of salicinoids based on the predicted structures, represented as text strings, using the Simplified Molecular Input Line Entry System (SMILES).

We calculated the following chemical traits for the three datasets of metabolites: i) concentration in a sample, ii) richness in a sample, iii) structural diversity in a sample, iv) structural variation between the samples. The concentration of metabolites in a sample was calculated as the peak area divided by the sample weight. The richness of metabolites was calculated as the number of metabolites in the given sample. The structural diversity of metabolites in a sample was calculated as functional Hill diversity equivalent to Shannon diversity using the CHEMODIV R-package (Petrén et al., 2023). Briefly, the predicted SMILES and the function 'compDis' were used to calculate dissimilarity among individual metabolites, which were then used to quantify the structural diversity of metabolites in the samples using the function 'calcDiv'. Structural variation between the samples was calculated using chemical structural-compositional similarity (CSCS) matrices (Sedio et al., 2017). The CSCS metric considers the similarity between individual metabolites based on the cosine of the angle between their consensus tandem mass spectra. Structurally related metabolites thus have a non-zero contribution toward the similarity between the samples. To calculate the CSCS metric, the data were uploaded to the Global Natural Products Social (GNPS) Molecular Networking platform (Wang et al., 2016). They were used to generate a molecular network using the "feature-based molecular networking" method (Nothias et al., 2020), as described in Sedio et al. (2021). The output was used to calculate the CSCS metric for every pair of samples in the three datasets to quantify the structural variation among samples. The metabolomics analyses failed for 11 samples, possibly due to poor sample quality or issues during their extraction. Only 149 samples were thus used in the following statistical analyses.

5.3. Statistical analysis

To provide background information for interpreting our other results, we initially tested whether the genotypes differed in the concentration, richness, or structural diversity of the total set of metabolites we detected, flavonoids, and salicinoids. To do so, we used Linear Mixed Effect Models (LMEs) in the package'lme4' (Bates et al., 2015) in R 4.2.0 (R Core Team, 2022). The concentration, richness, and structural diversity of the metabolites were log-transformed and used as response variables in the models. Genotype was used as a fixed effect and plot as a random effect. We performed post-hoc pairwise tests using a Tukey adjustment in the package 'emmeans' (Lenth et al., 2020) to compare the differences among the genotypes. We did not run a separate model for the concentration of the total set of metabolites we detected since we did not consider it an ecologically or functionally meaningful variable.

To test our first hypothesis, we first used Generalized Linear Mixed Effect Models (GLMMs) with a beta error term and logit link to test the correlation between herbivory damage and genotype. Herbivory was used as the response variable, genotype as the fixed effect, and plot as the random effect. The analysis was performed in the R package 'glmmTMB' (Brooks et al., 2017). Next, we used GLMMs with a beta error term and logit link to test for the correlation between herbivory damage and the concentration, richness, or structural diversity of the total set of metabolites we detected, flavonoids, and salicinoids. Z-scores were used for the tested chemical variables since they appeared on very different scales. Herbivory was used as the response variable. The genotype was included as a fixed effect in the null model since we were mainly interested in the effect of chemistry independent of the effect of genotype. AIC was used to compare the null model with a model including both the genotype and either concentration, richness, or structural diversity of metabolites in the given plant individual as fixed effects. The plot was used as a random effect.

We then correlated herbivory damage to the concentration of individual metabolites we detected. Since multiple metabolites were detected with the untargeted metabolomics, we used the LASSO regression to select the metabolites with non-zero coefficients that best correlated with the variation among the plants (Salazar et al., 2018). For this, all metabolites appearing in less than 10% of the samples were first removed, which resulted in a dataset comprised of 7384 metabolites. To fit the LASSO model, a 10-fold cross-validation method was used in the package "glmnet" (Friedman et al., 2010). We then used GLMMs with a beta error term, logit link and forward selection to select the best model explaining herbivory damage based on individual LASSO-selected metabolites. Herbivory was used as the response variable and the concentration of individual LASSO-selected metabolites as fixed effects. Z-scores were used for the metabolites since they appeared on very different scales. As in the previous analysis, the genotype was included as a fixed effect in the null model. The plot was used as a random effect.

To test our second hypothesis, we first used GLMMs with a beta error term and logit link to test the correlation between herbivory damage and plot composition. Herbivory was used as the response variable. Either

genotype diversity (ranging from 1 to 4) or genotype composition (the 15 possible genotype mixtures) of the plot was used as a fixed effect. Plot was used as a random effect. Next, we used GLMMs with a beta error term and logit link to test the correlation between the chemical variation in the plot and herbivory damage to the plants within. To do so, Principal Coordinate Analysis (PCoA) was first used to transform the CSCS matrices into a set of axes describing the structural chemical variation between the samples based on the total set of metabolites we detected, flavonoids, or salicinoids. The chemical variation between the samples was visualized with PCA in Canoco 5 (ter Braak and Smilauer, 2012). The coordinates of individual plants on the first two PCA axes were extracted and the mean coordinates for all possible combinations of genotypes and plot compositions were obtained. This procedure thus provided mean PCA axis 1 and 2 coordinates for each combination of genotype and type of mixture. Using these means, the standard deviation in the coordinates between plants in each of the studied plots was then calculated. It was used as our measure of chemical variation in the plots. Separate GLMM analyses were performed for the total set of metabolites we detected, flavonoids and salicinoids and axis 1 and axis 2 coordinates, using the chemical variation in the plots as a fixed effect and herbivory damage as a response variable. As before, the genotype was included as a fixed effect in the null model, which was then compared with a model that included both the genotype and standard deviation in the measured chemical traits as fixed effects. Plot was used as a random effect. We then performed separate models for each genotype to explore the effects of chemical variation in the plot on herbivory damage in more detail. Herbivory damage was used as a response variable. Axis 1 and axis 2 coordinates for the total set of metabolites we detected, flavonoids, and salicinoids were used as fixed effects, and plot as a random effect.

To test our third hypothesis, we used Redundancy Analysis (RDA) to test for the effect of genotype, plot diversity, and plot composition on the variation in the total set of metabolites we detected, flavonoids, or salicinoids. We used the PCoA axes generated in our previous analysis as response variables and plot as a covariate. The genotype, genotype diversity of the plot (ranging from 1 to 4), genotype composition of the plot (the 15 possible genotype mixtures), and the interactions between the genotype and the two remaining variables were used as explanatory variables. The variables best explaining the variation in chemical composition were selected with forward selection based on generating pseudo-F distribution with 9999 Monte-Carlo permutations in Canoco 5 (ter Braak and Smilauer, 2012). To further explore the effect of plot composition on the investment into specialized metabolites, the analysis was repeated with concentration, richness, and structural diversity of the total set of metabolites we detected, flavonoids, and salicinoids as response variables (same as above, we did not consider the concentration of the total set of metabolites we detected). We then used RDA with forward selection to identify the variables best explaining variation in these chemical traits, using the same approach as in the case of the previous analysis with PCoA axes.

CRediT authorship contribution statement

Gibson Aubona: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Priscila Mezzomo:** Writing – review & editing, Writing – original draft, Investigation. **Brian E. Sedio:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition. **Michael Staab:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Martin Volf:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, 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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2024.114249.

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