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The frequency and chemical phenotype of neighboring plants determine the effects of intraspecific plant diversity

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Abstract

Associational effects, whereby plants influence the biotic interactions of their neighbors, are an important component of plant–insect interactions. Plant chemistry has been hypothesized to mediate these interactions. The role of chemistry in associational effects, however, has been unclear in part because the diversity of plant chemistry makes it difficult to tease apart the importance and roles of particular classes of compounds. We examined the chemical ecology of associational effects using backcross-bred plants of the *Solanum pennellii* introgression lines. We used eight genotypes from the introgression line system to establish 14 unique neighborhood treatments that maximized differences in acyl sugars, proteinase inhibitor, and terpene chemical diversity. We found that the chemical traits of the neighboring plant, rather than simply the number of introgression lines within a neighborhood, influenced insect abundance on focal plants. Furthermore, within-chemical class diversity had contrasting effects on herbivore and predator abundances, and depended on the frequency of neighboring plant chemotypes. Notably, we found insect mobility—flying versus crawling—played a key role in insect response to phytochemistry. We highlight that the frequency and chemical phenotype of plant neighbors underlie associational effects and suggest this may be an important mechanism in maintaining intraspecific phytochemical variation within plant populations.

KEYWORDS

acyl sugars, associational effects, chemotypic diversity, intraspecific phytochemical variation, plant–insect interactions, proteinase inhibitors, terpenes

INTRODUCTION

Intraspecific diversity has been shown to have a major influence on community patterns and species interactions in both terrestrial and aquatic systems (Crutsinger et al., 2006; Jackrel et al., 2016; Morrison & Hay, 2011; Wimp et al., 2005). A key challenge, however, has been resolving the traits and mechanisms that underlie the effects of intraspecific diversity on ecology (Crutsinger, 2016). For terrestrial plants, a type of diversity that is thought to influence ecology is via associational effects, when the biotic interactions experienced by a plant are influenced by the traits of a neighboring plant (Barbosa et al., 2009; Hambäck et al., 2014). Associational effects are believed to be driven at least in part by differences in secondary chemistry between neighboring plants (Dyer et al., 2018; Kessler & Kalske, 2018; Wetzel & Whitehead, 2020). Variation in phytochemistry among individuals within populations has demonstrable effects on community structure of higher trophic levels (Bustos-Segura et al., 2017; Glassmire et al., 2019; Schuman et al., 2015). However, the limitation here is that focusing on a single class of compounds or grouping hundreds of metabolites into a single metric (i.e., phytochemical diversity) overlooks the diverse functions of the many metabolites within individual plants (Glassmire et al., 2020; Wetzel & Whitehead, 2020). An alternative approach is to separate metabolites by their functional role (i.e., mode of action) on interacting insects. This approach will facilitate our understanding of how insect community structure is influenced by phytochemistry and allow us to tease apart the ecological effects of intraspecific chemical variation among plants on their interactions with insects.

A limitation of past studies of intraspecific plant diversity has mostly focused on manipulating the genotypic richness of plant communities while overlooking the role of chemical diversity (Barbour et al., 2016; Moreira & Mooney, 2013; Root, 1973; Schuman et al., 2015). Those experiments that use genotypic monocultures and polycultures to test genetic diversity suggest that high plant genetic diversity contributes to reductions in pest outbreaks and herbivore consumption (Crutsinger et al., 2006; Johnson et al., 2006) and alters interactions between herbivores and predators (Wetzel et al., 2018). However, although polycultures sometimes work better than monocultures in reducing insect pests, the traits underlying the benefits of polycultures are typically not well understood, even though insects interact with traits, not genetic diversity per se (Bustos-Segura et al., 2017; Moreira et al., 2016; Schuman et al., 2015). Identifying the key traits that underlie the effects of plant genetic diversity (i.e., genotypic monocultures vs. polycultures) on higher trophic levels represents an important gap in our understanding of intraspecific plant diversity and the use of cultivar mixtures for agricultural sustainability.

Measuring responses by arthropods in various guilds or with different traits provides important resolution to how intraspecific variation influences arthropod diversity, as arthropods in different taxonomic groups and guilds have different relationships with chemical traits (reviewed in Moreira et al., 2016). Mobility is a particularly important trait for determining how insects respond to associational resistance mediated by the chemical composition of neighboring plants. For instance, direct phytochemical defenses, such as acyl sugars located on the trichome hairs of plants, interact with insects in close proximity, whereas indirect defenses, such as terpenes emitted into the air, interact with insects from far away. Moreover, insects having differences in mobility—flying versus crawling—will interact with direct versus indirect phytochemical defenses in unique ways. Specifically, flying parasitoids that have wider search ranges and can detect cues from long distances may depend more on indirect defenses. Whereas crawling spined-soldier bugs, for instance, that are searching for prey on the host plant may depend more on direct defenses. Thus, mobility differences of insects, which affects the search range and distance of chemical cue detection, changes the way flying insects respond to the same kind of compound or mixture of compounds compared with crawling insects; and this is true both in various guilds or within the same guild (Bommarco & Banks, 2003; Pearse et al., 2018). One way to measure insect responses to phytochemistry is to incorporate multiple kinds of sampling methods to collect insects based on mobility traits (Schmidt et al., 2008). Resolving the effects of intraspecific diversity on insect trait differences requires experiments using multiple sampling methods to fully capture a broad range of the insect community.

In this study, we used an experimental design to manipulate chemical traits according to functional groups and test how the associational effects of neighboring chemical trait diversity influence the abundance of insect herbivores and predators in an agricultural field setting. Plant neighborhoods with varying chemical diversity were constructed based on three chemical classes that are putatively effective tomato plant defenses (acyl sugars, proteinase inhibitors, and terpenes). Moreover, chemical diversity was fully crossed with the number of introgression line (ILs) in each plot (e.g., genotypic monocultures, dicultures, and polycultures) using eight genotypes from the *Solanum lycopersicum* × *S. pennellii* backcrossed ILs (Eshed & Zamir, 1994). These ILs map chemical traits to genotypes and are a manageable way to manipulate chemical variation because collectively they represent substantial phytochemical variation, but any two genotypes have subtle genetic variation and subtle phenotypic differences, making them nearly identical (Schilmiller et al., 2010, 2012). Furthermore, we sampled the broad insect community using two methods that targeted different kinds of insects based on their mobility. We addressed three questions using experimental plant communities: (1) How does diversity within each chemical class—acyl sugars, proteinase inhibitors, and terpenes—influence arthropod communities? (2) How well does chemotypic richness across all three chemical classes predict arthropod community patterns? (3) Do greater numbers of introgression genotypes predict arthropod community patterns? (4) Does plant density predict arthropod community patterns? These questions were asked with respect to the two mobility classes of insects: mobile flying insects (caught with sticky traps) and less mobile crawling insects (caught with beat sheets). We predicted that classes of chemicals have unique functions that can differentially affect herbivores and predators based on their mobility. Our results shed light on how herbivores and predators with different life-history traits respond to different chemical mixtures of experimental plant communities.

METHODS

Study system

The *Solanum pennellii* IL system comprises 83 genotypes that are backcrosses between tomato cultivar M82 (*S. lycopersicum*) and the wild tomato species *S. pennellii* (Eshed & Zamir, 1994). Each IL is genetically identical to M82 except that each has a single homozygous chromosomal segment introgressed from *S. pennellii*. The system has been frequently used for mapping genotype to phenotype, meaning the phenotypic effects of each introgression are well-studied (Caruso et al., 2016; Schilmiller et al., 2010, 2012). For selecting specific ILs to use in our experiment, the chemical traits we focused on were acyl sugars, proteinase inhibitors, and terpenes because they are a combination of direct and indirect phytochemical defenses that mediate interactions between tomato plants and herbivores and predators. Acyl sugars, typically forming chemical globules on trichomes, are sticky, deterrent compounds that can physically slow feeding, reducing nutrient uptake and development (Luu et al., 2017). They can also serve as cues for predators by chemically tagging the body and frass of herbivores (Weinhold & Baldwin, 2011). Proteinase inhibitors are another type of direct defense that reduces the quality of plant tissue for insect herbivores by inhibiting the digestion of plant proteins (Jongsma & Bolter, 1997; Zhu-Salzman & Zeng, 2015). Finally, terpenes can serve as volatile, indirect defenses that have attractant and repellant odors for colonizing herbivores and predators (Degenhardt et al., 2003; Kessler & Kalske, 2018).

Using previously collected LC-TOF-MS chemical data (Schilmiller et al., 2010), we selected seven IL genotypes and the parental M82 genotype to maximize intergenotypic chemical dissimilarity within three classes of phytochemicals with different putative ecological roles: acyl sugars, proteinase inhibitors, and terpenes (Appendix S1: Tables S1 and S2). Seven ILs were selected based on maximizing chemical dissimilarity within acyl sugars and terpenes. Briefly, we calculated non-metric multidimensional scaling (NMDS) distance matrix using Euclidean distance for each chemical class initially using 66 ILs based on LC-TOF-MS peak abundances of identified compounds (*refer to* Glassmire et al., 2020 *for methods on calculating chemical dissimilarity*). We ultimately selected seven lines that maximized chemical dissimilarity within acyl sugars, terpenes, and proteinase inhibitors compared with the parental M82 genotype. For proteinase inhibitors, we used a radial immunodiffusion assay to measure wound-induced proteinase inhibitor expression in ILs (*see methods in* Li et al., 2003). The amount of proteinase inhibitors was quantified for each genotype and used to calculate the difference in proteinase inhibitor concentration between neighbors and focal plant for each plant neighborhood (Howe & Sugimoto, unpublished data). Because all tomato plants had proteinase inhibitor II, we chose the genotype having the lowest amounts of proteinase inhibitor to use in our diculture treatment and calculated the relative amount of proteinase inhibitor for all neighborhoods. Overall, selecting lines in this way enabled us to establish plots that differed in the type and amount of chemical diversity among plants (Figure 1).

Each of the seven ILs we selected differs from the parental M82 genotype in key ways (Appendix S1: Table S1; Schilmiller et al., 2010). Of the three genotypes we selected for their differing terpene chemotypes, IL 1-4 has a unique alpha thujene monoterpene, IL 2-2 has 40% and 60% reduced abundance of monoterpenes and sesquiterpenes, and IL 10-3 has 75% reduced abundance of sesquiterpenes, relative to M82. Among the genotypes we selected for their differing acyl sugar chemotypes, IL 8-1-1 produces an elevated number of acyl sugar compounds, IL 5-3 has 80% reduced abundance of acyl sugar production, and IL 11-3 has 90% reduced abundance of acyl sugar production. The proteinase inhibitor chemotype, IL 3-2, has 80% reduction in the accumulation of proteinase inhibitor II compared to the parental M82 genotype (Sugimoto & Howe, unpublished data).

We started all plants from seed (C. M. Rick Tomato Genetics Resource Center, Davis, CA, USA) in 10-cm pots in a climate-controlled greenhouse (16-h daylight) for five weeks before transplanting into 194 plots in

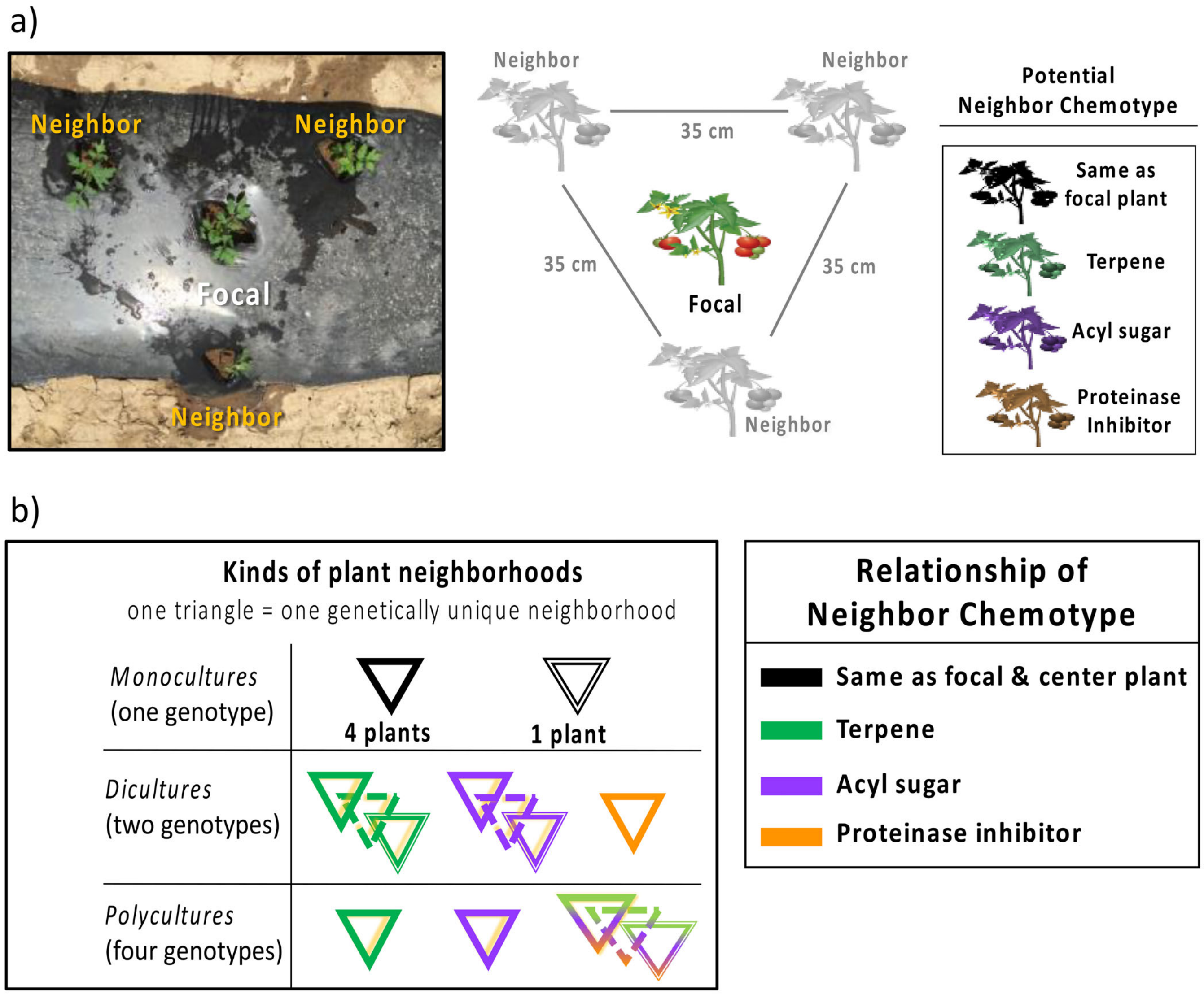

FIGURE 1 The field experiment used a factorial design combining plant genetic diversity (monocultures, dicultures, and polycultures) and chemotype (terpene, acyl sugars, proteinase inhibitors, and multi-trait). (a) The plant setup for each neighborhood (photo and experimental schematic by Andrea Glassmire). Focal plants were consistent, while neighbor plant genotypes varied based on genetic and chemotypic diversity. (b) The 14 treatment combinations (triangles). Unique tomato genotypes were used to create the neighborhood chemotype, denoted by color (green = terpenes, purple = acyl sugars, orange = proteinase inhibitors). Multi-trait neighborhoods included one terpene genotype (green hue), one acyl sugar genotype (purple hue), and the lowest proteinase inhibitor genotype (orange hue) (*refer to* Appendix S1: Table S2 *for specific genotype pairings and description of associated chemotype*).

two organic fields at Kellogg Biological Station (Hickory Corners, MI, USA) for the field experiment on June 6, 2018. Spacing in the field was 35 cm between stems within plots and 3 m between centroids across plots (Figure 1). After transplanting to the field, the ambient insect community naturally colonized the plants.

Plant neighborhoods

We created 14 experimental plant neighborhoods that varied in the type and/or amount of chemotypic diversity, chemotypic richness, genotypic richness, and plant density (Figure 1; Appendix S1: Table S2). Treatments varied in their (1) type of *chemotypic diversity* (variation among plants in acyl sugar, proteinase inhibitor, or terpene chemotype); (2) amount of *chemotypic richness* (monocultures represented a dense stand of one chemotype vs. polycultures that represented an even distribution of all three chemotypes simultaneously); (3) *genotypic richness* (one, two, or four genotypes); and (4) *plant density* (single plant vs. four plants established in monoculture, diculture, and polyculture treatments). In the center of every plot, an M82 plant served as the receiving focal plant, from which we took all samples and measurements. Using one genotype as a focal plant eliminated host plant genotypic variation and allowed us to focus on the effects of neighborhood composition.

The 14 neighborhood treatments in our design included one single plant neighborhood consisting of a sole M82 focal plant with no surrounding plants (one plant genotype); one monoculture neighborhood with three M82 neighbors surrounding the M82 focal (one plant genotype); seven unique diculture neighborhoods consisting of a focal M82 surrounded by three plants of one of our seven IL genotypes (two plant genotypes); and five unique polyculture neighborhoods with a focal M82 surrounded by three plants of three different IL genotypes (four plant genotypes; Figure 1). Appendix S1: Table S2 describes how each plant neighborhood differs in genotype number, chemotypic diversity, and the total replication number in greater detail. For the polyculture treatments, one included all three of the terpene genotypes, one included all three of the acyl sugar genotypes, and three included one genotype from each of the three chemical classes. For all polyculture neighborhoods previously described, the difference in proteinase inhibitor concentration between the neighbor plants and the center focal plant was calculated.

The treatment with a sole focal plant allowed us to isolate the effects of the identity of plant neighbors from the mere presence of plant neighbors or plant density more generally, an important component of understanding associational effects (Hambäck et al., 2014; Underwood et al., 2014). The seven diculture treatments allowed us to examine the effect of neighbors of a single genotype that differed primarily in a single chemical class. The two types of polyculture treatments allowed us to isolate the effects of increasing genotypic diversity from the effects of increasing chemical diversity within and among chemical classes. Taken together, comparing across these treatments allowed us to ask about the effects of increasing diversity within different chemical classes and to isolate the effects of increasing genotypic diversity from the effects of increasing chemotypic diversity.

Response variable measurements

On each focal plant in each plot, we quantified the insect community using two separate sampling methods—sticky traps and the beat sheet method—because it is established that these methods sample different kinds of insects based on mobility (Schmidt et al., 2008; Wade et al., 2006). Specifically, sticky traps capture flying life-stages, such as parasitoid wasps, and are effective over a 24-h period (Schmidt et al., 2008). However, larger, walking predators, like damsel bugs and green lacewings, can escape from sticky cards, and so the beat sheet method is more effective at collecting larger and/or less mobile predators and pests in agricultural row crops (Wade et al., 2006). On August 6, 2018, visiting insects were collected from each focal plant using 14 × 23.5 cm sticky traps (Great Lakes IPM Inc., Vestaburg, MI) placed 3 cm above the plant and collected 48 h later. On August 15–16, 2018, all insects residing on each focal plant were collected using a 0.61-m PVC pipe to knock insects into a beat sheet, where they were then collected with an aspirator, and stored in 70% ethanol. We collected a total of 7439 insects from *sticky trap* samples, with 31% identified to the herbivore feeding guild, 39% identified to the predator feeding guild, and the remaining 30% were unable to be identified (Appendix S1: Figures S1–S5). The dominant herbivores collected from sticky traps were leafhoppers (Hemiptera: Cicadellidae), aphids (Hemiptera: Aphididae), and frit flies (Diptera: Chloropidae). The dominant predators collected from sticky traps were chalcid wasps (Hymenoptera: Chalcididae), bethylid wasps (Hymenoptera: Bethylidae), and tachinid flies (Diptera: Tachinidae). A total of 3459 insects were collected from *beat sheet* samples, with 20% identified to the herbivore feeding guild, 46% identified to the predator feeding guild, and the remaining 34% were unable to be identified (Appendix S1: Figures S1–S5). The dominant herbivores collected from the beat sheet were stinkbugs (Hemiptera: Pentatomidae), plant and leaf bugs (Hemiptera: Miridae), and froghoppers (Hemiptera: Cercopidae). The dominant predators collected from the beat sheet were lady beetles (Coleoptera: Coccinellidae), stilt bugs (Hemiptera: Berytidae), and damsel bugs (Hemiptera: Nabidae).We collected above-ground vegetative biomass from each focal plant on August 28, 2018. Dried biomass was a proxy for plant growth to determine whether plant productivity was linked to insect abundance.

Statistical analyses

We analyzed insect abundance using generalized linear models via a Poisson distribution followed by likelihood ratio tests using the "lme4" R package (Bates et al., 2015; R Core Team, 2018). We checked the output of our models to confirm that the residual deviance is about equal to the residual deviance df. Specifically, we focused on herbivore and predator abundances, separately analyzing insects collected by sticky trap and beat sheet because these methods collected different components of the insect community (*refer to NMDS of insect communities based on sampling method*, Appendix S1: Figure S1). Sticky traps are designed to intercept flying insects, whereas the beat sheet method is designed to capture walking insects (Szendrei et al., 2011), and we assumed flying insects interact with the plant host and chemical cues in unique ways compared with walking insects (*sticky trap insects* Appendix S1: Figures S2 and S3, *beat sheet insects* Appendix S1: Figures S4 and S5). We included plant biomass and field as fixed effect variables in all models to account for differences in plant productivity among treatment combinations and differences between the two fields. All other fixed effect predictor variables differed among models depending on the question being addressed: (Q1) To examine the effects of *chemotypic diversity* within the terpene chemical class, we subset the dataset to the terpene neighborhoods and compared models with and without (null) fixed effects for plant diversity. We did the same for the acyl sugar and proteinase inhibitor chemical classes. (Q2) We examined the effect of *chemotypic richness*, across all three chemical classes, by subsetting the dataset to the polyculture treatments and comparing models with and without (null) fixed effects for chemotypic diversity. Restricting this analysis to polycultures allowed us to examine chemical diversity while controlling for genotypic diversity, because they all contained four genotypes while differing in chemical diversity. (Q3) We examined the effects of genotypic richness and plant density by comparing models with and without (null) a fixed effect for genotypic richness and plant density. All models were followed up using Tukey multiple comparisons to compare treatment levels using the R package "multcomp" (Hothorn et al., 2008). Finally, in all models we separated out the single plant treatment, allowing us to isolate the effects of neighbor identity from neighbor presence.

RESULTS

(Q1) How does diversity within each chemical class influence insect abundance?

We found that arthropod community patterns on our focal plants were strongly influenced by the specific chemical phenotypes of neighboring plants, rather than the total amount of genetic and chemical diversity in general. Moreover, diversity within each chemical class had differing effects on arthropods.

Sticky trap insect abundance

Among the *terpene* diversity treatments, herbivore abundances on the focal M82 plant increased by 26% on sticky traps when plant neighbors in polycultures had a low richness of terpene compounds (Figure 2a; Appendix S1: Table S3; $\chi^2 = 12.85$, df = 4, $p = 0.012$), suggesting that neighborhoods with low levels of terpene diversity attracted many kinds of herbivores that spilled over onto the M82 focal plant. In contrast, we did not find a significant effect across any of the terpene diversity neighborhoods on predator abundances (Figure 2a; Appendix S1: Table S3; $\chi^2 = 2.21$, df = 4, $p = 0.697$). For *acyl sugar* diversity neighborhoods, we did not find a significant effect on herbivore (Appendix S1: Table S3; $\chi^2 = 4.74$, df = 4, $p = 0.32$) or predator abundances (Figure 3a; Appendix S1: Table S3; $\chi^2 = 6.30$, df = 4, $p = 0.178$). Perhaps more mobile herbivores and predators, like leafhoppers and parasitoid wasps, can assess acyl sugars and quickly leave instead of having to cope with the acyl sugars. Finally, among the *proteinase inhibitor* diversity treatments, focal plants had fewer herbivores by 33% in sticky trap samples when their neighbors had slightly reduced levels of wound-inducible proteinase inhibitors (Figure 4a; Appendix S1: Table S3; $\chi^2 = 15.16$, df = 4, $p = 0.0044$). In contrast, we did not find a significant effect on predator abundances in the sticky traps (Figure 4a; Appendix S1: Table S3; $\chi^2 = 4.23$, df = 4, $p = 0.38$). These results suggest that herbivores and predators with greater mobility may have differential responses to proteinase inhibitors, and that herbivores concentrate on plant neighborhoods with lower proteinase inhibitor activity.

Beat sheet insect abundance

Among the *terpene* diversity neighborhoods, we did not find a significant effect on herbivore abundance (Figure 2b; Appendix S1: Table S4; $\chi^2 = 1.40$, df = 4, $p = 0.84$). In contrast, predator abundances were 35% lower when neighbors in dicultures had reduced mono- and sesquiterpenes (Figure 2b; Appendix S1: Table S4; $\chi^2 = 9.55$, df = 4, $p = 0.049$), suggesting that reduced mono- and sesquiterpenes may have made the patch, as a whole, less attractive to large predators. Among the *acyl sugar* diversity neighborhoods, we did not find a significant effect on herbivore abundance in beat sheet collections (Appendix S1: Table S4; $\chi^2 = 5.01$, df = 4, $p = 0.29$). Perhaps larger bodied herbivores experience acyl sugars once they are established and cope with the acyl sugars because it could be too energetically costly to relocate. In contrast, predator abundances were 41% lower in beat sheet samples when neighbors in polycultures had multiple genotypes with reduced acyl sugars (Figure 3b; Appendix S1: Table S4; $\chi^2 = 19.56$,

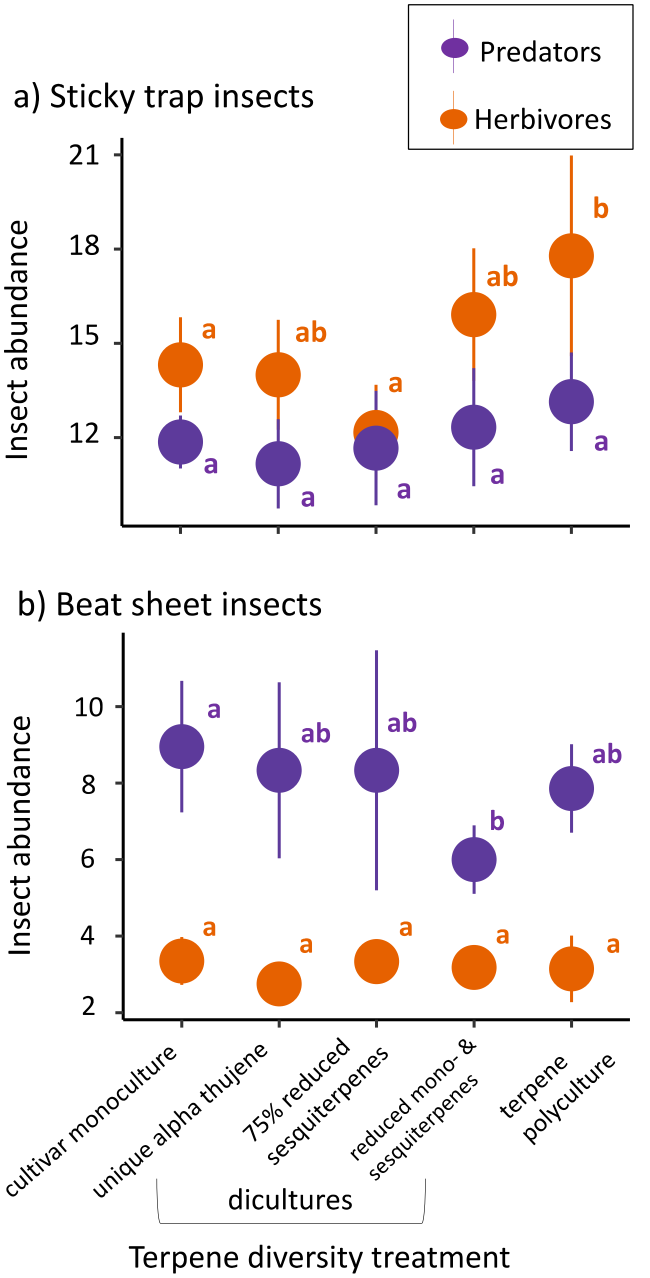

FIGURE 2 Chemotypic diversity effects on the abundance of insects collected via (a) sticky traps, and (b) the beat sheet method. Terpene neighborhoods were created by combining plant genotypes with varied mono- and sesquiterpenes. Insects were sampled from the cultivar focal genotype. Plots show mean ± SE. Lowercase letters represent significant differences among treatment combinations ($p < 0.05$) and all statistical summaries for the multiple comparisons can be found in Appendix S1: Tables S6 and S7.

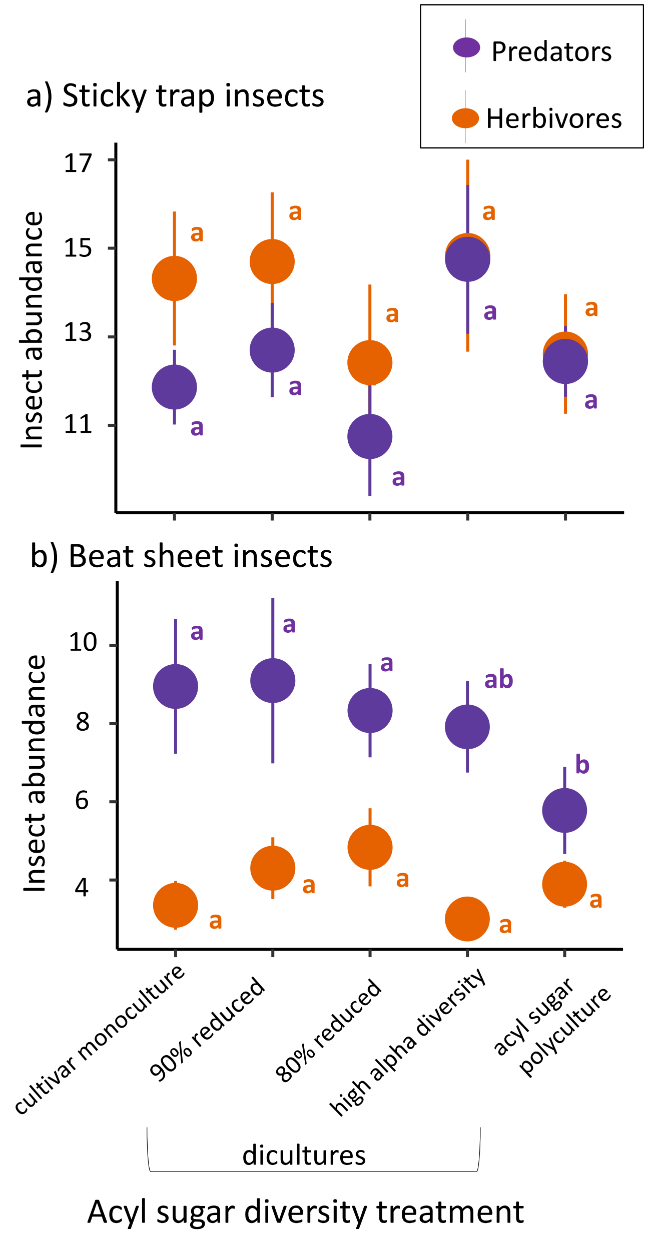

FIGURE 3 Chemotypic diversity effects on the abundance of insects collected via (a) sticky traps, and (b) the beat sheet method. Acyl sugar neighborhoods were created by combining plant genotypes with varied acetyl and acyl structural groups. Insects were sampled from the cultivar focal genotype. Plots show mean ± SE. Lowercase letters represent significant differences among treatment combinations ($p < 0.05$) and all statistical summaries for the multiple comparisons can be found in Appendix S1: Tables S6 and S7.

df = 4, $p = 0.00061$). This result suggests larger predators rely on high levels of acyl sugar diversity to track and locate prey. Finally, among the *proteinase inhibitor* diversity treatments, we did not find a significant effect on herbivore abundance in beat sheet collections (Figure 4b; Appendix S1: Table S4; $\chi^2 = 7.16$, df = 4, $p = 0.13$). In contrast, predator abundances were 26% higher when neighboring plants had the lowest proteinase inhibitor levels (Figure 4b; Appendix S1: Table S4; $\chi^2 = 23.22$, df = 4, $p = 0.00011$). This result suggests that predators are cueing in on less defended plants, which likely harbor bigger herbivores compared with those herbivores feeding on plants with high levels of proteinase inhibitors that reduce the availability of dietary amino acids.

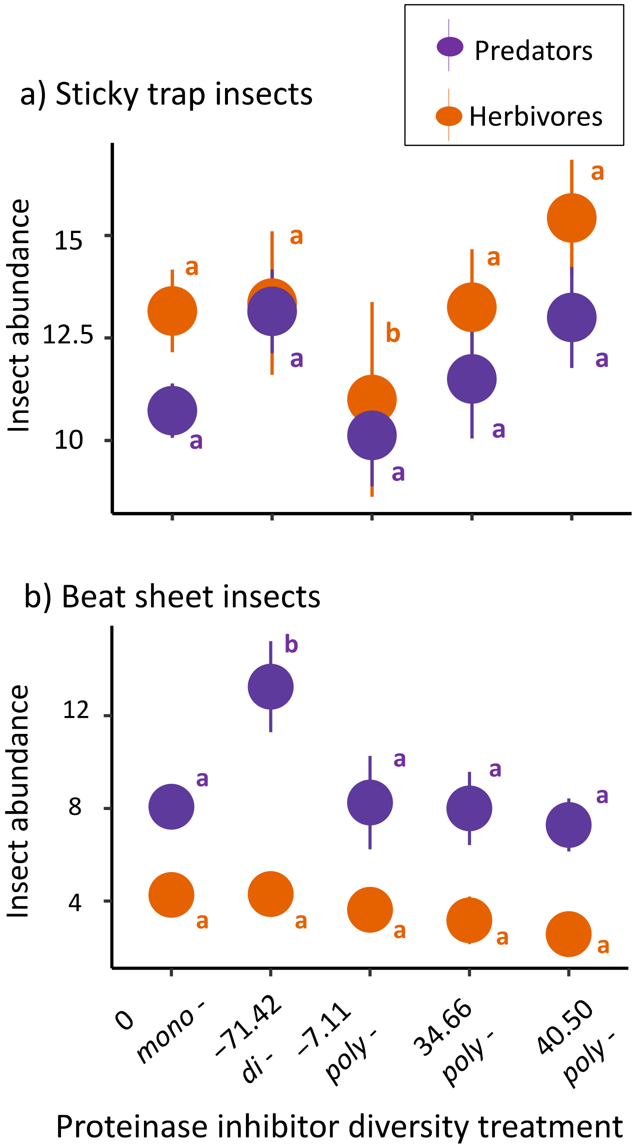

FIGURE 4 Chemotypic diversity effects on the abundance of insects collected via (a) sticky traps, and (b) the beat sheet method. Proteinase inhibitor (PI) neighborhoods were created by combining plant genotypes with varied expression of wound-inducible proteinase inhibitor. Specifically, the PI values represent the difference in the amount of proteinase inhibitor among neighboring plants compared with the focal plant. Insects were sampled from the cultivar focal genotype. The monoculture had a proteinase inhibitor level "0" because it was the same genotype as all the neighbors. Plots show mean ± SE. Lowercase letters represent significant differences among treatment combinations ($p < 0.05$) and all statistical summaries for the multiple comparisons can be found in Appendix S1: Tables S6 and S7.

(Q2) Does total chemotypic richness predict insect abundance?

Insect abundance

Our polyculture treatments held genotypic diversity constant while presenting insects with diversity in one or three chemical classes. In contrast to the strong effects of diversity within single chemical classes, we found no evidence that the number of chemical classes with diversity in a neighborhood influences herbivore abundance (Appendix S1: Figures S6 and S7, Tables S3 and S4; *sticky trap* $\chi^2 = 2.66$, $\text{df} = 1$, $p = 0.10$; *beat sheet* $\chi^2 = 1.34$, $\text{df} = 1$, $p = 0.25$) or predator abundance (Appendix S1: Figures S6 and S7, Tables S3 and S4; *sticky trap* $\chi^2 = 0.84$, $\text{df} = 1$, $p = 0.36$; *beat sheet* $\chi^2 = 3.68$, $\text{df} = 1$, $p = 0.055$).

(Q3) Do greater numbers of introgression genotypes predict insect abundance?

Insect abundance

Using Tukey multiple comparisons, we found no consistent differences in herbivore or predator abundances across our monoculture versus single (*estimate* $= 0.17$, $p = 0.35$), monoculture versus diculture (*estimate* $= 0.054$, $p = 0.86$), and monoculture versus polyculture (*estimate* $= 0.050$, $p = 0.89$) treatments for sticky trap insects (Figure 5a; Appendix S1: Tables S3 and S6). Similarly, there was not a significant difference in herbivore abundances for beat sheet insects (Figure 5b; Appendix S1: Tables S4 and S7). However, there was a significant difference between dicultures and polycultures for beat sheet predators (*estimate* $= -0.24$, $p = 0.0010$), but not between monocultures–dicultures (*estimate* $= -0.020$, $p = 0.99$) nor monocultures–polycultures (*estimate* $= -0.026$, $p = 0.18$) (Figure 5b; Appendix S1: Tables S4 and S7). Overall, these results suggest that in general the chemotype of the neighboring genotypes may matter more than the number of introgression genotypes.

(Q4) Does plant density predict arthropod community patterns?

The presence of three neighboring plants compared with none, however, had a major effect on the insect community on M82, increasing predators in sticky traps by 31% (Figure 5a; Appendix S1: Table S3; $\chi^2 = 8.14$, $\text{df} = 3$, $p = 0.043$), and predators in beat sheet samples by 24% (Figure 5b; Appendix S1: Table S4; $\chi^2 = 32.53$, $\text{df} = 3$,

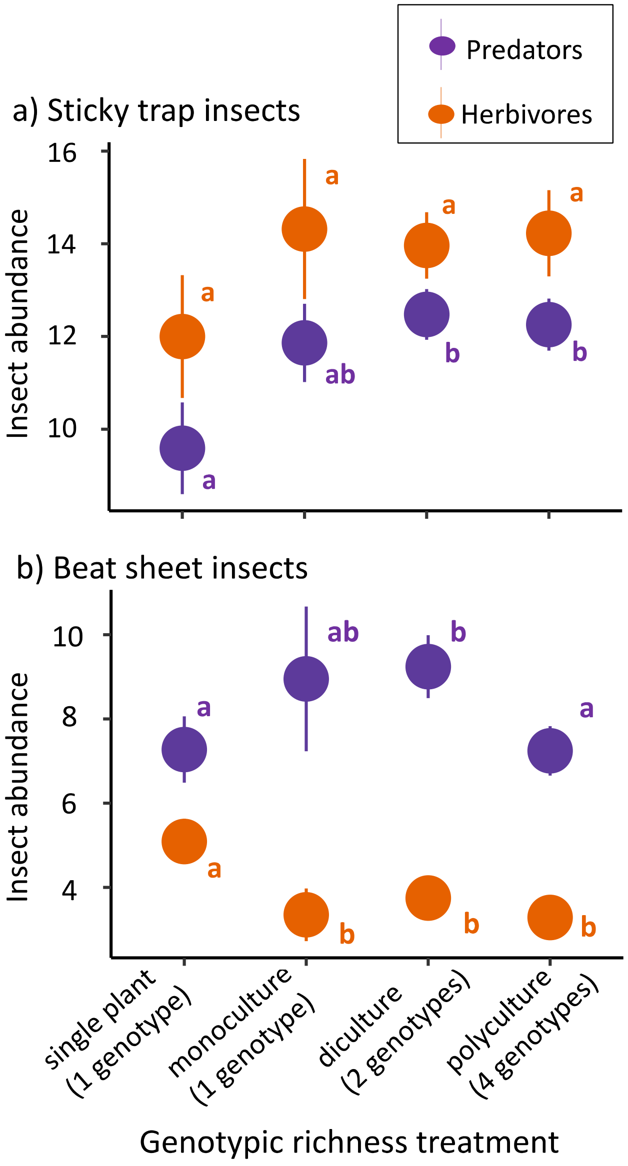

FIGURE 5 Genotypic diversity effects on the abundance of insects collected via (a) sticky traps, and (b) the beat sheet method. Insects were sampled from the cultivar focal genotype. Plots show mean ± SE. Lowercase letters represent significant differences among treatment combinations ($p < 0.05$) and all statistical summaries for the multiple comparisons can be found in Appendix S1: Tables S6 and S7.

$p = 4.045\text{e}{-07}$), and decreasing herbivores in beat sheet samples by 44% (Figure 5b; Appendix S1: Table S4; $\chi^2 = 7.90$, df = 3, $p = 0.048$), relative to the single plant treatment.

DISCUSSION

Using a set of ILs to experimentally manipulate chemical diversity within and among three chemical classes, our experiment allowed us to tease apart the effects of different types and amounts of plant neighborhood diversity on plant–arthropod interactions. Our results revealed that the diversity of specific chemical traits in a plant neighborhood, rather than the total chemotypic diversity, influenced the abundance of herbivores and predators on a focal plant. Specifically, diversity within different chemical classes had differing and often contrasting effects on the abundance of herbivores and predators on a nearby focal plant. Furthermore, variations in mobility of herbivores and predators demonstrated that flying insects responded differently than walking insects to chemical cues. Below, we discuss what our results indicate about the role of specific chemical traits in driving the associational effects of plant neighbors on plant-associated arthropod communities, and explain the implications of our results for our general understanding of the ecology of intraspecific plant diversity.

Chemical traits underlying associational effects

Though there is a large literature showing the prevalence of associational effects within plant neighborhoods, these interactions have been difficult to predict because the presence and direction of associational effects are highly variable among plant genotypes (Andow, 1991). Our results demonstrated that chemical diversity plays a key role, and whether the focal plant was susceptible or not to insects depended on chemical class identity and insect functional guild. Our trait-focused approach to constructing plant neighborhoods allowed us to start to resolve the effects of specific chemical traits, helping the field move to a trait-based understanding of these interactions.

Herbivores and predators interact with plants using plumes of volatile mixtures that vary in composition depending on the chemotype mixture of the neighborhood. Terpenes play a key role in the bouquet of cues used by insects to assess the suitability of plants (reviewed in Kessler & Kalske, 2018). Neighbor plants having low amounts of monoterpenes increased the vulnerability of the focal plant to mobile herbivores, indicating that complexity in terpene odors at high levels poses a challenge for flying herbivores by disrupting their ability to locate, recognize, or accept a particular host plant (i.e., *semiochemical-diversity hypothesis*, Beyaert & Hilker, 2014; Dicke & Baldwin, 2010). Additionally, several studies have indicated that herbivores use simple odor cues to locate host plants (Dahlin et al., 2018), supporting our results that plant neighborhoods with reduced amounts of monoterpenes are susceptible to mobile herbivores.

Surprisingly, flying parasitoids that depend on locating prey in complex odor environments did not respond to any of our terpene diversity neighborhoods. Foraging inefficiency could be due to how experienced parasitoid wasps are to host-infested plants versus non-host-infested plants and how abundant these plants are in a given neighborhood (Vosteen et al., 2019). Another possibility is these parasitoids are so highly specialized that they can hone in on host-specific cues that are unrelated to and not masked by any blends of plant terpenes. For large-bodied and less mobile predators collected using the beat sheet method, we found that predator abundances significantly decreased in plant neighborhoods with reduced mono- and sesquiterpenes. Interestingly, these were diculture neighborhoods, meaning that there was a high frequency of neighboring plant genotypes with reduced mono- and sesquiterpenes. The high frequency of plants with reduced terpenes made it difficult for large-bodied predators to track and locate their prey on the focal plants. Predators detect their host by cueing in on specific "signals," particularly mono- and sesquiterpenes, against a background of "noisy" compounds because small differences in attractive compounds having a strong contrast relative to background volatiles facilitate detection of reliable cues (i.e., *olfactory contrast hypothesis* Vet & Dicke, 1992). Our results suggest that large-bodied, less mobile predators are deterred when plant neighborhoods do not have a strong terpenoid "signal."

Neighboring plants with variation in acyl sugar traits also demonstrated an underlying associational effect on the focal plant, suggesting that acyl sugars are a locator cue for large-bodied, less mobile predators searching for prey. Polycultures with neighboring plants having an overall reduction in the types and kinds of acyl sugars reduced abundance in less mobile, large-bodied predators but not for flying predators. It is known that predators can cue in on the volatile fatty acid chains of acyl sugars to locate prey on tomato plants (Weinhold & Baldwin, 2011). Our results suggest that blends in the kinds and types of acyl sugars also lure predators to plant neighborhoods and that predators may be particularly attracted to a mixture of acyl sugars, each having a unique chain structure with either a sucrose or glucose core. However, when neighborhoods have reductions in acyl sugar diversity then that also affects predator abundance. It is not surprising that we did not find a difference in the abundance of mobile predators because insects with greater mobility can assess acyl sugars and quickly leave instead of having to cope with the acyl sugars. Oddly, we did not find any effect of acyl sugars on herbivore abundance in either the sticky traps or the beat sheet method. This could be because the biggest hurdle acyl sugars pose to herbivores is that they reduce feeding efficiency by sticking to insect mandibles and slowing development growth (Luu et al., 2017) rather than repelling herbivores.

Our finding that neighboring plants with reduced levels of proteinase inhibitors led to higher abundances of less mobile, large-bodied predators on focal plants suggests that proteinase inhibitors have an indirect positive effect on predators via herbivores. Plant proteinase inhibitors reduce the digestibility of proteins in herbivores by blocking insect gut proteinases (Jongsma & Bolter, 1997). Because defensive proteinase inhibitors were less abundant in those neighborhoods, herbivores may have been thriving and provided more options for predators. However, the indirect effects of proteinase inhibitors on predator performance are typically not addressed in the literature due to a focus on herbivores (Casaretto & Corcuera, 1995; Fan & Guo-Jiang, 2005). Our results suggest there is a positive consequence of reduced levels of proteinase inhibitors on predators by attracting them to plant neighborhoods and may act to increase the top-down pressure of predators on herbivores.

The ecology of associational effects and neighborhood diversity

Not all chemical diversity of plant neighborhoods had the same associational effect on herbivore and predator abundances on the focal plant, but rather the specific identity of chemical class was a better predictor than total chemotypic diversity. Recent studies have adopted experimental approaches to manipulating trait diversity using biosynthetic knockouts in wild tobacco (Schuman et al., 2015) or wild cabbage genotypes, with a polymorphism in the amount of foliar glucosinolates (Bustos-Segura et al., 2017). These studies have shown conclusively that a single class of defenses causes changes in insect ecology, but this makes it difficult to link multiple functions of phytochemical diversity to ecological outcomes. Here, we uniquely filled in this knowledge gap by demonstrating that chemical dissimilarity underlying associational effects depended on the ecological role of the chemical class, and how frequently those specific chemotypes occur in the neighborhood. Increasing diversity in a single compound class and plant density had a greater magnitude of effect on insect communities than increasing diversity across multiple chemical classes simultaneously.

The result that dicultures more often than polycultures resulted in measurable associational effects suggests that a high frequency of a particular chemical phenotype has the potential to influence insect community structure (Hauri et al., 2022). Predator abundances in beat sheet samples were higher in neighborhoods with a high frequency of plants of the same chemotype (i.e., dicultures). Conversely, predator abundance was reduced when plants in a neighborhood had different genotypes (i.e., polycultures) suggesting that a high number of genotypes may act to dilute the frequency of any one chemotype and make it hard for predators to distinguish. In contrast, diculture neighborhoods act to concentrate the chemical phenotype in the neighborhood because there was a 3:1 ratio favoring the frequency of the three neighbors having the same chemotype even though there are two genotypes. Future studies should consider the frequency of chemotypes in a neighborhood and whether the size of the neighborhood acts to concentrate or dilute the dominant chemotype. Furthermore, our results also suggest that plant density regulated herbivore abundance rather than genotype richness. Herbivores from beat sheet insects increased threefold in single plant neighborhoods compared with all other neighborhoods having four plants (mono-, di-, and polycultures). One explanation is that having more plants in a neighborhood provides a greater area for insects to disperse across, which consequently lowers the total abundance on any one plant (reviewed in Hambäck et al., 2014). The dilution effect on herbivore abundance resulting from increased plant density (i.e., from one to four plants) should be considered for future studies. Overall, research on the effects of plant diversity on multitrophic community interactions has been limited by not identifying the underlying mechanisms of such effects (Bustos-Segura et al., 2017; Moreira et al., 2016; Schuman et al., 2015). Here, we demonstrate that intraspecific plant diversity had strong effects across multiple trophic levels, and this was not entirely attributed to an increase in the number of plant genotypes, but rather was mediated by the chemotype ratios of the neighboring plants.

Conclusions

At any one time, there is likely a complex web of associational effects within a plant neighborhood. We found that chemical differences among neighboring plants spill over to the focal plant to structure insect communities. However, our results highlight that chemical dissimilarity in itself did not cause a consistent increase or decrease in herbivore and predator abundances. Rather, we found that the associational effects of chemical dissimilarity depended on the identity of certain classes of chemical traits and the mobility of the insect. Specifically, dissimilar trait neighborhoods having high levels of monoterpenes will increase the performance of plants by increasing the diversity of predators in communities. Moreover, the ratios of chemotypes used for intra-crop plantings should be considered for sustainable agriculture because our results suggest that polycultures may be diluting the influence of the key chemical trait. Overall, a key implication of this work is that the chemical class and its frequency of occurrence is important in maintaining intraspecific phytochemical variation. Our results demonstrate that a trait-based approach to understanding associational effects will enhance our understanding of plant–insect interactions.

AUTHOR CONTRIBUTIONS

Andrea E. Glassmire and William C. Wetzel conceived the experiment. Andrea E. Glassmire, Kayleigh C. Hauri, Daniel B. Turner, and Luke N. Zehr collected data. Koichi Sugimoto and Gregg A. Howe performed proteinase inhibitor assays. Andrea E. Glassmire, Daniel B. Turner, and William C. Wetzel performed statistical analyses. Andrea E. Glassmire wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Glassmire et al., 2024) are available in Figshare at https://doi.org/10.6084/m9.figshare.25922821.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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