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Compartmentalization of specialized metabolites across vegetative and reproductive tissues in two sympatric *Psychotria* species

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Abstract

Premise: The specialized metabolites of plants are recognized as key chemical traits in mediating the ecology and evolution of sundry plant–biotic interactions, from pollination to seed predation. Intra- and interspecific patterns of specialized metabolite diversity have been studied extensively in leaves, but the diverse biotic interactions that contribute to specialized metabolite diversity encompass all plant organs. Focusing on two species of *Psychotria* shrubs, we investigated and compared patterns of specialized metabolite diversity in leaves and fruit with respect to each organ's diversity of biotic interactions.

Methods: To evaluate associations between biotic interaction diversity and specialized metabolite diversity, we combined UPLC-MS metabolomic analysis of foliar and fruit specialized metabolites with existing surveys of leaf- and fruit-centered biotic interactions. We compared patterns of specialized metabolite richness and variance among vegetative and reproductive tissues, among plants, and between species.

Results: In our study system, leaves interact with a far larger number of consumer species than do fruit, while fruit-centric interactions are more ecologically diverse in that they involve antagonistic and mutualistic consumers. This aspect of fruit-centric interactions was reflected in specialized metabolite richness—leaves contained more than fruit, while each organ contained over 200 organ-specific specialized metabolites. Within each species, leaf- and fruit-specialized metabolite composition varied independently of one another across individual plants. Contrasts in specialized metabolite composition were stronger between organs than between species.

Conclusions: As ecologically disparate plant organs with organ-specific specialized metabolite traits, leaves and fruit can each contribute to the tremendous overall diversity of plant specialized metabolites.

KEYWORDS

Barro Colorado Island, chemical traits, interaction diversity, phytochemistry, Rubiaceae, specialized metabolites

Specialized metabolites of plants constitute a major component of the ecological and evolutionary framework of plant and animal biodiversity. Through mediating plants' ecological interactions and evolutionary relationships with consumers, parasitoids, pollinators, and seed-dispersers, specialized metabolites hold within their structural and functional diversity the potential to generate and reinforce the species richness of plants and the organisms with which they interact (Levey et al., 2007; Heil, 2008; Dicke and Baldwin, 2010; Richards et al., 2015; Courtois et al., 2016; Endara et al., 2017; Stevenson et al., 2017). To date, much of the development of theory and accumulation of evidence regarding evolutionary linkages between plant–biotic interactions and specialized metabolite diversification has stemmed from studies of plant taxa or communities and their specialist leaf-consuming herbivores (e.g., Becerra and Venable, 1999; Kursar et al., 2009; Becerra, 2015; Richards et al., 2015; Endara et al., 2017; Uckele et al., 2021). In addition, most studies have focused on taxonomic differences in specialized metabolite diversity without

considering how this diversity might differ across hierarchical levels of ecological organization.

In pursuing a plant-level perspective on specialized metabolite trait evolution—one that integrates organ-level patterns and processes—recent work has identified the interaction diversity hypothesis (IDH) from among other evolutionary hypotheses as a promising conceptual framework. In brief, the IDH posits that "selection acts independently on different compounds that confer different advantages in different pairwise interactions" (Whitehead et al., 2021, p. 1206), and thus the diversity of specialized metabolites in a given plant species is an emergent consequence of the diversity of biotic interactions amid which the species has evolved (Berenbaum and Zangerl, 1996; Iason et al., 2011; Whitehead et al., 2022). It is important to note that the IDH was originally conceived to address the diversity of putatively defensive specialized metabolites in leaves, compared among taxa. In this scenario, the IDH predicts that plant lineages accrue specialized metabolite diversity through iterations of selection for novel metabolites by pairwise interactions with antagonists (Berenbaum and Zangerl, 1996). When applied to a scenario of ecologically disparate plant organs within an individual (e.g., animal-dispersed fruit vs. leaves; Whitehead et al., 2022), the IDH predicts that distinct selective pressures of each antagonistic or mutualistic interaction act independently from one another on the chemical deterrents and/or attractants in the pertinent plant tissue. This scenario is hypothesized to result in a modular "tool kit" of many specialized metabolites, each suited to a distinct ecological interaction. If pairwise interactions associated with a specific plant tissue remain constant, irrespective of species identity, the IDH predicts a more similar level of diversity of specialized metabolites within tissues among individuals compared to across tissues within individuals. Finally, diversity of specialized metabolites could change with development of the plant. To date, most studies have focused on interspecific comparisons among leaves, with less known on variation in diversity of specialized metabolites across tissues, organs, and individuals. Supporting these conceptual advances have been technological and analytical advances in the field of metabolomics, allowing quantitative and comparative evaluations of specialized metabolites at tissue, organ, organismic, and community levels (Wang et al., 2016; Sedio, 2017; Aron et al., 2020; Walker et al., 2022).

Overlapping with the conceptual extension of the IDH in recent years has been an increasing recognition of fruit and its constituent tissues as an important component of specialized metabolite diversity, particularly in plants which rely on animals for seed dispersal (Cipollini and Levey, 1997; Whitehead et al., 2022). Fruits of zoochorous (animal-dispersed) species are subjected to exceptionally complex arrays of biotic selective pressures as compared to other organs within the same species. Specifically, zoochorous fruits are tasked with defending against numerous guilds of biotic antagonists, such as seed predators, nondispersing pulp consumers, and fungal pathogens, while attracting seed-dispersing mutualists upon ripening (Cipollini and Levey, 1997; Nevo and Ayasse, 2020; Whitehead et al., 2022). Considering the ecological context of zoochorous fruit, the IDH supports a prediction of highly specialized metabolite diversity in fruit pericarp relative to other tissues in zoochorous plant species. This prediction has been borne out by a handful of studies at the scale of chemical class (Whitehead and Bowers, 2013; Whitehead et al., 2013) and the metabolome (Schneider et al., 2021). However, further case studies are called for (Whitehead et al., 2022) to establish generalities of the IDH for specialized metabolite diversity and variability across a range of biological levels. Specifically, while IDH has been supported by numerous studies examining interspecific differences in foliar specialized metabolite diversity and a handful of studies examining interspecific and intertissue patterns in fruit specialized metabolite diversity (reviewed by Whitehead et al., 2022), whether it can be applied across levels of ecological organization, from within individual to among species, has yet to be evaluated.

In this study, our goal was to understand how the multiple dimensions of diversity in specialized metabolites vary across levels of ecological organization—across tissues, individuals, and species—in the context of IDH. Across these different levels, we compared chemical richness—the count of unique metabolites, similarity in chemical composition and structure, and variation in chemical composition. By including two species of *Psychotria* (Rubiaceae), we broadened the taxonomic scope of IDH-based comparisons of specialized metabolite composition across vegetative and reproductive tissues. For our focal plant taxa, we selected a pair of syntopic species of *Psychotria*, a pantropical genus of mainly shrubs and small trees in the Rubiaceae. Fruits of neotropical *Psychotria* are bird-dispersed (Charles-Dominique, 1993; Poulin et al., 1999), and the plants of this genus likewise represents an important food source for frugivorous understory birds throughout the tropics (Snow, 1981). *Psychotria* and closely related genera in the tribes Psychotrieae and Palicoureeae also host a diverse assemblage of insect herbivores; for example, the 20 Psychotrieae and Palicoureeae species at Barro Colorado Island, Panama, host at least 115 species of insect herbivores (Sedio, 2013). With respect to specialized metabolites, the Psychotrieae and Palicoureeae are known for their production of a structurally diverse array of alkaloids. Indeed, a chemotaxonomic approach utilizing structural groupings of Psychotrieae and Palicoureeae alkaloids has recently been employed in an attempt to disentangle the evolutionary relationships within these tribes (Berger et al., 2021).

Notably, studies of the specialized metabolites of Psychotrieae and Palicoureeae have generally been limited to vegetative tissue. However, sufficient ecological data are available to allow us to posit hypotheses regarding the specialized metabolite composition of the fruits of Psychotrieae/Palicoureeae in our focal species pair, a subset of the 20 species at Barro Colorado Island. Among these 20

species, fruits are consumed by a diverse array of birds (Poulin et al., 1999; G. F. Schneider, Utah State University, unpublished data) but are apparently unpalatable to mammalian frugivores (Wright et al., 2016). Fruit pulp and seeds of these 20 species experience low pre-dispersal pest damage (Basset et al., 2021) and are involved in a low number of antagonistic species interactions, with only seven known fruit antagonist species (Basset et al., 2021) in contrast to the 115 known leaf antagonist species previously mentioned (Sedio, 2013) (Appendix S1). The avian-specific seed dispersal and low fruit damage rates are consistent with patterns observed in plant taxa known to contain distinctive specialized metabolites in fruit tissues (summarized by Levey et al., 2007), which led us to hypothesize that *Psychotria* fruit may contain distinctive specialized metabolites. In general, we expected tissue type to explain differences in chemical diversity rather than species and individual identity. Based on the IDH, the low species richness of fruit-associated antagonists (2 pulp-feeders and 5 seed predators; Basset et al., 2021) as compared to leaf-associated antagonists (115; Sedio, 2013), led us to predict that fruit pulp and seeds will have lower richness of specialized metabolites than in leaves. Because specialized metabolite variability can impose behavioral and metabolic costs on consumers (Adler and Karban, 1994; Salazar et al., 2016; Massad et al., 2017; Pearse et al., 2018; Whitehead et al., 2022), we predicted that fruit pulp will have lower variation in chemical richness among individuals and higher specialized metabolite similarity in terms of composition and structure than in leaves or seeds as a result of selection to reduce costs for mutualistic consumers. Finally, we predicted that tissue type is a better predictor of chemical compositional variation than is species.

MATERIALS AND METHODS

Study site

Barro Colorado Island (9°9′N, 79°51′W), hereafter referred to as BCI, encompasses a lowland, moist tropical forest with average annual rainfall of 2600 mm (Leigh et al., 1996). The rainfall on BCI arrives mainly between May and December, with a pronounced dry season between January and April (Leigh et al., 1996). The vegetative and reproductive phenology of the woody plants of BCI exhibit seasonality, with leaf and fruit production both peaking in the early wet season (Leigh et al., 1996) and fruit maturation peaking between the early dry season and early wet season, though the latter varies widely by species (Wright et al., 1999; Zimmerman et al., 2007).

Study species

The two species selected for this study, *Psychotria limonensis* K. Krause and *Psychotria marginata* Sw. (Rubiaceae), are distinguished from the other *Psychotria* species of BCI—and linked to one another—by their reproductive phenology. Though only distantly related within the genus (Sedio et al., 2012), these species undergo fruit maturation in the early wet season, while all other Psychotrieae and Palicoureeae of BCI produce fruit in the late wet season (Poulin et al., 1999). Thus, *P. limonensis* and *P. marginata* represent replicates of a distinct point of ecological intersection between *Psychotria* and leaf- and fruit-consuming invertebrates and pathogens. Both species have been included in surveys of insect herbivores (Sedio, 2013) and seed predators and fruit pulp consumers (Basset et al., 2021). Of the 36 species of herbivores and two species of seed predators found feeding on *P. limonensis* and the 21 species of herbivores, one species of seed predator, and one species of fruit pulp consumer found feeding on *P. marginata*, the two *Psychotria* species share eight species of herbivores and none of the fruit antagonists in common (Sedio, 2013; Basset et al., 2021).

Sampling methods

From 16 individual plants of each species, we collected expanding leaves, mature (fully expanded) leaves, and ripe fruit from each plant in May–August 2019. All three sample types were collected from the same plant simultaneously. Only undamaged leaves and fruit were collected. Expanding leaves were identified by their distal position on the branch and lighter color with respect to mature leaves and were collected when their leaf area reached approximately 50–70% of that of the neighboring mature leaves. Sufficient material was collected to yield at least 100 mg (dry mass) of each sample type, including 100 mg each of pulp (both species have soft exocarp, which was combined with the mesocarp) and seeds, including the endosperm and embryo with lignified endocarp. All plants were located in qualitatively similar light environments in the forest understory, within 10 m of trails and with no canopy gaps larger than those resulting from trail maintenance.

Once collected, the tissues were immediately placed in a chilled and insulated cooler and taken to the laboratory to be processed for extraction. Leaf samples were weighed and then placed in a −80°C freezer. Fruit samples were separated into pulp and seeds, weighed, and placed in −80°C freezer. With the low thickness of all sample types (intact fruit diameter ≤5 mm), freezing at −80°C was sufficiently rapid to prevent tissue damage due to ice crystal formation. After a minimum of 24 h at −80°C, the samples were transferred to vacuum flasks and freeze-dried for 72 h. After drying, samples were weighed and ground into powder, then returned to a −80°C freezer for storage until transport to Utah State University for secondary metabolite extractions.

Chemical extractions and untargeted metabolomics

Chemical extractions

Plant secondary metabolites were extracted at Utah State University. For each sample, 80 mg of homogenized powder was weighed into a 1.8 mL polypropylene screw-top tube using a microbalance. To isolate the broadest possible range of phytochemicals, the extraction solvent used was 99.9% v/v ethanol with 0.1% v/v formic acid. Each sample was extracted with a total of 7.0 mL of the extraction solvent, in five iterations of the following procedure. A glass syringe was used to add 1.4 mL of extraction solvent to the 1.8 mL tube containing the sample. The sample was mixed with the extraction solvent for 5 min in a vortexer at 1500 rpm and then centrifuged for 5 min at 13,800 × *g*, then the supernatant was removed and added to a 20 mL glass scintillation vial. The supernatant from each of the five iterations was combined in the same 20 mL vial. The combined extract was dried at room temperature using a vacuum-centrifuge. The dried extract was then weighed and stored at −20°C until analysis.

UPLC-MS and post-processing

We used untargeted mass spectrometry (MS)-based metabolomics to characterize specialized metabolite diversity. LC-MS data were collected using an Acquity I-class UPLC coupled to a Waters Xevo G2-S quadrupole time-of-flight mass spectrometer (Waters Corp., Milford, MA, USA) at the Chemistry Mass Spectrometry Facility at the University of Utah. For analysis, dried extracts were resuspended at 10 mg/mL in 75:25 water:acetonitrile + 0.1% v/v formic acid, with 2.0 μg/mL stevioside hydrate (Sigma-Aldrich Inc., St. Louis, MO, USA) as an internal standard. The extract was then sonicated for 10 min, after which a 20 μL aliquot was taken and diluted 10-fold with 75:25 water: acetonitrile + 0.1% v/v formic acid. The resultant solution was then vortexed and centrifuged (10 min, 13,000 × *g*), and an aliquot (180 μL) was transferred to an LC-MS vial for analysis. Solvent blanks were injected at regular intervals during data collection. The autosampler temperature was 10°C and the injection volume was 1.5 μL. The column employed was a reverse-phase Acquity BEH C18 with dimensions of 2.1 mm ID × 150 mm and 1.7 μm particle size (Waters Corp.) maintained at 35°C at a flow rate of 0.5 mL/min. Solvent A was water with 0.1% v/v formic acid, and solvent B was acetonitrile with 0.1% formic acid (LCMS grade; Fisher Chemical, Pittsburgh, PA, USA). A linear solvent gradient was used between hold points; time points and solvent A % at gradient endpoint or during hold point were as follows: 0–1.0 min held at 98%; 2.0 min, 96%; 11.0 min, 60%; 16.0 min, 30%; 17.5 min, 2%; 17.5–18.0 min, held at 2%; 20 min, 98%; 20–22.5 min held at 98%.

Full-scan mass spectra and DDA fragmentation spectra were collected separately to ensure accurate quantitation of molecular ion relative abundance. Both types of spectra were collected with ESI ionization set to positive mode. The instrument parameters for single-MS mode were as follows: peak data recorded in centroid mode; 0.185 s MS scan time; 30–2000 Da mass range; no collision energy; 125°C source temperature; 3 V capillary voltage; 30 V sample cone voltage; 350°C desolvation temperature; nitrogen desolvation at 500 L/h; 10 μL/min lockspray flow rate; 0.1 s lockspray scan time; 20 s lockspray scan interval; 3 lockspray scans to average; 0.5 Da lockspray mass window; 3 V lockspray capillary voltage. The instrument parameters for MS/MS mode were the same as in single-MS mode except for the following: argon collision gas; 10–30 V low mass collision energy ramp; 50–100 V high mass collision energy ramp; 30–2000 Da mass range; 30–40 V sample cone voltage; 1 s lockspray scan time; 10 s lockspray scan interval; 50 lockspray scans to average. Acquisition of MS/MS data was initiated at individual ion intensity of 5000 counts s^{-1} and discontinued when base peak intensity (BPI) fell below 5000 counts s^{-1} or after 5 s. All m/z acquired while MS/MS was activated were temporarily excluded from reacquisition for 10 s.

Alignment, deconvolution, and annotation of molecular and adduct ions were conducted using the XCMS and CAMERA packages (Smith et al., 2006; Tautenhahn et al., 2008; Benton et al., 2010; Kuhl et al., 2012) in R version 4.2.1 (R Core Team, 2022).

Tandem mass spectrometry fragmentation data processing

The tandem mass spectrometry described above generated fragmentation spectra for all fragmentable molecular ions in our specialized metabolite extracts. These fragmentation spectra are diagnostic of molecular structure, and through pairwise comparison can be used to generate a network linking putative compounds to one another based on their structural similarity (Wang et al., 2016; Sedio et al., 2017; Aron et al., 2020). The methods used for this process were described by Schneider et al. (2021) with the exception of an updated GNPS workflow: METABOLOMICS-SNETS-V2, version "release_30".

Statistical analyses

To compare diversity of specialized metabolites across levels of ecological organization in the context of IDH, we analyzed (1) the median and variance of chemical richness, (2) means of chemical similarities, and (3) variation in chemical similarities among tissue types and species based on untargeted metabolomics. Finally, we visualized and quantified how well tissue types and species identity explain variation in chemical composition.

Chemical richness

We compared the median and variance of chemical richness across the following groups: (1) between *P. limonensis* and *P. marginata* with tissue types pooled within each species to assess species-level differences in diversity irrespective of tissue identity and (2) among tissue types with species pooled within tissue type to assess tissue-level differences in diversity irrespective of species identity. We first used a Shapiro–Wilk test to ascertain the normality of the chemical richness data. We found the data to be left-skewed and nontransformable and thus used the nonparametric Brown–Mood test to test for differences in median chemical richness among groups and post hoc pairwise comparisons (controlling for the false discovery rate; Benjamini and Hochberg, 1995). These tests were conducted using the R packages coin (Hothorn et al., 2008) and rcompanion (Mangiafico, 2022). To compare the variance of chemical richness, we used Levene's test in the R package car (Fox and Weisberg, 2019; R Core Team, 2022).

Chemical similarity

Metabolome-scale chemical similarity among samples and groups of samples was quantified using two complementary distance-based metrics, Bray–Curtis chemical compositional similarity (BCS) and chemical structural similarity (CSS). The BCS metric represents the mean variability in the pairwise chemical composition across samples, while CSS represents the mean variability in the pairwise similarities of molecular structures.

Bray–Curtis chemical compositional similarity

We calculated the pairwise BCS between all samples using the R package vegan (Oksanen et al., 2022). In this context, BCS is independent of chemical structural similarity between compounds, quantifying similarity based on the extent to which compounds that are found in at least one sample are shared between samples and the relative ion abundances of each shared compound (Sedio et al., 2017; Schneider et al., 2021). The BCS was originally designed to quantify dissimilarity, but it can be converted to a similarity index simply by subtracting the dissimilarity value from 1.

Chemical structural similarity

We used the GNPS molecular networking output described above and an R-based workflow adapted from Sedio et al. (2018) to calculate the pairwise molecular structural similarities of all samples, referred to as chemical structural similarity (CSS). Unlike the BCS approach, this approach accounts for the putative structural similarity of specialized metabolites, using comparisons of MS/MS fragmentation patterns to quantify similarity. Our workflow was described in detail by Schneider et al. (2021). Briefly, for each sample-pair comparison, the samples' structural similarity is calculated by taking the mean of all pairwise comparisons of compound-level molecular fragment similarity. These nested pairwise comparisons are used to generate a sample-by-sample distance matrix similar in format to that of BCS.

Analysis of chemical similarity means

We compared the means of BCS and CSS across groups at four distinct levels dictated by potential sources of biological variation: between species, among tissue types to assess differences among species and tissue types; within species, among tissue types to assess differences among tissue types within species; between species, within tissue type to assess differences between species within tissue types; and within species, within tissue type to assess differences among individuals. To test for differences in group means, we used one-way permutational ANOVA tests in the R package lmPerm version 2.1.0. (Wheeler and Torchiano, 2016). These were followed by post hoc pairwise permutation tests controlling for false discovery rate (Benjamini and Hochberg, 1995) using the pairwisePermutationMatrix function in the R package rcompanion (Mangiafico, 2022).

Analysis of chemical similarity variation

To test for any effects of species, tissue type, and individual plant identity on the distribution of BCS, we used a series of PERMANOVA model tests (Oksanen et al., 2022). PERMANOVA assumes that the samples are exchangeable under a true null hypothesis and is sensitive to differences in multivariate dispersion, but can be used where variance is heterogeneous across groups as long as the treatments or observations are of a balanced design (Anderson and Walsh, 2013; Anderson, 2017). To assess multivariate dispersion in our data, we used a permutation test (N = 999 permutations, function betadisper in the R package vegan) that quantified homogeneity of multivariate dispersion from the overall centroid, an analogue of Levene's tests for homogeneity of variances, across the four tissue types. This was followed by a post hoc pairwise permutation test to assess pairwise differences in multivariate location and dispersion between tissue types. These tests indicated that variance was heterogeneous across tissue types (detailed results in Results section), but because tissue groups were balanced in our study design, we proceeded with the PERMANOVA.

First, we tested the effects of species, tissue, and their interaction on chemical compositional similarity using a fixed-effects model. Next, we tested whether tissue-level variation was nested within plant-level variation for each species by comparing fixed-effect models—with tissue as the only fixed effect—to mixed-effect models with plant ID

added as a random effect (separate models for *P. limonensis* and *P. marginata*). Finally, we tested the effect of plant-level variation on chemical compositional similarity within each species, with plant ID as a fixed effect and species as a random effect in a mixed-effect model. In all models, the number of permutations used was $N = 999$. Individual plants for which any of the four tissues were not collected (due to lack of vegetative or reproductive growth) were omitted from the latter two analyses ($N = 4$ *P. limonensis*, 4 *P. marginata*). All PERMANOVAs were conducted using the function adonis2 in the R package vegan version 2.6-2 (Oksanen et al., 2022).

The CSS data were omitted from the PERMANOVA because they were characterized by presence/absence of compounds. Given the low levels of variation in compound presence/absence established in our preliminary analyses, the CSS data were not informative of within-group variation. In addition, the homogeneity of multivariate dispersion test was not conducted for the CSS due to the reduced sample size from which molecular structural data were collected.

Variation in chemical composition

Our final set of analyses examined the patterns of variance in the chemical compositional data and the factors influencing these patterns. We used partial least-squares discriminant analysis (PLS-DA) to reduce the dimensionality of the chemical compositional data to identify and quantify the principal components of variance. Samples were grouped in the following models: (1) species-grouped: species identity with tissue identity withheld, (2) tissue-grouped: tissue identity with species identity withheld from the model, and (3) tissue-with-species grouped: both species and tissue identity assigned. This analysis was conducted through the MetaboAnalyst version 5.0 online workflow (Xia and Wishart, 2016).

Unless otherwise stated, all tests were conducted using R version 4.2.1 (R Core Team, 2022).

RESULTS

Chemical richness

Across all sample groups (i.e., expanding leaves, mature leaves, ripe pulp, and mature seeds from *Psychotria limonensis* and *Psychotria marginata*), our UPLC-MS analysis yielded 2722 molecular features annotated as putative compounds, hereafter referred to as compounds. Combining the two species to summarize tissue-level results, we found that 48.6% of the 2722 compounds were present in all four tissue types, while 18.7% occurred only in expanding and mature leaves, 8.4% occurred only in fruit pulp and seed, and the remaining 24.3% occurred in a subset of both fruit and leaf tissues (Figure 1).

We found no significant difference in median chemical richness between the two species overall (pooling tissue types within species; $Z = -0.0928$, $P = 0.926$; Figure 2). However, comparing among tissue types with species

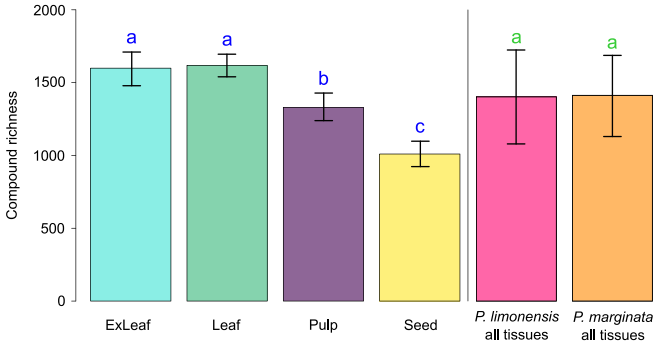

FIGURE 2 Group-level variation in compound richness. Tissues are coded as follows: ExLeaf = expanding leaf; Leaf = mature leaf; Pulp = ripe pulp; Seed = mature seed. Left: Compound richness by tissue types with species pooled within tissue type; right: Compound richness by species with tissues pooled within species. Different letters above error bars indicate significant differences ($P \leq 0.05$) between groups. Error bars denote ±1 SD.

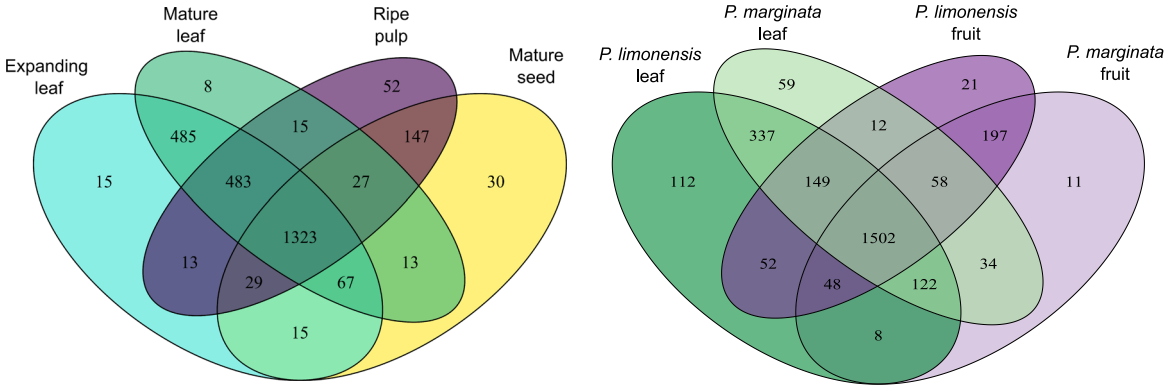

FIGURE 1 Venn diagrams of specialized compound richness. Left: Compound richness by tissue identity with species combined. Right: Compound richness by species and organ identity, with expanding and mature leaves combined into "leaf" and pulp and seed combined into "fruit".

TABLE 1 Results of Levene's tests for homogeneity of variance across groups conducted with each pairwise combination of tissues.

Tissue	Expanding leaf	Mature leaf	Ripe pulp
Expanding leaf	—	$F_{1,26} = 2.44$	$F_{1,24} = 1.12$
		$P = 0.131$	$P = 0.300$
Mature leaf	$F_{1,26} = 2.44$	—	$F_{1,29} = 0.006$
	$P = 0.131$		$P = 0.942$
Ripe pulp	$F_{1,24} = 1.12$	$F_{1,29} = 0.006$	—
	$P = 0.300$	$P = 0.942$	
Mature seed	$F_{1,24} = 1.27$	$F_{1,29} = 0.003$	$F_{1,24} = 1.1837$
	$P = 0.271$	$P = 0.955$	$P = 0.2874$

pooled, we found a significant difference in tissue-level medians overall ($\chi^2 = 93.93$, df = 3, $P < 0.001$) and, in each post hoc pairwise comparison between tissue types, except for that of the two leaf development stages (expanding leaf–mature leaf: adjusted $P = 0.695$; all other comparisons: adjusted $P < 0.001$; Figure 2). Using Levene's test to evaluate the homogeneity of variance in compound occurrence between species, we found that variance in compound occurrence between *P. limonensis* and *P. marginata* was marginally heterogeneous ($F_{1,111} = 3.71$, $P = 0.057$). Levene's indicated significant heterogeneity of variance among the four tissue types overall (among tissue types with species pooled; $F_{3,109} = 2.82$, $P = 0.042$); however, we found no significant heterogeneity in any post hoc pairwise comparison of tissues (Table 1).

Chemical similarity

Analysis of chemical similarity means

Overall, the permutational ANOVA indicated highly significant differences ($P < 0.001$) in mean similarities among the following comparison groups: within species, among tissue types (Figure 3A, B); between species, within tissue type (Figure 3C, D); and within species, within tissue type (Figure 3C, D). In within species, among tissue type comparisons, mean BCS and CSS values for expanding and mature leaves were the most similar to one another (adjusted $P < 0.001$ in all comparisons, Figure 3A, B), while mean BCS values of mature leaves and seeds were the least similar to one another (adjusted $P < 0.002$ in all comparisons, Figure 3A). Mean CSS values of mature leaves and seeds shared the lowest among-tissue similarity with expanding leaves vs. mature seeds and mature leaves vs. ripe pulp (adjusted $P < 0.05$) (Figure 3B). Mean CSS values of the two reproductive tissues tended to be just as similar to each other within species as compared to either of the vegetative tissues (except for the mature leaf vs mature seed comparison, Figure 3B). In within species, within tissue type comparisons, mean BCS and CSS values of expanding and mature leaves were most similar to one another (adjusted $P < 0.001$ in all comparisons, Figure 3C, D), while mean BCS values of mature leaves and seeds were the least similar (adjusted $P < 0.002$ in all comparisons, Figure 3C), and means CSS values of mature leaves and seeds shared the lowest similarity scores with expanding leaves vs. mature seeds and mature leaves vs. ripe pulp (adjusted $P < 0.05$, Figure 3D). In comparisons between species within tissue types, the two vegetative tissue types had lower mean BCS similarity than did the two reproductive tissue types (adjusted $P < 0.001$, Figure 3C, Appendix S2). Mean CSS similarity between species, within tissue type and within species, within tissue type were equivalent among tissue types (Figure 3D). In post hoc pairwise permutation tests, comparing the pairwise BCS of each tissue within and across the two *Psychotria* species (between species within-tissue; within species within-tissue types), we found that all four tissues had significantly higher mean similarity within species than between species (adjusted $P < 0.001$, Figure 3C). For CSS, these comparisons tended to be nonsignificant, except for mature leaves (adjusted $P < 0.05$) (Figure 3D).

Analysis of chemical similarity variation

Testing the effects of species, tissue, and their interaction on BCS using PERMANOVA demonstrated that both factors, along with their interaction, had significant effects on chemical compositional similarity. Tissue type ($F_{3,112} = 47$, $R^2 = 0.49$, $P < 0.001$) explained 6-fold or more of the variation in chemical compositional similarity than did species ($F_{1,112} = 22$, $R^2 = 0.07$, $P < 0.001$) or the tissue-by-species interaction ($F_{3,112} = 7.9$, $R^2 = 0.08$, $P < 0.001$). Examining the nesting of tissue-level within plant-level variation, we found that nested models were not discernible from non-nested models in either species (*P. limonensis*: $F_{3,31} = 18$, $R^2 = 0.66$, $P < 0.001$; *P. marginata*: $F_{3,35} = 19$, $R^2 = 0.65$, $P < 0.001$), indicating a negligible level of nesting for tissue-level BCS variation within plant-level BCS variation. Indeed, when examined as a fixed effect, plant-level variation itself did not contribute significantly to BCS trends within species ($F_{16,67} = 19$, $R^2 = 0.17$, $P > 0.999$). Following up our PERMANOVA tests with analyses of BCS variance across tissues with species pooled within tissue type, we found significant heterogeneity of the corresponding multivariate dispersions ($F_{3,109} = 9.95$, $P < 0.001$) (Figure 4).

Variation in chemical composition

These data comprise the sample-level occurrence and relative ion abundance of the 2722 compounds described above, with samples grouped by tissue and/or species depending on the analysis.

We constructed three PLS-DA models for each of the different groupings: species-grouped model, tissue-grouped

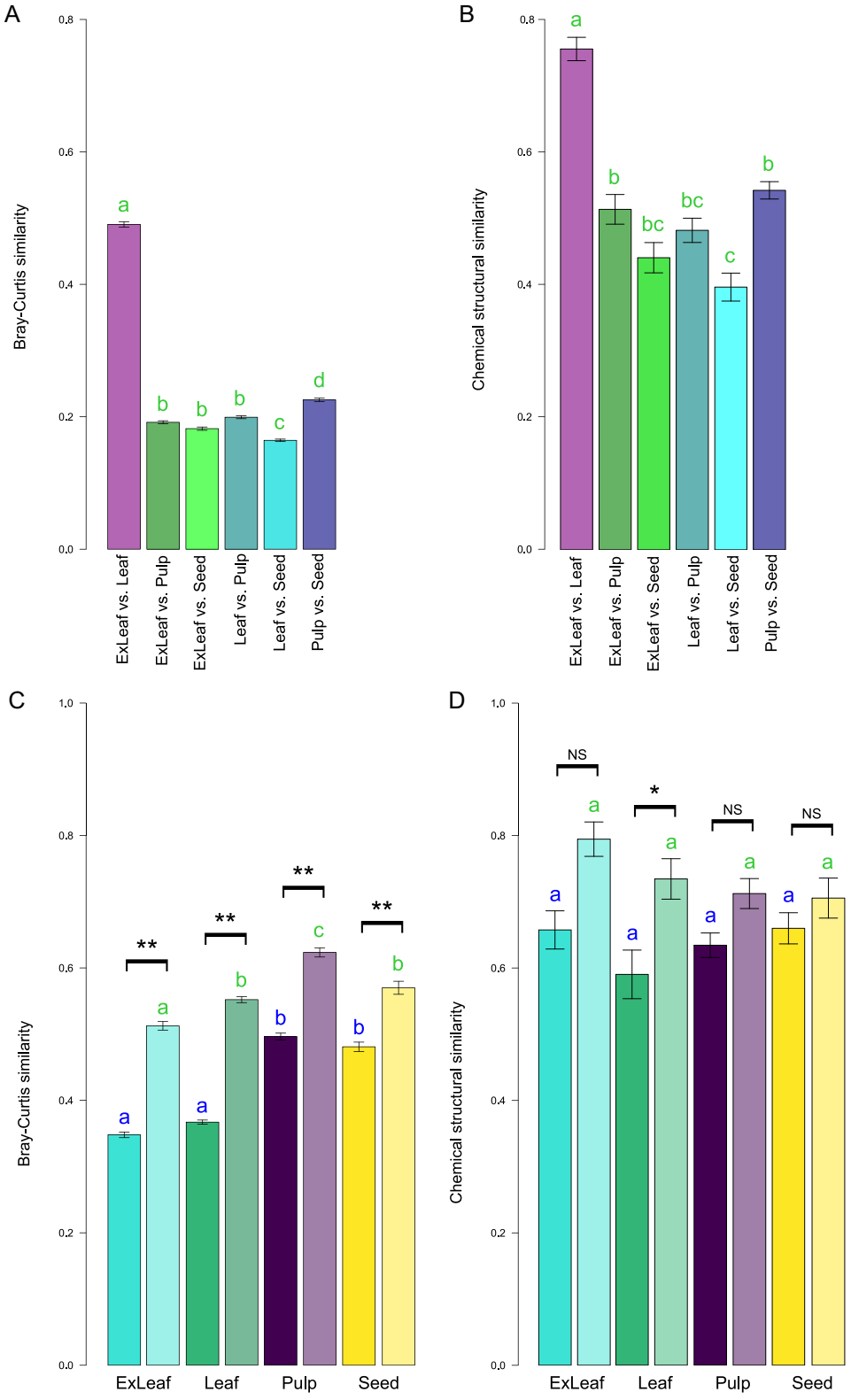

FIGURE 3 Bray–Curtis and chemical structural similarity of samples within and across species and tissues. Tissues are coded as follows: ExLeaf = expanding leaf; Leaf = mature leaf; Pulp = ripe pulp; Seed = mature seed. Error bars denote ±SE of mean. (A) and (B) are within species, among tissue comparisons; different letters above error bars indicate significant differences (adjusted $P \leq 0.05$) between groups. (C, D) Within-tissue comparisons, both between- and within-species with between species in dark shading and within species in light shading. Different letters above error bars indicate significant differences (adjusted $P \leq 0.05$) between groups, with letter colors indicating separate statistical tests. Blue letters indicate comparisons of between species groups and green letters indicate comparisons of within species groups. Brackets above error bars indicate statistical significance of between-species vs. within-species comparison for each tissue type; NS = not significant, *adjusted $P \leq 0.05$, **adjusted $P < 0.001$.

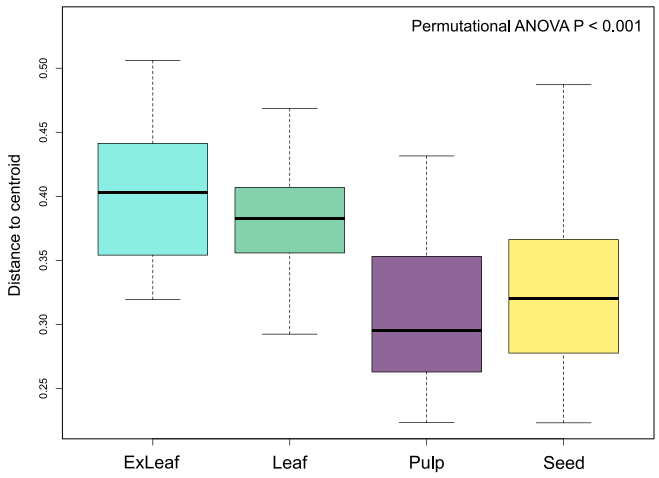

FIGURE 4 Comparison of heterogeneity of chemical composition by tissue. Species are pooled within tissue type. Tissues are coded as follows: ExLeaf = expanding leaf; Leaf = mature leaf; Pulp = ripe pulp; Seed = mature seed. Boxes indicate median ± interquartile range (IQR). Whiskers indicate spread of data within 1.5× the IQR.

model, and tissue-with-species-grouped models (Figure 5). In the species-grouped model, which maximized chemical compositional variance between species, the primary component "component 1" accounted for only 11.9% of total variance and did not produce a statistically significant separation distance between the two groups (Bohn–Wolfe permutation test, $P = 0.176$, number of permutations = 1000). Further, the first component in this model explains 7.3% less total variance in the data than does the second component, which explained 19.2% of the total variance. The PLS-DA model by definition sets component 2 to be orthogonal to component 1. As observed in Figure 5, component 1 reflects between-species variance in chemical composition, whereas component 2 reflects within-species variance in chemical composition. Thus, the species-grouped model suggests that within-species variance is greater than between-species variance in chemical composition across *P. limonensis* and *P. marginata*. In contrast to the species-grouped model, the tissue-grouped and tissue-with-species-grouped models yielded first components accounting for more total variance than each model's subsequent components (Figure 5). In addition, the tissue-with-species-grouped model produced a first component with loadings that showed greater separation between vegetative and reproductive tissue types than between species (Figure 5). Finally, the last two models both produced a statistically significant separation distance between groups (both models: $P < 0.001$, number of permutations = 1000).

DISCUSSION

As a nexus of antagonistic and mutualistic interactions distinct from those centered on leaves, zoochorous fruits have gained increasing attention from researchers seeking an organismal perspective on the ecology and evolution of plant specialized metabolites (Schneider et al., 2021; Whitehead et al., 2022). Numerous case studies comparing organs of a given species have revealed or corroborated facets of organ-specific specialized metabolite composition (reviewed by Kessler and Halitschke, 2009; Whitehead et al., 2013; Berardi et al., 2016; Schneider et al., 2021). These studies, as well as cases of pleiotropy where key metabolites are shared among organs (Adler et al., 2012; Keith and Mitchell-Olds, 2019), have provided evidence of organ-level selective pressures as a key evolutionary dynamic in yielding the overall species- and community-level diversity of plant specialized metabolites. Though limited in taxonomic scope, our study builds on this body of work by contributing to the evidence of inter-organ differences in chemical richness and variance as well as the presence of organ-specific metabolites. Perhaps most notably, our study is the first to document the scale of inter-organ variation in specialized metabolites in comparison to variation among individuals within species, with the former greatly exceeding the latter. Finally, our study supports our system-specific hypotheses of specialized metabolite composition based on the interaction diversity hypothesis (IDH), though more species across a variety of ecosystems will need to be studied to assess the generality of IDH in explaining specialized metabolite diversity from a whole-plant perspective.

Using untargeted metabolomics, we examined the biological patterns of variation of specialized metabolites across leaves and fruit of two neotropical *Psychotria* species. As a whole, our comparisons of leaf- and fruit-associated specialized metabolites indicate that chemical variation between vegetative and reproductive tissues, both within and between species, substantially exceeds analogous variation across all other biological scales represented in our study. This pattern of chemical variation was consistent across multiple metrics: richness of compounds, PLS-DA multivariate main components, compositional similarity, and chemical structural similarity. Such a pattern of variation is consistent with the specialized metabolite composition of each organ being shaped—at least in part—by the biotic interactions specific to that organ.

If we are to regard the specialized metabolite composition of each organ as a distinct multivariate trait, we must first establish whether these putative traits (1) occupy distinct neighborhoods of trait space and (2) vary independently of one another across individuals. With sufficient sample size and high-resolution chromatography and mass spectrometry, we were able to compare metabolome-scale trait values and intra- and inter-organ variation of specialized metabolite composition. We quantified multivariate specialized metabolite composition using the complementary methods of distance based BCS and CSS (Figure 3A–D) and PLS-DA dimension reduction (Figure 5A–C). Analysis of mean BCS and CSS confirmed that specialized metabolite composition was less similar among different organs

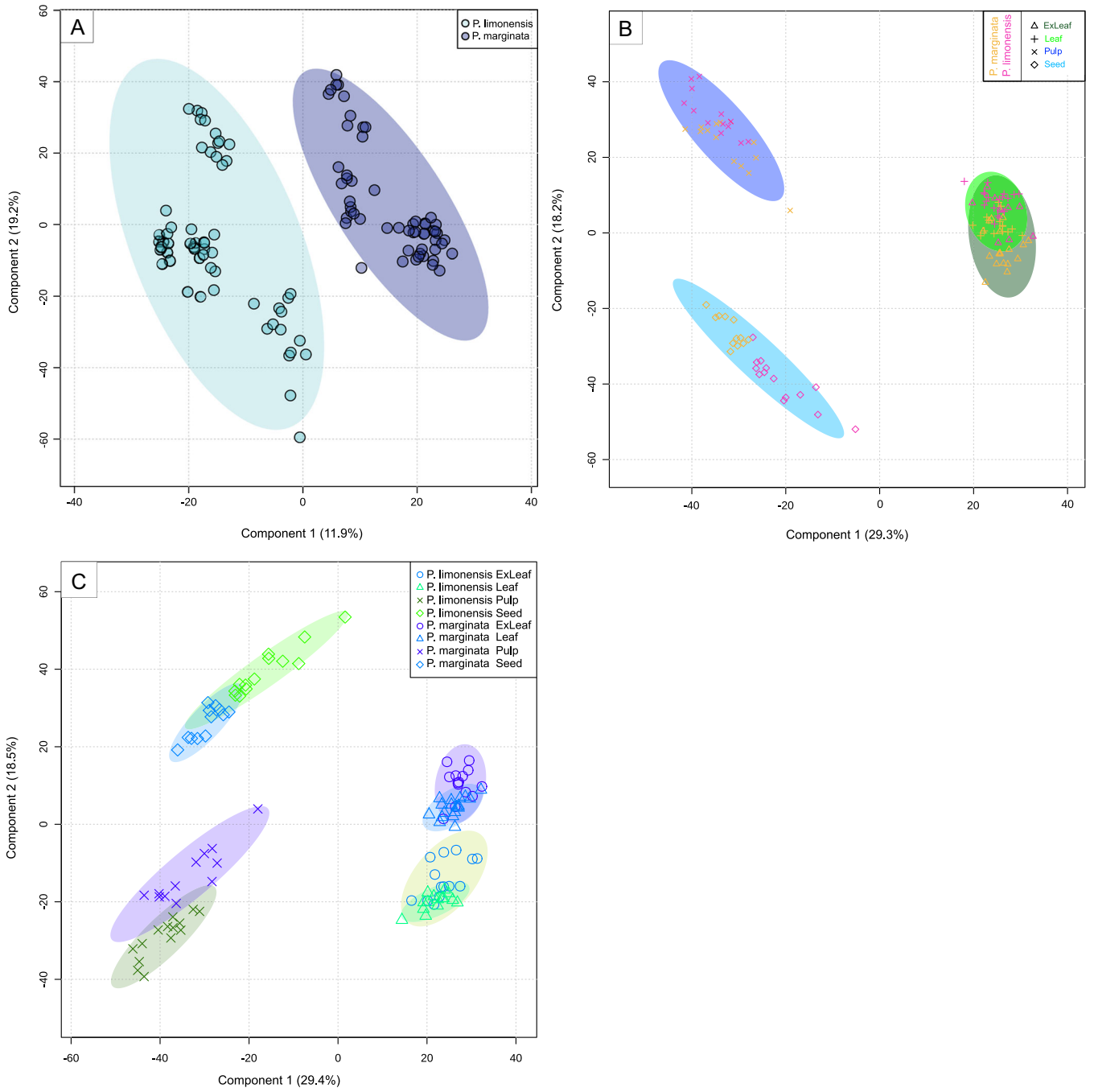

FIGURE 5 PLS-DA principal component plots for (A) chemical composition by species, (B) tissue, and (C) tissue-with-species. Tissues are coded as follows: ExLeaf = expanding leaf; Leaf = mature leaf; Pulp = ripe pulp; Seed = mature seed. Shading indicates 95% confidence intervals of group variance.

of a given species than across replicates of a given organ across individuals and even across species (Figure 3A–D). In addition, based on the results from the PERMANOVA of BCS data, we were able to confirm that plant identity had no significant influence on organ-level variation in chemical composition, indicating that leaf and fruit chemical composition varied independently of one another regardless of whether the samples were taken from the same plant. PLS-DA confirmed that the specialized metabolite composition of leaves, fruit pulp, and seeds all occupy different neighborhoods of multivariate trait space (Figure 5B, C).

Regarding our system-specific, IDH-based hypotheses, the chemical compositional characteristics of leaves and fruit were sufficiently distinctive to allow informative contrasts. Taken together, our quantitative measures of specialized metabolite relative abundances and molecular structural relationships showed that leaves had a larger number of unique chemical compounds as compared to fruit (Figure 1) and higher degrees of variation in

metabolite relative abundances among individuals and between species (Figures 3 and 4). Both of these results supported our predictions, attributable to the higher richness and taxonomic diversity of interactions in which leaves are involved as compared to fruits in these *Psychotria* as well as the predicted costs of chemical variation to consumers.

While fruit tissues had fewer unique chemical compounds than leaves, the number of compounds found only in fruits was still in the hundreds (Figure 1). Further, the between-species chemical structural variation was comparable to that of leaves (Figure 3C, D), indicating that leaf-specific and fruit-specific metabolites have similar degrees of structural divergence with respect to ubiquitous metabolites. Despite the lower taxonomic richness and phylogenetic diversity of fruit-centric interactions as compared with leaf-centric interactions in this system, these results are consistent with IDH. We interpret interaction diversity as reflecting the strength and sign of interactions as well as the richness and diversity of the species involved. While quantifying the strength of the antagonistic and mutualistic interactions in this system is beyond the scope of our study, the fact that fruit are involved in both antagonistic and mutualistic interactions suggests that fruit are exposed to substantial interaction diversity as reflected in the selective pressures on fruits' specialized metabolites. Developing the mathematical underpinnings of IDH to quantitatively incorporate the aspects of interaction diversity which we have discussed will represent an important component of future work in this field.

CONCLUSIONS

Although our study represents only two species, it contributes to a growing body of evidence that zoochorous fruit and leaves can represent distinct compartments in the ecology and evolution of plant specialized metabolites. Such partitioning of chemical composition based on localized selective pressures is likely applicable to other ecologically distinct organs and tissues as well, such as flowers (Berardi et al., 2016) and roots (Wang et al., 2015), and our ongoing research seeks to integrate all such compartments into a whole-plant perspective. The genetic and phylogenetic dynamics of the genomic connections of these compartments remain unelucidated, but recognizing specialized metabolite composition as a set of associated organ- or tissue-level traits is a crucial step toward understanding their micro- and macroevolutionary basis.

AUTHOR CONTRIBUTIONS

C.A.C., N.G.B., and E.M.J. designed the field study. C.A.C. collected field samples and conducted chemical extractions. G.F.S. designed and conducted chemical analyses. G.F.S. designed and conducted the statistical analyses with contributions from N.G.B. G.F.S. led the writing of the initial draft with contributions from N.G.B. All authors contributed to revising the manuscript.

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DATA AVAILABILITY STATEMENT

UPLC-MS data and molecular network files are available through GNPS MassIVE at https://doi.org/10.25345/C5930P49R. All other data and R code are available through Zenodo at https://doi.org/10.5281/zenodo.7779100.

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SUPPORTING INFORMATION

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

Appendix S1. Rarefaction and extrapolation analysis of fruit antagonist species richness.

Appendix S2. Among-tissue comparisons of BCS and CSS across separate *Psychotria* species.

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