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RESEARCH ARTICLE

Functional Traits 2.0: The Power of Metabolomics for Plant Ecology

Tree species richness differentially affects the chemical composition of leaves, roots and root exudates in four subtropical tree species

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Abstract

1. Plants produce thousands of compounds, collectively called the metabolome, which mediate interactions with other organisms. The metabolome of an individual plant may change according to the number and nature of these interactions. We tested the hypothesis that tree diversity level affects the metabolome of four subtropical tree species in a biodiversity–ecosystem functioning experiment, BEF-China. We postulated that the chemical diversity of leaves, roots and root exudates increases with tree diversity. We expected that the strength of this diversity effect differs among leaf, root and root exudates samples. Considering their role in plant competition, we expected to find the strongest effects in root exudates.
2. Roots, root exudates and leaves of four tree species (*Cinnamomum camphora*, *Cyclobalanopsis glauca*, *Daphniphyllum oldhamii* and *Schima superba*) were sampled from selected plots in BEF-China. The exudate metabolomes were normalized over their non-purgeable organic carbon level. Multivariate analyses were applied to identify the effect of both neighbouring (local) trees and plot diversity on tree metabolomes. The species- and sample-specific metabolites were assigned to major compound classes using the ClassyFire tool, whereas potential metabolites related to diversity effects were annotated manually.
3. Individual tree species showed distinct leaf, root and root exudate metabolomes. The main compound class in leaves was the flavonoids, whereas carboxylic acids, prenol lipids and specific alkaloids were most prominent in root exudates and roots. Overall, plot diversity had a stronger effect on metabolome profiles than the local diversity. Leaf metabolomes responded more often to tree diversity level than exudates, whereas root metabolomes varied the least. We found no uniform

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or general pattern of alterations in metabolite richness or diversity in response to variation in tree diversity. The response differed among species and tissues.
4. *Synthesis*. Classification of metabolites supported initial ecological interpretation of differences among species and organs. Particularly, the metabolomes of leaves and root exudates respond to differences in tree diversity. These responses were neither linear nor uniform and individual metabolites showed different dynamics. More controlled interaction experiments are needed to dissect the causes and consequences of the observed shifts in plant metabolomes.

KEYWORDS
chemical diversity, ecometabolomics, LC-qToF-MS, plant–plant interactions, root exudate, secondary metabolites, specialized molecules, subtropical forest ecology, tree interactions

1 | INTRODUCTION

Forests are important ecosystems that provide many ecosystem services. To humans, they are an important resource for food, firewood and timber (Jang et al., 2019). Forests represent rich natural pharmacies containing species with known or potential medicinal or nutritional value (Karjalainen et al., 2009). In addition, they harbour an enormous number of different organisms, from microbes to large mammals (Barlow et al., 2018). This is particularly true for (sub) tropical forests, which contain 96% of all tree species on Earth (Poorter et al., 2015). Consequently, tropical forests sustain high levels of biodiversity, in particular insects and other arthropods. Tree species diversity commonly has a positive effect on ecosystem services. Several studies show positive relationships between tree species richness and biomass production (Guillemot et al., 2020), carbon storage (Poorter et al., 2015; Steur et al., 2020) and insect diversity (Huang et al., 2018; Schuldt et al., 2019). Considering the current biodiversity crisis fuelled by anthropogenic changes in land-use and CO_2 levels (Trumbore et al., 2015), it is important to understand how ecosystem diversity relates to ecosystem functions.

Specialized molecules play an important role in interactions between plants and their biotic environment. These molecules, often called secondary metabolites, such as alkaloids, phenolic compounds or volatile organic compounds, mediate resistance to herbivores and pathogens, attract pollinators and beneficial root microbes, and serve as allelopathic compounds to suppress competing plant species (Raguso et al., 2015). In contrast to ubiquitous compounds, such as amino acids and sugars, the profiles of secondary metabolites are commonly species specific (Endara et al., 2015; Gargallo-Garriga et al., 2020; Sedio et al., 2017). Chemical diversity in plants can drive the diversity of the associated trophic levels, and is therefore an important mechanism underlying organismal diversity in tropical forests (Richards et al., 2015). Recently, untargeted metabolomics has emerged as a powerful approach to study the chemical diversity in plants. Metabolomics is principally an untargeted technology, which aims to measure as many metabolites as possible in one extract without prior knowledge of its chemical composition (Macel & van Dam, 2018). This independence also allows the application of metabolomics in ecology, which gave rise to the field of ecometabolomics (Gargallo-Garriga et al., 2020; Peters et al., 2018). Ecometabolomic approaches enhance our understanding of the role of plant metabolites in individual plant interactions as well as in ecosystem functioning, for example as a functional trait in plant diversity (Sardans et al., 2020; Walker et al., editorial of this issue).

When it comes to the analyses of trees, so far, untargeted metabolomic approaches have been mostly used to quantify chemical diversity related to phylogenetic distances in large tropical tree genera (Endara et al., 2015; Salazar et al., 2018; Sedio et al., 2017), chemical diversity within canopies (Sedio et al., 2017, 2019; Wiggins et al., 2016), herbivore-induced responses or variation in leaf metabolomes among seasons (Gargallo-Garriga et al., 2020). Among others, these studies showed that plant metabolomes are not static, and change in response to biotic and abiotic environmental variation. For example, attacks by above-ground or below-ground herbivores and pathogens may shift the metabolome of plants towards producing more (defensive) metabolites (van Dam & Heil, 2011; Macel et al., 2014). In other terms, plant metabolomes are plastic, and change within their genetic boundaries and depending on the type and number of interactions in their environment. Indeed, ecometabolomic analyses of grasses and frobs in biodiversity and ecosystem functioning (BEF) studies showed that metabolomes of individual plant species change with plant diversity level in their surrounding (Huberty et al., 2020; Ristok et al., 2019; Scherling et al., 2010). These diversity-driven effects on plant metabolomes could be caused directly by above-ground and below-ground plant–plant interactions (Bixenmann et al., 2016) or indirectly via plant–soil feedbacks. When plant–plant interactions lead to competition, this may reduce the levels of primary metabolites, such as amino acid levels (Scherling et al., 2010) or increase the production of allelopathic metabolites (Fernandez et al., 2016). Soil feedbacks occur because plant species and plant communities facilitate specific soil microbial communities (Bardgett & van der Putten, 2014). Individual plants exposed to these different soil communities can respond by changing their metabolomic profiles (Huberty et al., 2020; Ristok et al., 2019).

Other than for grassland species, diversity-related effects on tree metabolomes have not been studied at great depth. For example, targeted metabolomics approaches revealed that total phenolic concentrations in birch *Betula pendula* leaves increased with plot diversity level (Poeydebat et al., 2020). Different phenolic compounds, however, can have very different effects on herbivores and pathogens (Whitehead et al., 2021). Untargeted metabolomics can provide more detail on the identity and composition particular phenolics, as well as on the presence of other compound classes, such as alkaloids, which play important roles in ecological interactions (Peters et al., 2018).

To test whether tree metabolomes are affected by surrounding tree diversity levels, we analysed root exudate, root and leaf metabolomes of four subtropical plant species in the experimental field site of BEF-China (Bruelheide et al., 2014; Huang et al., 2018; Trogisch et al., 2021). Although previous studies on tree metabolomes focused on leaf metabolomes, we explicitly included analyses of roots and their exudates. This is important, because roots and shoots have different functions, and function in different biotic and abiotic environments (van Dam, 2009). Roots fulfil important physiological functions, such as water uptake, macro- and micro-nutrient foraging and storage. Niche differentiation, in the form of morphological root responses (Sun et al., 2017) and the uptake of different forms of *N* (complementarity), may alleviate nutrient limitation and maintain tree diversity (Lang et al., 2014). Moreover, roots produce exudates, which are secreted into the rhizosphere and contain a large variety of metabolites (van Dam & Bouwmeester, 2016). Depending on soil conditions, root exudates may contain metabolites that increase mineral nutrient bioavailability through rhizosphere priming effects (Dijkstra et al., 2013) and increase the attraction of mutualistic interactions with beneficial micro-organisms, such as mycorrhizal fungi (Ferlian et al., 2018). Both root and exudate metabolites can deter or resist pathogenic microbes, invertebrate herbivores and parasitic plants, as well as suppress competing plant species (Baetz & Martinoia, 2014; Zeng, 2014). Leaves, on the other hand, are responsible for the assimilation of carbohydrates. In dense forest canopies, the leaves compete for light with their direct neighbours. Intraspecific and interspecific competition may have different effects on the canopy structure (Guisasola et al., 2015), which may cause differences in the ecophysiological status of trees in monocultures versus more diverse tree plots (Grossiord, 2020). Lastly, leaves also respond to herbivores and pathogens by producing more defensive metabolites (Agrawal et al., 1999). The level of diversity in tree stands may affect the levels of herbivory experienced by trees (Schuldt et al., 2010; but see Wang et al., 2020) and microbial pathogens (Rutten et al., 2021). We therefore expect that root, root exudate and leaf metabolomes all respond directly, that is, in response to their neighbours, or indirectly, that is, via other biotic interactions, to tree diversity levels in their environment.

Root exudates, roots and leaves of the four selected tree species were sampled in plots with different levels of diversity, ranging from monocultures over 2, 4 and 8 species to 16 and 24 species per plot. We also recorded the local species diversity among the trees directly surrounding the target trees. This allowed us to test the effect of surrounding tree diversity levels (local diversity) and total plot diversity on the metabolomes of leaves, roots and root exudates. We hypothesized that roots, root exudates and leaves showed species-specific metabolomic profiles and that within species, root and root exudate metabolomes would be most similar. Because root exudates may travel only very short distances (Finzi et al., 2015), the closest neighbours likely have a greater influence on roots and root exudates than remote trees. Therefore, we also expected that local diversity would have a larger effect on plant metabolomes than plot diversity. We also postulated that metabolite diversity would increase with tree diversity level because the diversity of chemical or physical signals coming from the neighbours increases. Finally, we postulated that the metabolomes of root exudates would respond stronger to species diversity than roots or leaves, because of the positive effects of diversity on the production of fine roots (Sun et al., 2017), which might also enhance root exudation.

2 | MATERIALS AND METHODS

2.1 | Field location

This study was carried out in the Biodiversity–Ecosystem Functioning Experiment China (BEF-China), which has been set up and managed since 2009 (Bruelheide et al., 2014), (Huang et al., 2018). It is located in Jiangxi Province, China (29°08′–29°11′N, 117°90′–117°93′E) on two sites, A and B, which were planted in 2009 and 2010, respectively. A former monoculture timber forest was replanted with local tree species in monoculture and mixed stands using a 'broken stick' design. Using a pool of 40 tree species, extinction scenarios were simulated with tree richness levels of 1, 2, 4, 8, 16 and 24 species on a total of 566 plots of 25.8 m × 25.8 m and 400 trees each. The trees are planted on a rectangular grid with a 1.29 m distance. Thus, every tree has eight potential neighbours at a distance of 1.29 m or 1.82 m (diagonal). This study was carried out on site B, which ranges in altitude from 113 to 182 m and with slopes from 15 to 43 degrees. The site has a subtropical climate. From 1971 to 2000, the mean annual temperature was 16.7°C and the mean annual precipitation 1,800 mm. This increased to 17.9°C and 2,076 mm, respectively, during 2013–2017. January is the coldest month with a mean temperature of 0.4°C and July is the hottest with a mean temperature of 34.2°C. The (natural) vegetation is characterized by subtropical forest with a mixture of evergreen and deciduous species (Bruelheide et al., 2011).

2.2 | Target tree species

For our analyses, we targeted four different tree species planted in BEF-China Site B (Figure S1). These were selected for having at least three or more replicates in the plots with the different diversity levels. Moreover, we selected trees from different families to avoid phylogenetic bias. All four tree species are evergreens.

Cyclobalanopsis glauca (Thunb.) Oerst. (homotypic syn. of *Quercus glauca* Thunb.), Fagaceae, or the 'ring-cup oak', is native to subtropical and warmer temperate zones of Asia. It contains a variety of tannins (catechins, procyanidins and gallic acids), triterpenoids, flavonoid glycosides and steroids (cycloartanols and stigmastanes; Kamano et al., 1976; Shen et al., 2012; Sheu et al., 1992; Suga & Kondo, 1974; Wakamatsu et al., 2020). *Schima superba* Gardner & Champ., Theaceae, is a dominant broad-leaved tree distributed over temperate Asia. Chemical analyses showed that *Schima* spp. contain triterpenoids and saponins as well as flavonoids and anthocyanins (Deng et al., 2010; Kitagawa et al., 1975; Liang et al., 2019; Liu et al., 2019; Wu et al., 2015, 2019; Yang et al., 2018; Yu et al., 2019). *Daphniphyllum oldhamii* (Hemsl.) K. Rosenthal, heterotypic synonym of *Daphniphyllum pentandrum* Hayata, Daphniphyllaceae, is native to temperate Eastern Asia. Species in the genus *Daphniphyllum* contain Daphniphyllum alkaloids, a diverse group of specific alkaloids with unusual ring structures (azaspirodecane derivatives) with different backbones, probably derived from squalene and mevalonate (Kobayashi & Kubota, 2009). Furthermore, lignans and flavonoids have been described (Chao et al., 2018; Gan et al., 2007; Kobayashi et al., 2003; Mu et al., 2006, 2007; Shao et al., 2004; Takatsu et al., 2004). *Cinnamomum camphora* (L.) J. Presl, camphor, Lauraceae, is native to Japan and Taiwan and cultivated or naturalized in temperate and tropical regions worldwide. This plant is well known for its monoterpenoids (bicyclic, acyclic, menthane monoterpenoids, e.g. camphor, camphene, terpineol, pinene and borneol). *C. camphora also* contains tri- and sesquiterpenoids, neolignans, steroids, benzenoids, flavonoids and oxolanes (KNApSAcK database, Afendi et al., 2012).

2.3 | Sampling design

The sampling took place from 19 October until 28 October 2019. We took our samples in 11 of the 13 plots in the BEF-China experiment containing our target species. We sampled root exudates, roots and leaves of the above-mentioned four tree species in plots with species richness levels 1 (4 plots), 2 (two plots), 4 (1 plot), 8 (one plot) or 16 species (one plot) (=plot diversity groups D1, D2, D4, D8, D16). As there was not a sufficient number of replicates to be found in the available D16 plot, five samples were collected in two D24 plots and treated as one plot diversity group together with D16 (D16/24). In monoculture plots (1 per species), we sampled three randomly selected trees. In the two-species plots, 4–8 replicates were sampled for each species. In the plots with 4, 8, 16/24 species, we sampled 1–7 trees per species. The detailed sampled species combinations and sampling replicate numbers are shown in Figure S1 and in the sampling data (https://doi.org/10.5281/zenodo.5255811). In total, we sampled 84 trees, yielding 84 samples for root exudates, roots and leaves each.

After scrutiny of the metabolomes, it became apparent that 9 of the 84 root (exudate) samples were not from the target trees. Pre-experiments showed that under field conditions, the root excavated for exudate collection is not always originating from the target tree, even when taking all possible care. Eight of the root and root exudate samples could be reassigned to one of the other three species based on their metabolome. One sample could not be reassigned; thus, for the roots, 83 samples remained for statistical analyses, for the exudates 81 (2 were lost during sample preparation) and for the leaves the complete 84.

2.4 | Determination of local diversity

The position of each target tree and neighbourhood species were recorded at the time of sampling using the grid coordinates from the plot (see Figure S1 and sampling data https://doi.org/10.5281/zenodo.5255811). We defined the local neighbourhood as 12 adjacent squares containing two target trees in the middle and the 10 surrounding trees. These data were used to determine the local diversity.

2.5 | Leaves, roots and root exudate collection

For the collection of root exudates, we followed the protocol of Phillips et al. (2008), with slight modifications. The root exudates were collected in the middle between two trees. We excavated the roots from the upper 10 cm of mineral soil, starting from the base of the tree, to identify terminal fine root strands (<2 mm). Adhering soil particles were removed with demineralized water. Roots were dried using paper tissue and placed into a 30-ml glass syringe, which was then filled with new, unused glass beads (500–750 μm, acid washed, ACROS Organics, ThermoFisher, New Jersey, USA/Geel, Belgium). The outlet of the glass syringe was attached to a plastic tube with a plastic 50 Ml syringe attached to it. The glass syringe with the roots was filled with nutrient solution (0.50 mM NH_4NO_3, 0.10 mM KH_2PO_4, 0.20 mM K_2SO_4, 0.15 mM $MgSO_4$, 0.30 mM $CaCl_2$) from the top. The solution was sucked through the plastic syringe and discarded. The root was left to equilibrate for 20 min, after which fresh nutrient solution was added and the procedure was repeated. After this second washing step, the glass syringe was closed with cotton wool and sealed with parafilm. Everything was wrapped in aluminium foil and left for 48 hr. After 48 hr, the roots were cut-off and the syringes, which contained the root and glass beads, were placed in plastic bags and brought into the laboratory. In addition, randomly chosen leaves were sampled for each tree from the shaded part of the crowns.

To recover the root exudates, a 0.22-μm sterile filter was placed on a vacuum pump manifold over a 60-mL glass/plastic vial. The glass syringe with the roots and the glass beads was mounted on top of the manifold and filled with 60 Ml nutrient solution. Then, the vacuum pump was started and the exudate solution was collected into the vial. Five millilitres of the exudate solution of each sample were aliquoted for non-purgeable organic carbon (NPOC) analysis (see below). The remaining root exudate solution was frozen at

−20°C in 60-ml HDPE cryo-vials until freeze-drying but thawed for shipment. After the collection of exudate solution, the roots were cleaned with water to remove residual glass beads. Root and leaf samples were put in envelopes, oven-dried for 30 min under 105°C, and then oven-dried for 24 hr under 60°C.

2.6 | Sample preparation for LC-MS

Dried roots and leaves were extracted for LC-MS according to a standard protocol: Per 20 mg powdered material, 1 ml of extraction buffer (75% v/v methanol, HPLC grade, 25% v/v acetate buffer (2.3 ml acetic acid and 3.41 g ammonium acetate in 1 l 18 MΩ water, pH set to 4.8) plus 50 µl 100 mM IAA-Valin as internal standard) was added and shaken with ceramic beads in a tissue homogenizer (Retch MM400, Retch GmbH, Haan, Germany) for 5 min at 30 Hz. Samples were centrifuged for 15 min at RT at 15,000*g*. The pellet was re-extracted with another 1 ml of extraction buffer per 20 mg starting material, centrifuged again and both supernatants were unified. Samples were diluted 1:5 with the extraction buffer, kept at −20°C overnight, centrifuged at 15,000*g* for 10 min and transferred to HPLC vials.

Exudates were freeze-dried after shipment, re-dissolved in 1 ml water and the bottles washed with 1 ml methanol. The samples were transferred to a new 2-ml reaction tube, completely dried (vacuum centrifuge 27°C) and we tried to re-dissolve them in a smaller volume of the extraction buffer. However, this was not possible, so 1 ml of water was added, dissolution was aided by vortexing, ultrasonic bath and heating to 40°C, the samples centrifuged, the remaining pellet again dissolved in another 1 ml of water, centrifuged again and both supernatants were unified. After that, the samples were concentrated, but not to complete dryness, in a vacuum centrifuge.

2.7 | NPOC measurements

Non-purgeable organic carbon (NPOC) was determined after removal of inorganic C (acidification and sparging) by catalytic thermal oxidation (at 680°C) and subsequent detection of CO_2 by an infrared gas analyser (TOC VCPN) on three analytical replicates.

2.8 | Normalization of exudate samples by NPOC before LC-MS analysis

Because exudate NPOC values depended heavily on the root morphology and differed significantly among species (Figure S2), we used NPOC values to dilute samples before analysis. This procedure normalizes for exudation rates and thus allows a better comparison of root exudation profiles over species and treatments. To correct for the NPOC, the volume of the concentrated samples was measured by taking it up with a pipette. The median NPOC of all samples was calculated (5.023 mg/l). The minimum was 0.84 mg/l, and the maximum was 33.66 mg/l. It was decided that the median concentration of carbon should correspond to 300 µl, and the required volume for each sample was calculated as follows: $\text{volume}_{\text{sample}}$ (µl) = $\text{NPOC}_{\text{sample}}$ (mg/l) × 300 µl/median NPOC (mg/l). The concentrated samples (remaining volume 50–200 µl) were filled up with 70% methanol to attain the calculated volume (range: 50–2011 µl).

2.9 | LC-MS measurements

Chromatographic separations were performed at 40°C on an UltiMate™ 3000 Standard Ultra-High-Performance Liquid Chromatography system (UHPLC, Thermo Scientific) equipped with an Acclaim® Rapid Separation Liquid Chromatography (RSLC) 120 column (150 mm × 2.1 mm, particle size 2.2 µm, ThermoFischer Scientific) using the following gradient at a flow rate of 0.4 ml/min:0–1 min, isocratic 95% A [water/formic acid 99.9/0.1 (v/v %)], 5% B [acetonitrile/formic acid 99.9/0.1 (v/v %)]; 1–2 min, linear from 5% to 20% B; 3–8 min, linear from 20% to 25% B; 8–16 min, linear from 25% to 95% B; 16–18 min, isocratic 95% B; 18–18.01 min, linear from 95% to 5% B; 18.01–20 min, isocratic 5% B. Data were recorded from 0 min to 18 min. The injection volume was 10 µl for exudates and 5 µl for roots and leaves.

Eluted compounds were detected from *m/z* 90 to 1,600 at a spectra rate of 5 Hz (line spectra only) using an ESI-UHR-Q-ToF-MS (maXis impact, Bruker Daltonics) in positive ion mode with data-dependent collision-induced dissociation (Auto-MSMS mode). The following instrument settings were applied: nebulizer on, 2.5 bar; dry gas, nitrogen, 11 l/min, dry temperature 220°C; capillary voltage, 4,500 V; end plate offset, 500 V; funnel 1 radio frequency (RF), 200 Volts peak-to-peak (Vpp); funnel 2 RF, 220 Vpp; in-source collision-induced dissociation (CID) energy, 0.0 eV; hexapole RF, 120 Vpp; quadrupole ion energy, 4 eV; quadrupole low mass, 100 *m/z*; collision gas, nitrogen; collision energy, 10 eV; prepulse storage, 7 µs. Stepping: on; basic mode; collision cell RF, from 400 Vpp to 1,000 Vpp; transfer time, from 30 to 70 µs, timing; 50%/50%, collision energy for MSMS, 80%, timing 50%/50%. Data-dependent CID settings: intensity threshold 600, cycle time, 1 s, active exclusion on after 2 spectra, release after 0.5 min, smart exclusion, off, isolation and fragmentation settings, size- and charge-dependent, width 3–15 *m/z*, collision energy 20–30 eV, charge states included: 1*z*, 2*z* and 3*z*.

Calibration of the *m/z* scale was performed for individual raw data files on sodium formate cluster ions obtained by automatic infusion of 1.66 µl/min of 10 mM sodium formate solution of NaOH in 50/50 (v/v) isopropanol water containing 0.2% formic acid at the end of the gradient (HPC mode).

Three mixed QCs were prepared (QC roots, QC leaves and QC exudates) and run after each 8th sample. A mix of eight commercial standards was also run after eight samples (MM8; Böttcher et al., 2007). We used the following blanks: injection blanks (ACN); extraction blanks (empty reaction tubes for roots and leaves with the extraction buffer; for exudates: empty reaction tubes with 1 ml water and 1 ml methanol), furthermore for exudates: water and collection buffer from the field campaign stored in the HDPE cryo vials as well as empty cryo-vials, all passed through the whole shipment,

freeze-drying and exudate preparation process. Roots, leaves and exudates were prepared and measured in separate batches to avoid cross-contamination. Within the tissues, all samples were randomized throughout the complete process of processing and measurement.

2.10 | Data processing

The LC-qToF-MS data were processed all together with Bruker Compass MetaboScape Mass Spectrometry Software, Version 5.0.0 (Build 683; Bruker Daltonik GmbH). Mass recalibration, peak picking, peak alignment, region complete feature extraction, and grouping of isotopes, adduct and charge states was performed with the T-ReX algorithm in Metaboscape. Settings: Peak detection: intensity threshold, 1,000 counts, minimum peak length, 7 spectra, feature signal, intensity. Minimum peak length for recursive feature extraction, 5 spectra. Retention time range, 0–18 min. Mass range, 90–1,600 *m/z*. MSMS import method, average, grouped by collision energy. Ion deconvolution: EIC correlation, 0.8, primary ion, [M+H]+, seed ions, [M+Na]+, [M+K]+, [M+NH_4]+, common ions, [M+H-H_2O]+ and T-ReX-Positive Recalibration Auto-Detect. Feature filters: Minimum number of samples: present in 3 of 302, minimum for recursive feature extraction: present in 3 of 302, group filter: present in at least 80% of at least one group (1 group = all replicates from 1 diversity level within each species and tissue). Features from Blanks were excluded when the maximum signal in samples divided by maximum signal in blanks was ≤3. Quality checks included stability of retention time and signal intensity, check for carry-over and check for correct species identity. With these settings, two feature tables were created: One that classified the plant diversity around the target trees by the diversity of the experimental plot ('plot diversity') and one that classified it by the local neighbourhood ('local diversity'). After processing, the dataset for plot diversity contained 39,077 features in total, to which 22,520 fragment spectra were assigned; 3,883 features from blanks were removed, leaving 35,194. For local diversity: total, 38,105, assigned fragment spectra 22,104; 3,649 features from blanks were excluded, leaving 34,456.

2.11 | Statistical analysis

For further statistics, the feature tables were exported to Metaboanalyst 4.0 (Chong et al., 2019). For the general comparison by principal component analysis (PCA) of the species-specific metabolomes (Figure 1, PCA), all species and tissues were analysed together. The data were IQR filtered before and Pareto scaled.

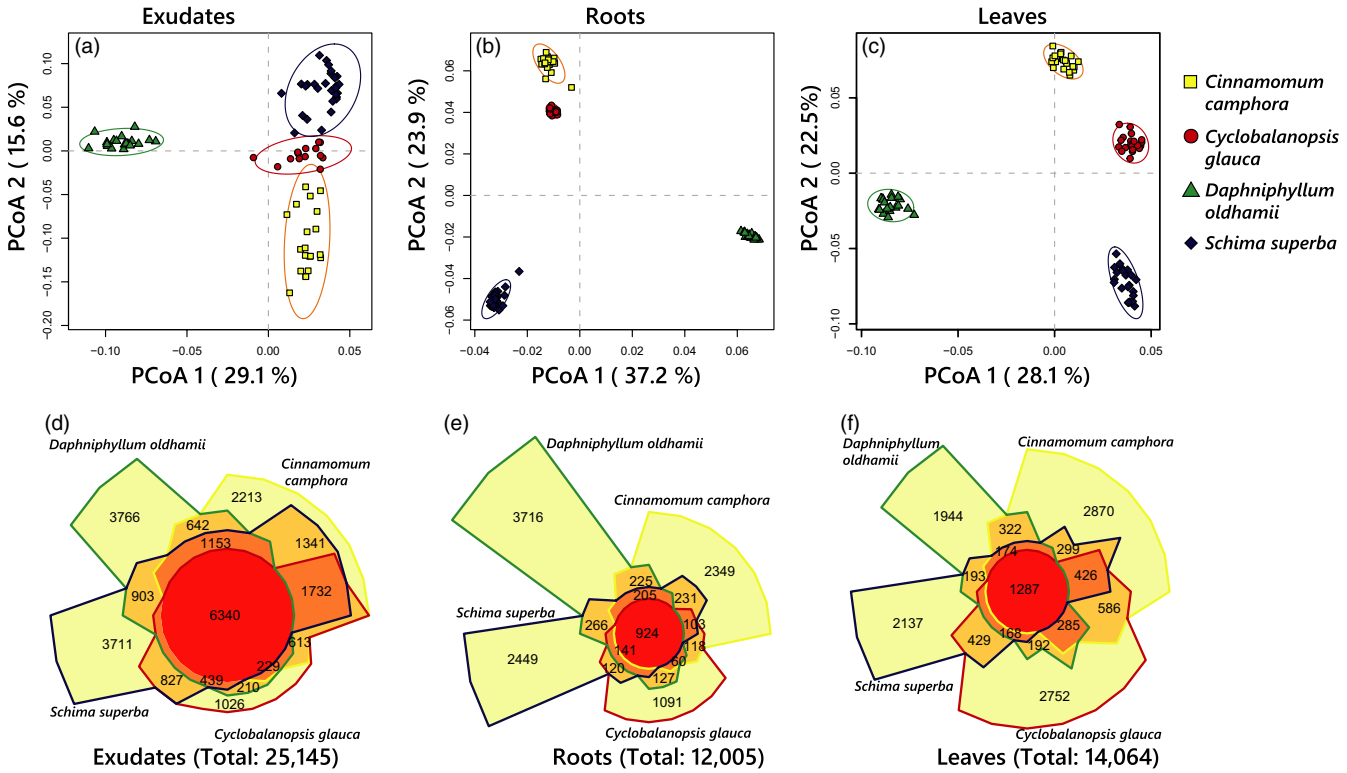


FIGURE 1 Principal coordinate analysis (PCoA) score plot of the first two principal components (PC) of features found in the exudates (a), roots (b) and leaves (c) of *Cinnamomum camphora* (yellow squares), *Cyclobalanopsis glauca* (red circles), *Daphniphyllum oldhamii* (green triangles) and *Schima superba* (blue diamonds). Numbers in brackets are the percentage of variation explained by the corresponding PC. Ellipses are the 95% confidence interval. Data were Pareto scaled. Second row: Venn diagrams (Chow–Ruskey type) of species specific and shared features in the exudates (d), roots (e) and leaves (f) of the four different tree species showing the number of mass features unique to a single species (light yellow) or shared between two (light orange), three (dark orange) or all species (red). The area of single sections corresponds to the proportion of the number of features compared to the total number

Chow–Ruskey diagrams were produced with 'intervene' (https://asntech.shinyapps.io/intervene/; Khan & Mathelier, 2017) counting a feature as 'present' when it was detected in at least two samples with an intensity of >/=1,000.

For partial least squares-discriminant analysis (PLS-DA), we created smaller feature tables including only the respective species and tissue metabolomes to avoid datasets with too many zeros. The data were also IQR filtered before and Pareto scaled. To group the samples in different diversity levels for PLS-DA, we used either the plot diversity or the local diversity. Because local diversity is maximized to 12 (see also Figure S1; Table S1) and varied due to tree dieback or misplantings, some local diversity levels had two or fewer replicates. To have at least $n = 3$ per local diversity level, we grouped the samples as follows: 1 species (monoculture), 2 species, 3 or 4 species, and 5 to 7 species mixtures. In the case of *Cy. glauca*, the latter two levels (3, 4 and 5) had to be combined to obtain a sufficient number of replicates, and there were no trees with local diversity level of 6 or 7. For *D. oldhamii*, the monoculture and two species samples were combined, because one of the monocultures was invaded by another tree species, leaving only two replicates for monocultures. To compare the data matrices, we performed multiple response permutation procedure (MRPP) with the R-package VEGAN (v.2.5-6; https://CRAN.R-project.org/package=vegan) in R (version 3.6.0; RCoreTeam, 2020).

We calculated metabolite richness as the sum of all features with an intensity >=1,000 and metabolite diversity as the Shannon diversity of all features with an intensity >=1,000 using the R-package VEGAN.

Principal coordinate analysis (PCoA) was calculated using R version 4.0.4 by using the function 'pco' in the package ecodist: (v. 2.0.7), using the data matrix of the metabolomics dataset with 'Bray–Curtis' as dissimilarity measure. The distance matrix was calculated using the package VEGAN (v. 2.5-7) and the function 'vegdist'. PERMANOVA was calculated using the 'adonis2' function in *vegan* with 'Bray–Curtis' as distance measure. ANOVA was calculated using the 'aov' function in R with the following model: Chemical richness/Chemical Diversity~Tissue*Species*Species Diversity (Local or Plot).

Spearman's rank-order correlation of metabolite richness or Shannon index with diversity was calculated using the function 'stat_cor' in the R package GGPUBR (v. 0.4.0) along with a nonlinear 'loess' fit.

2.12 | Compound annotation

First, all fragment spectra were matched in a parallel search against the following databases: NIST17 (The NIST Mass Spectrometry Data Center, U.S. Department of Commerce), MoNa (https://mona.fiehnlab.ucdavis.edu/), ReSpect (Sawada et al., 2012), Riken public databases http://prime.psc.riken.jp/compms/msdial/main.html#MSP), Mass Bank EU (https://massbank.eu/MassBank/Index), GNPS (https://gnps.ucsd.edu/) and an internal database (Döll, unpublished), using the MetaboScape Spectral Library Search function [Parameters: Filter: exact match of data base entry to precursor mass; tolerances (narrow wide): *m/z* 10–30 mDa, mSigma 20–100, MS/MS score 900–800]. Afterwards, we performed a manual annotation of species-specific compounds with features from literature and KNApSAcK database (see 'Target tree species') based on sum formula generation (Smart Formula algorithm, MetaboScape), in-source fragmentation patterns and spectral similarities to the mentioned databases. From these manual annotations, a small spectral library was created (197 species-specific compounds). This library was matched against the whole dataset with the MetaboScape Spectral Library Search function without the exact match of the precursor mass to find more species-specific compounds similar to the already annotated ones. For the classification approach, the whole dataset was searched against NIST17 without the exact match of the precursor mass. Tolerances were as above, and the search was limited to NIST 17 because of computational limitations. Relevant features selected after PCA (features with the highest loading on the separating principal components) or PLS-DA (20 highest VIPs—variable importance in projection on the first component) were manually annotated as above.

Annotated compounds were classified using an R workflow (Supporting Information, R Script). In brief, the annotated feature table was exported in.csv format including CAS numbers, if available, and read into R (v 4.0.3; RCoreTeam, 2020). We employed '*webchem*' (Szöcs et al., 2020) packages for automatic collection of the PubChem Compound Identification number (CID). In a subsequent step, the CID was then used to retrieve descriptors (SMILES and InChIKeys) from the PubChem database. The actual classification was performed employing the ClassyFire tool (Feunang et al., 2016) using the RAMCLUSTR package (Broeckling et al., 2014) in R for getting the chemical ontology information of the annotated compounds. Thus, 8,101 features were classified into 'Kingdom' (e.g. 'Organic compounds'), 'Superclass' (e.g. 'Phenylpropanoids and polyketides') and 'Class' (e.g. 'Coumarins and derivatives'). Sunburst plots were created in Excel 2019, aided by an R script (packages TIDYVERSE, FS, READXL, WRITEXL; see supplemental file: code_snippets_data analysis_MTBLS1968.R), by adding up the feature intensities for each feature from all the samples of one species and tissue.

3 | RESULTS

3.1 | Metabolome composition of exudates, roots and leaves is defined by species identity

When leaf, root and root metabolomes were analysed together in a principal coordinate analysis (PCoA), it was clear that the metabolomes of the different tissues clustered together (Figure S3a). Therefore, we proceeded our analyses per tissue type. PCoA showed that the metabolomes of the four tree species can be distinguished in each tissue type we sampled (Figure 1a–c). In general, the metabolome of exudates showed a larger variation, which is indicated by wider confidence bands and larger score distances within

FIGURE 2 Chow–Ruskey Venn diagrams (a) of features in the metabolomes of the four tree species *Cinnamomum camphora*, *Cyclobalanopsis glauca*, *Daphniphyllum oldhamii* and *Schima superba* that are unique to exudates (red), roots (green) and leaves (blue) and features that are shared between two or all of the different samples. The area of single sections corresponds to the proportion of the number of features compared to the total number. Second to fourth row: Sunburst Plots visualizing the metabolome compositions of exudates (b), roots (c) and leaves (d). The inner circle represents the *chemical superclass* according to the *ClassyFire* hierarchical classification while the outer circle represents the *chemical class*. Proportions of the circle represent the sum of the intensities of all features assigned to *chemical class*. *Chemical superclasses* are defined in the legend at the bottom of the figure and selected *chemical classes* are specified in the sunburst plots

the species (Figure 1a) than leaf and root metabolomes (Figure 1b–c). The total number of mass features (25,145) in root exudates was more than twice as high as in root (12,205 in Figure 1d–f) or shoot samples (14,064; Figure 1d–f). However, root exudates of the four species shared about 25% of their metabolites, whereas in roots only 7.7% and in shoots 9.1% of all the metabolites were found in all four species. In a PERMANOVA (Table S2), species identity was the main factor in separating the metabolomes in all three tissues and explained more than 70% of the variation for roots and leaves.

The metabolomes of *D. oldhamii* always clearly separated from the metabolomes of the other three species (Figure 1; Figure S3b–d). The compounds responsible for this separation likely are the Daphniphyllum alkaloids, which had the highest loadings on PC1 (Table S3 and S4; Figure 2b,c, third column). The leaf metabolomes of *D. oldhamii* contained relatively few alkaloids (Figure 2d, third column). There, the discriminating compounds were glycosides, most likely with a flavonoid backbone, a disaccharide, a putative coumarin and a steroidal compound (Table S5). The other three species mainly separated on PC2 (Figure S3b–d). Whereas the exudate metabolomes (Figure S3b) of *C. camphora*, *Cy. glauca* and *S. superba* showed some overlap, their leaf and root metabolomes clearly separated (Figure S3c–d).

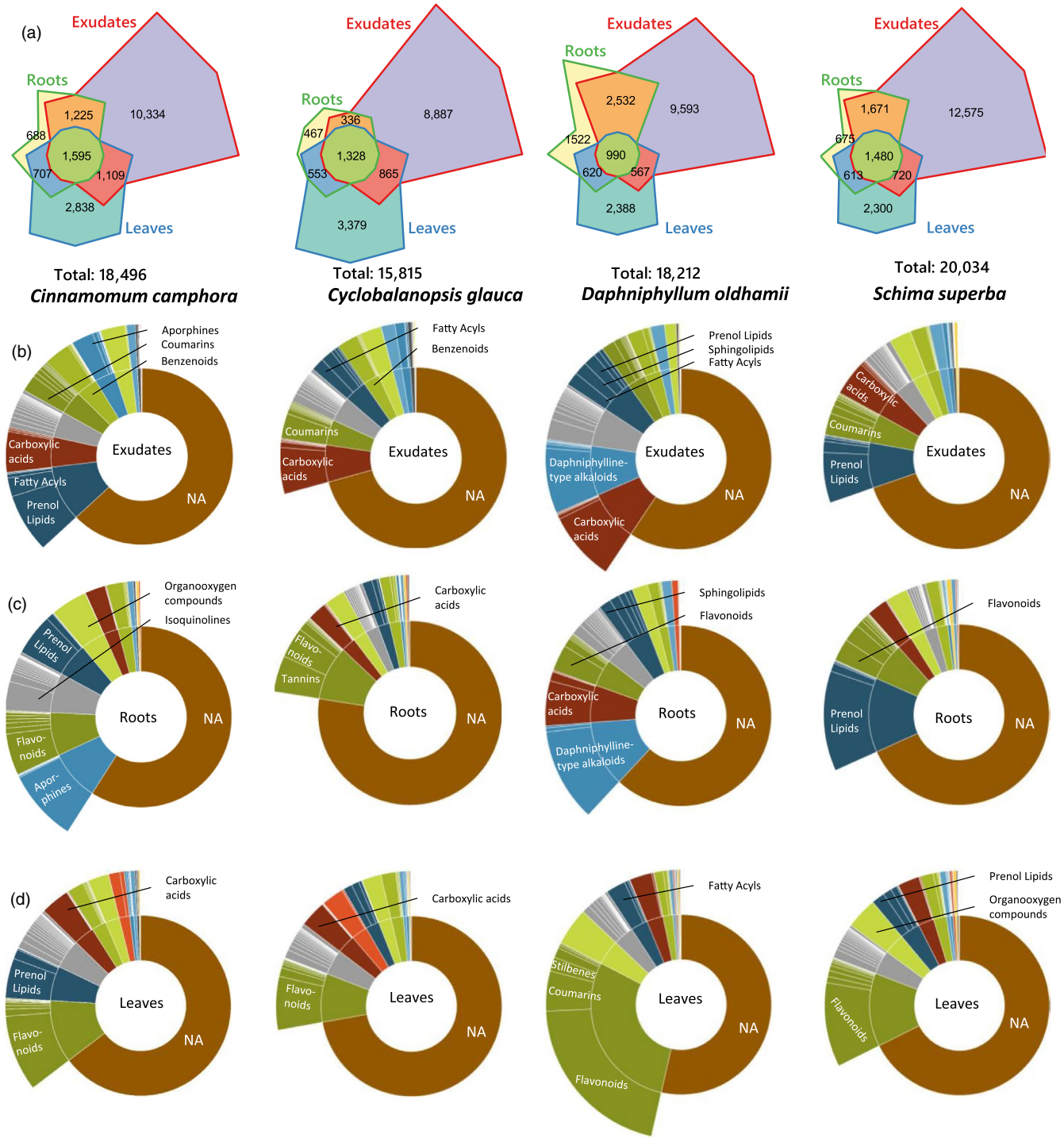
The presence of laurolitsine-like alkaloids separated both *C. camphora* exudates and root metabolomes from the other species (Table S3 and S4). Several terpenoids and a putative phenylpropanoid in *C. camphora* exudates (high loadings on PC2; Table S3), and a procyanidin, a putative reticuline, a sesquiterpene and an unknown metabolite in *C. camphora* roots were the most responsible for separating this species from the other two. The *C. camphora* leaves were typified by specific terpenoids. In roots and root exudates, saponines had the highest influence in separating *S. superba* from the rest (Table S3 and S4). A coumarin (in exudates) and a putative phenylpropanoid (roots) contributed to the separation. For *Cy. glauca* leaves, quinic acid, polyphenols and terpenoids were the discriminating metabolites with the highest loading. We could not identify discriminating features for roots and root exudates of *Cy. glauca*, because there was too much overlap with the metabolomic profiles of *C. camphora* and *D. oldhamii* in PC1 versus. PC2 (Figure S3).

When comparing samples within species, it became apparent that in all four species the exudates had the highest number of unique mass features, followed by leaves and roots (Figure 2a). The number of root specific features was generally <4%, with the exception of *D. oldhamii* (8.4%). In *D. oldhamii*, *C. camphora* and *S. superba*, the numbers of features shared between roots and exudates were greater than those shared between roots and leaves (Figure 2a).

To better characterize the metabolomes of exudates, roots and leaves, we used an automated approach to classify our tentatively annotated features per species using the ClassyFire tool (Feunang et al., 2016; see methods section). This tool uses structural information (e.g. SMILES, InChIs or IUPAC names) to categorize chemical entities into hierarchical chemical classes. Thus, we were able to categorize between 22% (roots *Cy. glauca*) and 46% (leaves *D. oldhamii*) of the *m/z* features into main compound classes (Table S7). The resulting plots reveal that carboxylic acids are a major common compound class in root exudates (Figure 2b). In roots, alkaloids, flavonoids and prenol lipids are among the major specific compound classes we could categorize. The leaf metabolomes of all four species are dominated by phenylpropanoids with flavonoids as the most abundant chemical class, followed by organoheterocyclic compounds (e.g. imidazopyrimidines, indoles, azaspirodecanes) or organo-oxygen compounds (e.g. alcohols and polyols, carbohydrates and conjugates, carbonyl compounds; Figure 2d). Prenol lipids were omnipresent, but most prominently present in roots and exudates of *S. superba* as well as in exudates and leaves of *C. camphora* (Figure 2b–c). Aporphines, a class of alkaloids, and the structurally related isoquinolines, were most prominent in *C. camphora* roots, but less in the exudates, whereas the classified metabolome of both *D. oldhamii* roots and root exudates largely of the typical Daphniphyllum alkaloids (Figure 2b–c, 3rd column). Neither in *C. camphora* nor in *D. oldhamii* leaves, alkaloids were as pronouncedly present as they were in their exudates or roots. The plots also show that on the level of compound class, the chemical profiles of roots and root exudates are more similar to each other than to leaf metabolomes (Figure 2b–d). This is also in line with the high number of overlapping features for roots and exudates (Figure 2a). Finally, we found coumarins to be present in *C. camphora* and *S. superba* exudates, as well as in *D. oldhamii* leaf metabolomes. Sphingolipids formed a notable part of *D. oldhamii* roots and root exudates, whereas the roots of *Cy. glauca* showed tannins and flavonoids as major classes (Figure 2d).

3.2 | Effects of plot and local tree diversity on metabolome composition

Because the metabolomes differed substantially among species and tissue types, we assessed the effect of plot and local tree diversity by species and tissue type. Partial least squares-discriminant analysis (PLS-DA) showed that plot diversity affected the metabolome composition in some, but not all species and tissue types. The exudate metabolomes of three of the four tree species (*Cy. glauca*, *D.*



- Alkaloids and derivatives
- Benzenoids
- Homogeneous non-metal compounds
- Hydrocarbon derivatives
- Hydrocarbons
- Lignans, neolignans and related compounds
- Lipids and lipid-like molecules
- Mixed metal/non-metal compounds
- NA – not annotated
- Nucleosides, nucleotides, and analogues
- Organic 1,3-dipolar compounds
- Organic acids and derivatives
- Organic nitrogen compounds
- Organic oxygen compounds
- Organic Polymers
- Organoheterocyclic compounds
- Organometallic compounds
- Organophosphorus compounds
- Organosulfur compounds
- Phenylpropanoids and polyketides

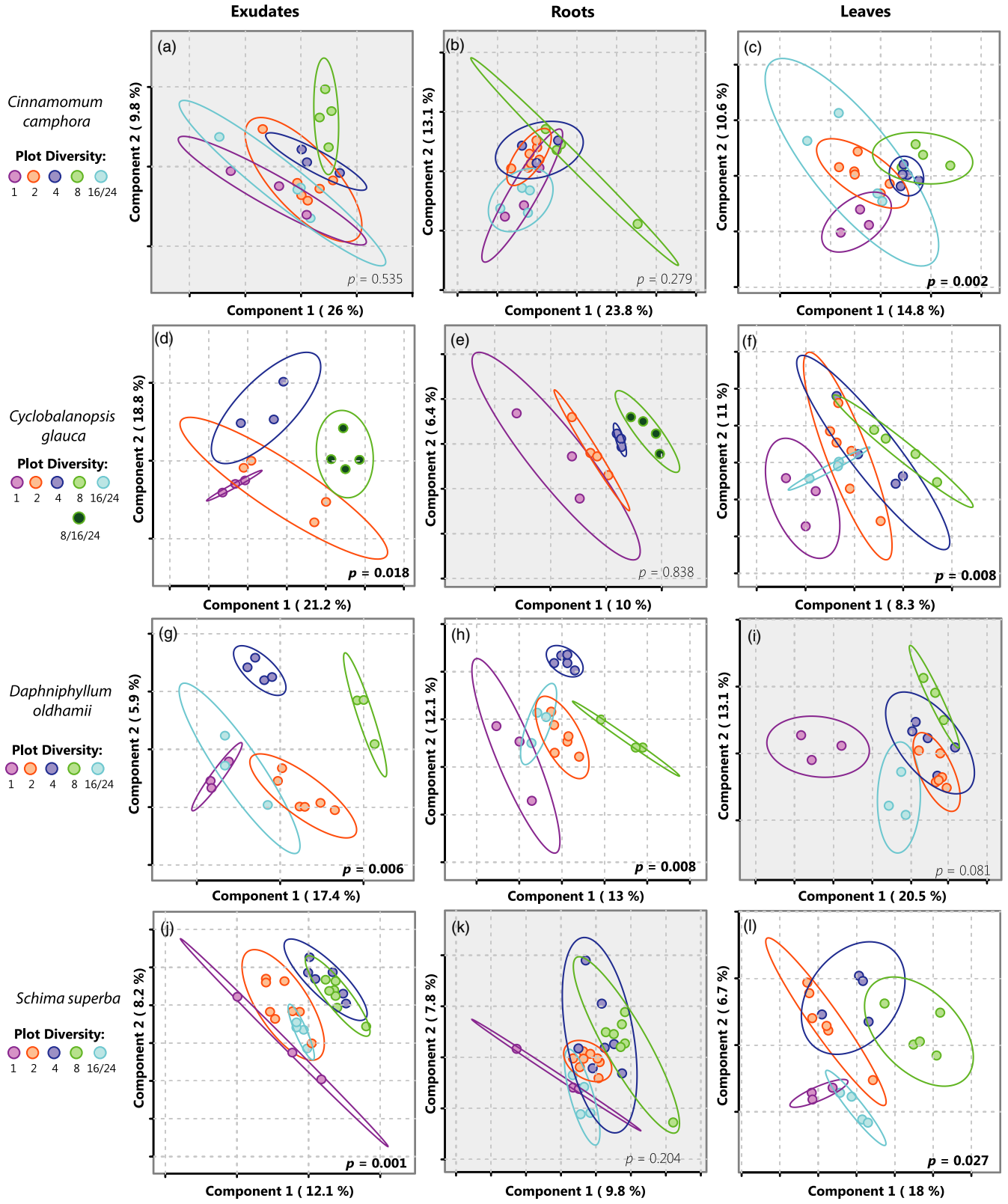


FIGURE 3 Partial least squares-discriminant analysis (PLS-DA) score plots of the features found in the exudates, roots and leaves of the four tree species *Cinnamomum camphora* (a–c), *Cyclobalanopsis glauca* (d–f), *Daphniphyllum oldhamii* (g–i) and *Schima superba* (j–l). Different colours indicate the tree species diversity in the investigated plot. Percentages at the axes indicate the variation explained by the single components. Ellipses are the 95% confidence interval. *p*-values are global values and are based on pairwise multiple-response permutation procedures (MRPP). Because of an insufficient number of replicates, diversity groups 8 and 16/24 in *Cyclobalanopsis glauca* were combined for statistical analysis. The data were Pareto scaled and features from blanks removed. *p*-values smaller than 0.05 are in bold and the corresponding plot is highlighted

oldhamii and *S. superba*) were significantly affected by plot diversity level (Figure 3; MRPP $p < 0.05$; Table S6).

In the roots, only the metabolomes of *D. oldhamii* varied significantly with plot diversity level, whereas the leaf metabolomes of *C. camphora*, *Cy. glauca* and *S. superba* all varied significantly due to plot diversity. The overall effect of diversity on leaf metabolome was corroborated by the PERMONAVA on the data of all four species, showing that local diversity only affected the leaf metabolome (Table S2). Pairwise comparison showed that on plot diversity level mostly the metabolome of the two species and four species mixtures were significantly different (Table S6). This was true for all three tissue types.

The effect of local diversity was less pronounced; only *S. superba* exudates showed a significant difference (Figure 4) while none of the root metabolomes was affected significantly. The leaf metabolomes of the same three species (*C. camphora*, *Cy. glauca* and *S. superba*) responding to plot diversity also showed a significant response to local diversity (Figure 4). In the pairwise comparison, it was always the two-species mixture that had significantly different metabolomes (Figure S7).

For all species and samples that showed a significant overall response to plot or local diversity, we picked the features with the highest VIP (variable importance in projection) value to analyse their response to tree diversity in more detail (Table S9, S10, and S11). Several of these features were annotated to compound classes that also constituted a large fraction of the respective metabolomes. In the exudates and roots of *D. oldhamii*, two of the typical Daphniphyllum alkaloids were responding to plot diversity, and in the exudates of *S. superba* the levels of saponins and phenylpropanoids varied with plot and local diversity (Figure 5b,d). In the leaves of *C. camphora*, *Cy. glauca* and *S. superba*, flavonoids (e.g. kaempferols, rutin and other quercetins) and terpenes were among the compounds responding to variation in diversity level.

Despite the presence of features responding to biodiversity level, there was no overall uniform response on species, tissue or compound level. For example, in *S. superba* leaves, quinic acid decreased in intensity with plot and local diversity, while in the same species rutin was increasing with increasing local or plot diversity (Figure 6c,f). Daphniphyllum alkaloids either showed a hump-shaped response or rather decreased at intermediate plot diversity levels in *D. oldhamii* exudates (Figure 5b). In roots, they either peaked at diversity level 8, or increased with diversity (Figure 5e). Within species and samples, we identified some common patterns. Saponin levels in *S. superba* commonly decreased (Figure 5c,d), whereas a farnesene-like sesquiterpene in *C. camphora* consistently increased with local and plot diversity (Figure 6a,d). In general, the peak intensity of the single features varied substantially across species and sample types, likely due to sampling issues and low replication rates.

3.3 | Effects of plant diversity on species chemical diversity

We analysed how chemical richness, that is, the number of mass features with intensity ≧1,000, or the chemical diversity, calculated as the Shannon diversity of the mass features, is affected by either local diversity or plot diversity. ANOVA [Chemical diversity or Chemical richness ~Tissue*Species*Species Diversity (Local or Plot)] tests showed that tissue type and species identity had a significant effect on chemical diversity and chemical richness (Tables S12 and S13). This was true for local and plot diversity. Chemical diversity was significantly influenced by plot diversity and the interaction of species and tissue, but not by the local diversity. Chemical richness is influenced by all aforementioned factors and additionally the interaction of tissue and plot diversity. Local diversity had only a marginal significant effect on chemical richness (Table S13, $p = 0.072$).

Spearman rank correlations revealed that metabolite richness was not significantly affected by plot diversity in any tissue type or tree species. The leaf metabolite richness of *Cy. glauca* ($p = 0.055$) and *D. oldhamii* ($p = 0.072$) showed a marginally significant response (Figure S4f,l). Local diversity only affected leaf metabolite richness of *Cy. glauca* ($p = 0.049$) and marginally so, the exudates of *S. superba* ($p = 0.057$; Figure S5f,J). In none of the samples, we found a significant correlation between Shannon diversity of mass features and local or plot diversity level (Figures S6 and S7).

4 | DISCUSSION

Overall, exudates, roots and leaves each showed clearly species-specific metabolomic profiles. Leaf and root metabolomes were more distinctive among species than root exudates. The prominent presence of specific alkaloids separated the *C. camphora* and *D. oldhamii* root and root exudate metabolomes from each other as well as from the other two tree species. As postulated, root and root exudate metabolomes were more similar to each other than to leaf metabolomes. Carboxylic acids formed a large part of all exudates, whereas phenylpropanoids, in particular flavonoids, dominated the classifiable subset of all leaf metabolites. All four tree species showed a metabolomic response to tree diversity in at least one of the metabolomes sampled. Both root exudates and leaf metabolomes responded more often to differences in tree diversity levels than roots. Plot diversity level had an overall larger effect on tree metabolomes than local diversity. Important features driving the differences in the metabolomes were saponines in *S. superba* exudates, *D. oldhamii* specific alkaloids in roots, kaempferols, quercetins and quinic acid in *S. superba* leaves, and sesquiterpenes in *C. camphora*.

Previous studies have shown that leaf metabolomes of various tropical species show a high level of species specificity (Richards et al., 2015; Salazar et al., 2018; Sedio et al., 2017, 2019). Here we showed that roots and their exudates also have distinct species-specific metabolomic profiles. Compared to leaves and roots, however, exudates had higher numbers of specific features with a higher intraspecific variance. Moreover, the profiles showed more overlap, despite the fact that we normalized peak areas over NPOC to mitigate differences in exudation rates. Additionally, species identity

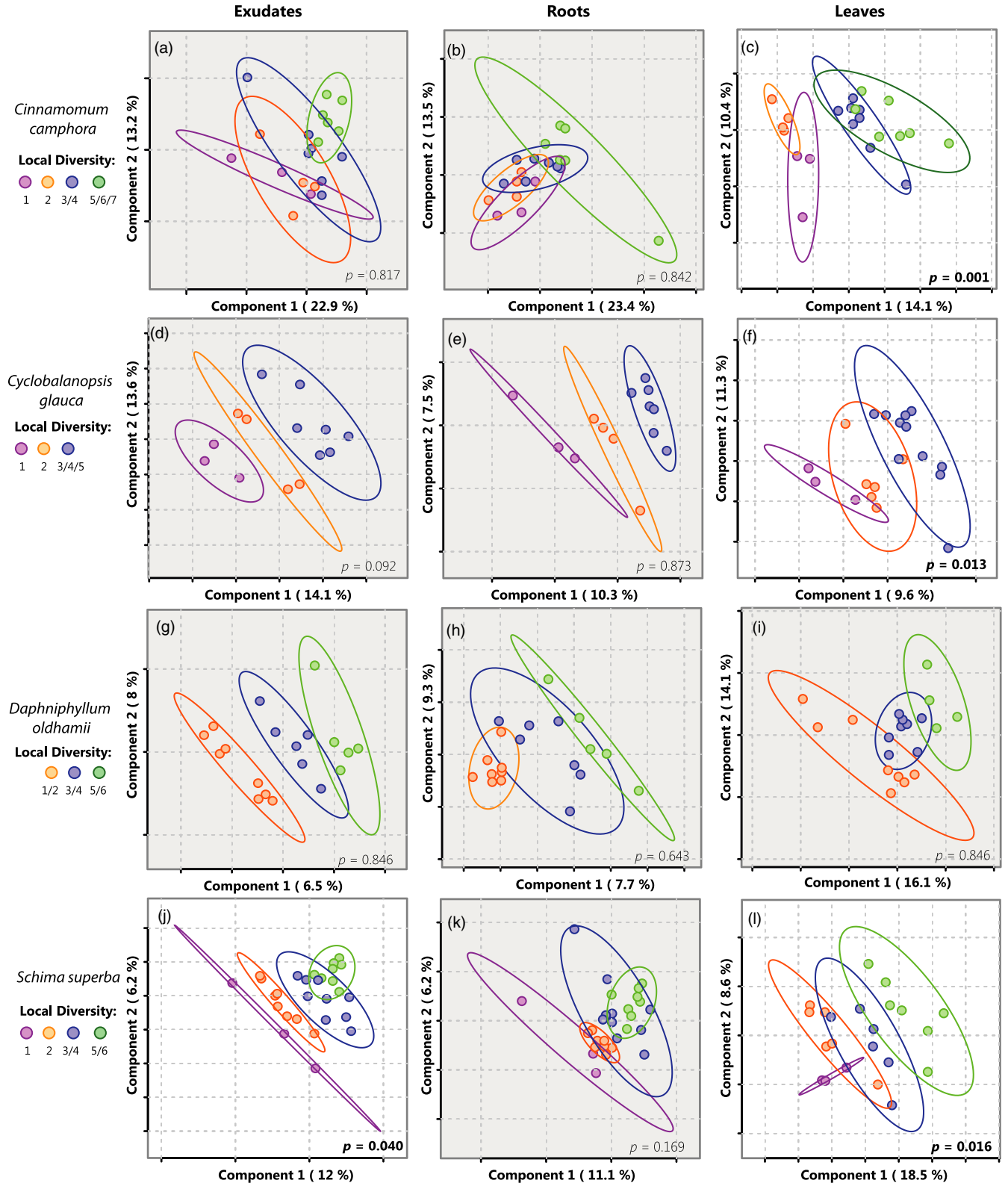


FIGURE 4 Partial least squares-discriminant analysis (PLS-DA) score plot of the first two components of the features found in the exudates, roots and leaves of the four tree species *Cinnamomum camphora* (a–c), *Cyclobalanopsis glauca* (d–f), *Daphniphyllum oldhamii* (g–i) and *Schima superba* (j–l). Different colours indicate the actual tree species diversity in the immediate local neighbourhood of the target tree (as opposed to 'plot diversity'). Percentages at the axes indicate the variation explained by the single components. Ellipses are the 95% confidence interval. Because of an insufficient number of replicates, some of the diversity groups in 'Local Diversity' were combined for statistical analysis. *p*-values are global values and are based on pairwise multiple-response permutation procedures (MRPP). The data were Pareto scaled and features from blanks removed. *p*-values smaller than 0.05 are in bold and the corresponding plot is highlighted

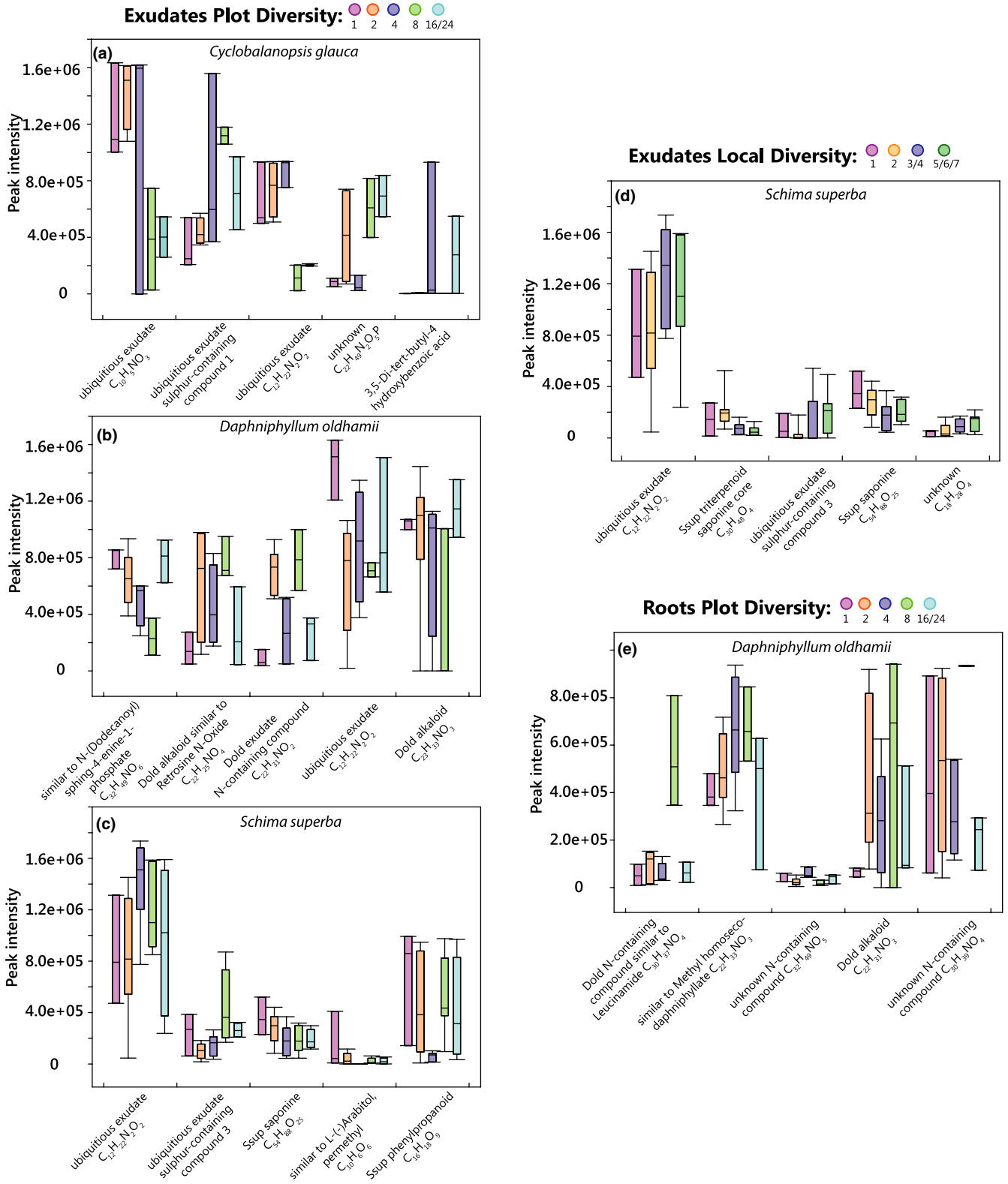


FIGURE 5 Boxplots of the top five VIPs (variable importance in projection) of PLS-DA that showed a significant global difference in the metabolome (MRPP $p < 0.05$) of exudates (a–c) and roots (e) in Figure 3 and in the metabolome (MRPP $p < 0.05$) of exudates in Figure 4d. ('Box': 25th to 75th percentiles and median, 'whiskers': 10th and 90th percentiles)

accounted for less variance in the exudates (Table S2). Likely, this is due to the fact that exudates were sampled in situ, which means that these samples inevitably contained compounds from microbes and organic matter in the rhizosphere. Additionally, (local) differences in nutrient status among plots unrelated to plot diversity may have caused differences in exudation patterns (Meier et al., 2020).

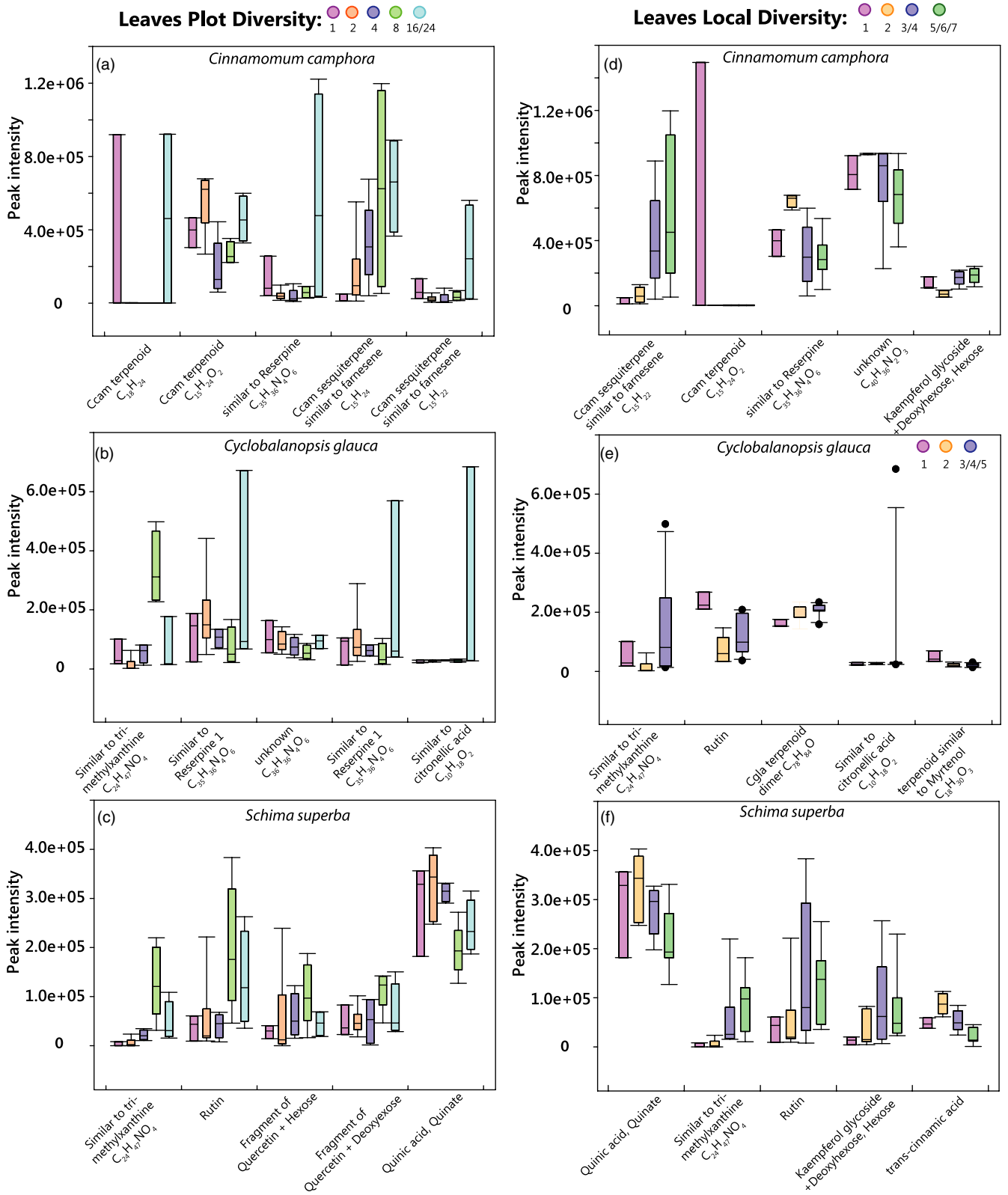


FIGURE 6 Boxplots of the top five VIPs (variable importance in projection) of PLS-DA that showed a significant global difference in the metabolome of leaves (MRPP $p < 0.05$) in Figures 3a–c and 4d–f. ('Box': 25th to 75th percentiles and median, 'whiskers': 10th and 90th percentiles, circles: outliers)

Commonly, much <10% of the mass features in metabolomic experiments can be assigned, even in model systems (da Silva et al., 2015). This limitation prohibits the ecological interpretation of differences in metabolomic profiles (Peters et al., 2018). Using bioinformatics tools to compare *m/z* features to online metabolite databases, we could broadly classify up to 45% of the features to

compound classes (Figure 2). This did not only allow us to derive broad ecological functions but also created a starting point for more targeted analyses combined with biological experiments to assess the ecological relevance of particular metabolites in more detail.

Carboxylic acids were one of the most abundant metabolite classes in the root exudates, which is in line with what has been found for forbs (van Dam & Bouwmeester, 2016). Similarly, carboxylic acids (mono-, di- and tricarboxylic acids) were also found in the root exudates of trees like birch and spruce (Sandnes et al., 2005). Carboxylic acids, like citrate or malate, play a role in P mobilization (Inderjit & Weston, 2003), which is likely the reason they are commonly found in root exudates. In *D. oldhamii*, the second largest (in exudates) and largest group (in roots) of annotated features were alkaloids. Daphniphyllum alkaloids are a structurally diverse group of metabolites, which were isolated from the bark of *Daphniphyllum* spp. They were also reported in leaves and fruits (Wu et al., 2013). Therefore, it is surprising that we found Daphniphyllum alkaloids in roots and root exudates, but not so much in leaves. One study showed that Daphniphyllum alkaloids may have insecticidal activities (Li et al., 2009), but because they are mostly studied for their medicinal properties, little is known about their exact ecological roles. The exudates and roots of *C. camphora* contained a considerable number of lauralitsine and reticuline-like alkaloids. These alkaloids, which are also mainly studied for their medicinal properties, were previously reported to be present in the roots (Custódio & Florêncio da Veiga Junior, 2014), but not in the leaves. Their ecological functions are not experimentally assessed. Considering that alkaloids in general serve as anti-herbivore defences (Mithofer & Boland, 2012), it is likely that the alkaloids in *D. oldhamii* and *C. camphora* roots and exudates serve as defences against root feeders and pathogens. Further experiments, preferably under controlled conditions, are needed to falsify this hypothesis. Prenol lipids made up a substantial fraction of annotated features in the exudates, roots and leaves of *C. camphora* and *S. superba*. This class contains molecules consisting of one or several isoprene (C5) units (Fahy et al., 2005). It includes mono- (C10) and sesquiterpenoids (C15), which are known for their roles in direct and indirect defence against herbivores and pathogens. In addition, also carotene (C40), which plays a role in photosynthesis (Fahy et al., 2005) and saponines which have been shown to modulate soil microbial communities (Fujimatsu et al., 2020), belong to this group. The roots of *Cy. glauca* contained tannins as a major class. Tannins are typical for the oak family, including *Cy. glauca* (Wakamatsu et al., 2020), which are known as defences to a broad range of herbivores (Barbehenn & Constabel, 2011). Our metabolomic analyses of these four chemically poorly described tree species are a starting point for testing their chemical ecology in more detail.

Root exudate and root metabolomes showed a high overlap in the number of features detected as well as in the chemical classes that could be annotated (Figure 2). In particular, *D. oldhamii* and *S. superba* exhibited a high similarity in root exudate and root metabolites. On the other hand, *C. camphora* showed a high overlap in the number of features, but the composition of root exudates and roots is very different. This might be due to the different ways in which metabolites are exuded by plants (Oburger & Jones, 2018) or the interspecific interactions with trees in the local neighbourhood (Xia et al., 2016). The metabolomes of root exudates shared also a number of features with the leaf metabolomes, which was not reflected in the composition of the annotated metabolomes. The shared features might belong to the group that could not be annotated and therefore makes interpretation more difficult. Leaf metabolites could also be introduced into the soil via leachate from leaf litter and show up in our exudate samples, despite cleaning and washing of roots prior to sampling. Alternatively, these compounds might be transported or synthesized and exuded, without them accumulating in the root.

In addition to the species-specific metabolomic profiles, we also found that the metabolomes of the different species and organs responded to differences in tree diversity levels. Especially exudate and leaf metabolomes varied with plot diversity level, whereas the fine roots we sampled had rather constant profiles. The latter is in line with a recent study, showing that the glucosinolate profiles of fine roots of *Brassica* spp. did not change in response to local or systemic herbivory (Tsunoda et al., 2018). Fine roots of trees have high turnover rates, and therefore trees may invest less in defending their fine roots (Bouma et al., 2001; Yanai & Eissenstat, 2002). This is supported by the fact that we found fewer organ-specific metabolites in roots than in leaves.

Other than expected, plot diversity had an overall stronger effect on exudate and leaf metabolome profiles than local diversity. Our hypothesis was based on the fact that direct neighbouring trees would have a stronger effect on our target trees than more remote trees in the plot. The BEF-China experiment was deliberately planned to have a high tree density (0.6 tree/m^2; Bruelheide et al., 2014), which means that 10 years after planting the roots may have grown sufficiently to contact also more remote trees. An effect of remote signalling, for example, via volatiles, might be another explanation. The effect on the exudate composition might be caused by signal transport from the leaves to the roots. Even in grasslands, where plants are growing in much closer proximity, the local neighbourhood also determined a small part of the variance in exudate composition (Dietz et al., 2019). In addition, trees may be connected via widespread mycorrhizal networks, thus influencing each other beyond the local neighbourhood (Courty et al., 2010). This may cause that overall plot diversity has a larger effect on root exudates than initially expected.

Only for *Cy. glauca* leaves, we found a consistent and positive effect of chemical richness with local ($\rho = 0.446$, $p = 0.048$) and plot ($\rho = 0.436$, $p = 0.054$) tree diversity. In addition, the number of exudate metabolites in *S. superba* showed a trend ($\rho = 0.346$, $p = 0.056$) of increasing with local diversity, whereas the number of metabolites in *D. oldhamii* leaves tended to decrease with plot diversity ($\rho = -0.41$, $p = 0.072$). Although such variable relationships between diversity level and chemical richness or diversity are in line with studies analysing grassland species (Ristok et al., 2019), the high variation among samples may also have limited our ability to detect general trends.

5 | CONCLUSIONS

We showed that plant diversity affects above-ground and below-ground metabolomes of four subtropical tree species. Overall, plot diversity as well as the local diversity has an impact on the metabolome of root exudates and leaves. Studies on tropical trees have linked metabolomic diversity to differences in insect community composition (Richards et al., 2015; Zu et al., 2020). Our observation that tree diversity affects leaf metabolomes and thus may affect herbivore communities on trees of the same species, but growing in plots with different diversity levels. Similarly, differences in root exudate composition may shape the microbial diversity in the rhizosphere (Haichar et al., 2008). We also showed that the response of specific metabolites to plot diversity is diverse and not strictly linear. In future studies, the results of our field analyses may be used to design experiments focussing, for example, more on single species and including additional factors like plot characteristics and species identity of the neighbouring trees with more replication. These future studies may benefit from our ecometabolomics workflow to identify chemical classes of metabolites that could be investigated with more targeted approaches.

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CONFLICT OF INTEREST

Nicole M. van Dam is an Associate Editor of *Journal of Ecology*, but took no part in the peer review and decision-making processes for this paper. The authors have no other conflicts of interest.

AUTHORS' CONTRIBUTIONS

A.W., M.L. and N.M.v.D. designed the study; M.L. sampled the exudates, root and leaf samples in the field in China, S.D. processed the samples and performed the chemical analyses. The metabolomic data were processed by A.W., S.D., A.S. and Y.P.; S.N. was responsible for data archiving and curation on MetaboLights; A.W., S.D. and N.M.v.D. took the lead in writing the paper with contributions of Y.P., A.S., M.L., X.X. and S.N.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/1365-2745.13777.

DATA AVAILABILITY STATEMENT

LC-MS data can be accessed at the MetaboLights data repository for metabolomics experiments (Haug et al., 2020) as MTBLS1968 (https://www.ebi.ac.uk/metabolights/MTBLS1968/). Sampling data and sample specific data as well as the R code for producing the sunburst plots from the ClassyFire results are available at Zenodo https://doi.org/10.5281/zenodo.5255811.

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SUPPORTING INFORMATION

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