

Metabolomic reprogramming drives the invasion success of *Anthemis cotula* L.

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Research Article

Keywords: Chemical ecology, Metabolome, Invasive species, Metabolic reprogramming, Asteraceae

Posted Date: June 6th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6715800/v1>

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Abstract

Plant invasions are increasingly recognised as mechanisms driven by biochemical adaptations, yet the role of metabolomic reprogramming facilitating invasion success remains underexplored. To investigate this, we cultivated *Anthemis cotula* from seeds collected across its native Mediterranean and non-native Himalayan ranges under controlled conditions and compared their growth traits and metabolomic profiles. While growth parameters showed no significant differences ($p > 0.05$), non-native plants exhibited higher metabolite richness, particularly in root exudates. Untargeted metabolomic profiling detected 14,224 metabolites in non-native and 13,066 in native plants. Leaves, flowers, and roots shared most metabolites with similar chemical diversity (richness, inverse Simpson, Shannon, and Pielou's evenness indices; all $p > 0.5$), clustering closely in PCA and NMDS analyses. Root exudates, however, showed the strongest biogeographic divergence (PERMANOVA, $p = 0.008$), with non-native plants producing unique compounds and native exudates exhibiting greater chemical evenness (Shannon, $p = 0.036$). Annotated metabolites were largely tissue-conserved, while unannotated metabolites showed pronounced geographic divergence. Non-native plants maintained ancestral above-ground chemistry but displayed significant divergence below ground, reflecting an adaptive shift in rhizosphere interactions. Molecular networking revealed denser shikimate–flavonoid clusters in non-native plants, with leaves and flowers rich in flavonoids and terpenoids, and roots and exudates featuring unique alkaloids, terpenoids, and shikimate-derived compounds. Hill diversity profiling showed non-native plants favoured rare metabolites, while native plants prioritized dominant, evenly distributed compounds.

This dual strategy-conserving above-ground metabolism while diversifying below-ground chemistry, without phenotypic shifts, indicates *A. cotula* remodels key metabolomic modules for invasion success. Our study offers new insights into invasion biology and identifies promising biochemical markers for predicting invasion potential.

Introduction

Plant invasions represent one of the most pressing threats to global biodiversity, with non-native species frequently outcompeting native flora through superior resource acquisition and novel defence strategies (Searcy et al., 2023). The ecological success of invasive plants stems from complex biochemical adaptations that remain incompletely understood (Adhikari et al. 2020). The Enemy Release Hypothesis (ERH) posits that invasive plants experience reduced pressure from natural enemies, such as herbivores, pathogens, and competitors in their introduced ranges, allowing them to allocate fewer resources to defence and more to growth and reproduction (Keane and Crawley 2002). For example, studies on the invasive *Lythrum salicaria* have shown reduced levels of certain defensive compounds in its introduced range (North America) compared to its native range (Europe), correlating with lower herbivore damage (Joshi and Tielbörger 2012). Contrasting evidence suggests that some invasive plant species develop enhanced or novel chemical defences, particularly against native competitors and herbivores that lack evolutionary exposure to the invader (Callaway and Ridenour 2004). This phenomenon, often termed the 'Novel Weapons Hypothesis (NWH)', proposes that invasive plants produce allelopathic compounds or

toxins that disrupt local ecosystems. For example, *Alliaria petiolata*, an invasive species in North America, releases glucosinolates that inhibit the growth of native plants and deter herbivores, giving it a competitive advantage (Cipollini 2016). Similarly, *Chromolaena odorata* and *Xanthium strumarium* exhibit greater phytochemical diversity in their invasive ranges compared to their native ones (Skubel et al. 2020). This paradox- whether invasive plants succeed by reducing their defences (ERH) or evolve novel chemical strategies (NWH) highlights critical gaps in our understanding of how biochemical plasticity contributes to invasion success. Invasive plants may shift resource allocation based on local conditions, such as soil nutrient availability, climate, and biotic interactions (Pyšek et al. 2012; Gaertner et al. 2014). Recent metabolomic studies reveal that invasive plants frequently undergo chemical profile shifts when establishing in non-native ecosystems (Akbar et al. 2024). For example, *Centaurea stoebe* produces root exudates that disrupt soil microbiomes to suppress native plants (Thorpe and Callaway 2011), while other species reallocate resources from defence to growth-promoting compounds (Macel et al. 2014). However, three key limitations persist in this field: (1) most studies compare different species rather than native/non-native populations of the same species, (2) few examine whole-plant metabolic responses across multiple tissue types, and (3) the functional consequences of observed chemical differences remain speculative. These knowledge gaps hinder our ability to predict invasion outcomes and develop targeted management strategies.

We used *Anthemis cotula*, an ideal model plant, to address these challenges. Native to Mediterranean Europe but invasive across the Pacific Northwest (PNW) USA, and Kashmir (India), (Adhikari et al. 2020), this ruderal Asteraceae species produces diverse secondary metabolites, including terpenes and flavonoids (Nissar et al. 2025), that may underlie its competitive dominance. Preliminary evidence suggests that its non-native populations exhibit altered herbivore resistance (Reshi et al. 2012), but no study has systematically compared their metabolomes or linked chemical differences to invasion mechanisms. Without understanding how biochemical traits change during range expansion, we cannot fully explain why some plants become invasive while others do not.

In the present study, we employed controlled growth chamber experiments and advanced metabolomic profiling to: (1) quantify differences in secondary metabolites between native and non-native *A. cotula* roots, leaves, flowers, and root exudates; (2) identify tissue-specific metabolic strategies associated with invasion success; and (3) evaluate whether chemical profiles support the Enemy Release or Novel Weapon hypotheses. By integrating these findings with existing ecological data, we provide a mechanistic framework for understanding how biochemical plasticity facilitates the spread of *A. cotula*. This work advances fundamental knowledge in plant invasion ecology while offering practical insights for managing one of the world's most problematic agricultural weeds.

Materials and methods

Achene (seed) collection

Achenes (hereafter seeds) of *Anthemis cotula* were collected from its native Mediterranean range (Hungary) and its introduced Himalayan range (Kashmir, India). We made efforts to obtain seeds from multiple locations across the native range; however, successful collection was only possible from Hungary. Seeds were collected from multiple plants per population to ensure genetic representation, with all material stored in breathable paper bags at 4°C until experimentation.

Growth chamber experiment

A controlled growth chamber experiment was set up to eliminate environmental confounding factors. Seeds were surface sterilized with 1% sodium hypochlorite for 5 min, followed by triple rinsing with autoclaved MilliQ water. Germination was initiated on moistened Whatman filter paper grade 1 in Petri dishes under optimized conditions (16h light/8h dark at 25°C). After 7 days, uniform-sized seedlings were transplanted into 200 mL pots containing a sterilized cocopeat: soil mix in a 1:1 ratio, with 15 replicates per origin group arranged in a complete randomized block design. Plants were maintained under consistent environmental conditions (photoperiod, temperature, humidity) with weekly pot rotation until flowering.

Growth parameter measurements

At the reproductive stage, we measured five key traits: (1) root length (base-to-root tip), (2) shoot length, (3) root biomass (oven-dried at 60°C), (4) shoot biomass, and (5) floral output (capitulum counts). Immediately after measurement, root, leaf, and flower tissues were flash-frozen in liquid nitrogen and stored at -80°C to preserve metabolic integrity for subsequent analysis.

Metabolite extraction

Frozen tissues (roots, leaves, and flowers) were ground to a fine powder under liquid nitrogen using a ceramic mortar and pestle. For each sample, 200 mg of powdered tissue was extracted with 1 mL of 70% methanol containing 200 ng of formononetin as an internal standard. The extraction followed a stepwise protocol: vortexing for 2 minutes, incubation at room temperature for 10 minutes, followed by centrifugation at 5,000 rpm for 5 minutes. The supernatant was then subjected to a second centrifugation at 15,000 rpm for 10 minutes. Samples were cryo-cleared at -80°C for 1–2 hours and centrifuged once more at 4°C for 20 minutes. Finally, 600 µL of the clear supernatant was transferred to LC-MS vials and stored at -20°C until analysis.

For metabolic profiling of root exudates without interference from soil metabolites, we used a hydroponic collection system to profile root exudates. After carefully washing and cleaning the roots of plants (3 native, 5 non-native) for any adhering particles, *A. cotula* plants were transferred to 500 mL containers with half-strength Hoagland solution. Light exclusion was maintained with aluminium foil wrapping during the 14-day collection period. Hoagland solution was replenished every 24 hours. The collected exudates were lyophilized (Labconco, -84°C), then reconstituted in 1 mL 70% methanol containing internal standard, filtered (0.45 µm nylon), and stored at -80°C until analysis.

LC-QTOF conditions for metabolite detection

We utilized a Sciex UPLC system equipped with a binary solvent and sample manager and coupled with a X500R Q-TOF mass spectrometer with an electron spray ionization (ESI) interface for metabolite characterisation. Chromatographic separation was achieved on a Gemini C18 column (5 μ m, 110A, 50 X 30mm) by applying a linear gradient of H₂O (A)-methanol(B), both containing 0.1% (v/v) formic acid. The gradient started with 5% methanol, rising to 95% methanol by 13.0 minutes, and returning to 5% methanol by 16.0 minutes, at a flow rate of 0.5L/min. The injection volume was 20 μ L, and the column oven temperature was maintained at 40°C. The mass spectrometer was operated in positive ionization mode using information-dependent acquisition (IDA). Source gas parameters were set as follows: ion source gas 1 at 50 psi, ion source gas 2 at 55 psi, and curtain gas at 40 psi. The collision-activated dissociation (CAD) gas pressure was maintained at 7 psi, and the ion source temperature was set to 400°C. The spray voltage was set to 5500V. For TOF MS scans, the mass range was set from 100 Da to 1000 Da, with an accumulation time of 0.25 seconds per spectrum. The Declustering Potential (DP) was set to 60 V, with a DP spread of 5 V. The Collision Energy (CE) was set to 25 eV, and the CE spread was 10 eV. In the TOF MS/MS mode, the mass range was extended from 50 Da to 1000 Da, with an accumulation time of 0.1 seconds per spectrum. The Declustering Potential (DP) was maintained at 60 V, with the Collision Energy (CE) at 25 eV and a CE spread of 10 eV.

Metabolite annotation

The raw LC-MS data files obtained were processed using MS-DIAL version 5.3.240617 for metabolite profiling and visualization (Tsugawa et al., 2015). The raw files were imported into MS-DIAL, and the profile data type was selected for both MS1 and MS/MS. Soft ionization was selected to suit the experimental conditions. Peak detection was performed using a minimum peak height threshold of 1000 amplitude, with a mass slice width set to 0.1 Da. The peaks were deconvoluted using the default sigma window value of 0.5, allowing for accurate separation of co-eluting compounds in complex samples. The MS/MS abundance cutoff was set to 10 amplitudes to filter out low-intensity signals, thereby enhancing the reliability of metabolite identification. Peak alignment across different samples was conducted with a retention time tolerance of 0.015 Da. The alignment was corrected for retention time drift and ensured accurate comparison between samples. Metabolite identification was done using MS-DIAL *in silico* MS/MS positive ion library with 326,575 records for positive mode. The identification focused on the [M + H⁺] adduct for positive mode, with MS1 tolerance set at 0.01 Da and MS₂ tolerance set at 0.025 Da. The reference matches, MS₂-acquired data, and blank filter were selected in the peak spot navigator window. The deconvoluted peak lists from each sample replicate were exported as text files for further analysis in R. We categorized metabolites into two distinct groups: reference-matched metabolites and unannotated (unidentified) metabolites that failed library matching. For reference-matched metabolites, we performed rigorous LC-MS spectral matching against the MSMS_Public_ExpBioInsilico_Pos_VS17 library using stringent thresholds (± 0.01 Da m/z tolerance, ± 0.1 min retention time window). Technical replicates were averaged to generate robust abundance values, followed by row-wise normalization to relative abundances and log-transformation to account for compositional effects.

Classification of unannotated metabolites

The unidentified metabolite fraction (unannotated metabolites) comprised features that failed library matching but passed quality filters (MS1 amplitude > 1000, presence in ≥ 3 replicates). These were consolidated into distinct molecular entities through peak binning (± 0.01 Da m/z , ± 0.2 min RT), retaining only the most intense peak per bin to avoid redundancy. This dual approach ensured comprehensive coverage of both characterized and novel metabolites in subsequent analyses. Blank filtration was performed by identifying and removing metabolites common in both blank and sample groups to eliminate potential contaminants. The datasets were further refined by retaining only the $[M + H]^+$ adducts to ensure consistency in ion mode analysis. To resolve duplicate metabolite entries, peaks were binned using a mass-to-charge ratio (m/z) tolerance of ± 0.01 Da and a retention time (RT) tolerance of ± 0.2 minutes, with the most representative peak retained per bin.

Classification of reference-matched metabolites

Metabolite identification was performed by matching experimental mass spectra against the reference library (MSMS_Public_ExpBioInsilico_Pos_VS17) using stringent criteria (m/z tolerance ± 0.01 Da). Technical replicates were averaged to obtain representative values for each metabolite-organ-range combination. Data were normalized to relative abundances (row-wise) and log-transformed wherever appropriate to account for compositional effects. Data preprocessing included the removal of contaminants detected in blank samples and the normalization of peak areas to relative abundances. These reference-matched metabolites were annotated with SMILES and InChIKeys using PubChem.

Data analysis and visualisation

To comprehensively compare metabolite profiles across plant organs and root exudates across native and non-native samples, we employed a multi-tiered analytical pipeline combining classical and modern ecological, chemical, and statistical approaches. To explore the overlap and uniqueness of metabolites among native and non-native plant samples, we generated UpSet plots using the Complex Heatmap package, providing a clear visualization of shared and distinct metabolites across conditions.

To assess chemical diversity, we calculated both conventional (richness, Shannon, Simpson, and Pielou's evenness) and Hill number-based diversity indices ($q = 0$ to $q = 3$) using the vegan and chemodiv packages. These indices allowed for robust quantification of alpha diversity (within-group diversity) with varying sensitivity to rare and abundant metabolites. Statistical comparisons between groups were performed using t-tests and Wilcoxon rank-sum tests, wherever appropriate. To evaluate beta diversity (between-group dissimilarity), we computed pairwise dissimilarities using PubChem fingerprints with the compDis() function from the chemodiv package, followed by Generalized UniFrac distance calculations. Diversity profiles were visualized with calcDivProf() to examine how diversity scaled with sensitivity parameters (q-values), capturing subtle compositional differences between sample groups.

Multivariate analyses were employed to reveal compositional patterns in metabolite profiles. Principal Component Analysis (PCA) was performed on Hellinger-transformed data to reduce dimensionality and visualize clustering among sample types. We also used Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis dissimilarity, and Principal Coordinates Analysis (PCoA) based on UniFrac distances, to capture compositional variation in a non-linear ordination space. To statistically validate group differences in multivariate space, PERMANOVA and ANOSIM (999 permutations) were performed.

Molecular network analysis

Chemical similarity networks were constructed to visualize structural relationships between metabolites. PubChem fingerprints were used to calculate pairwise similarity scores among compounds, with edges retained only for scores above 0.75. Networks were visualized using the Kamada-Kawai force-directed layout, implemented via the igraph package. Nodes were annotated by compound class using the NPClassifier taxonomy and coloured by sample type (organ, soil, range), with node size reflecting relative abundance and edge opacity indicating structural similarity. We created two levels of networks: (1) organ-specific subnetworks (leaf, flower, root, exudate), and (2) comparisons between native vs. non-native samples. Network robustness was evaluated using (a) sensitivity analysis by varying similarity thresholds (± 0.05), (b) permutation tests to assess topological deviation from random networks, and (c) subsampling validation (80% samples, 10 iterations) to ensure consistency of patterns.

Results

Growth performance

The individual plants grown from native and non-native seeds of *A. cotula* exhibited remarkably similar morphological characteristics and growth patterns. Detailed measurements across five key parameters, namely root length ($p = 0.44$), shoot length ($p = 1$), root biomass ($p = 0.7$), shoot biomass ($p = 0.7$), and capitula production ($p = 0.92$) revealed no statistically significant difference (based on non-parametric Wilcoxon test) between the two groups (Figs. 1–3).

Metabolite profiles

Our untargeted metabolomics approach detected a total of 14,224 molecular features in non-native plants compared to 13,066 in native plants, with 6,263 compounds shared between both groups (Fig. 4) (Table S1). This represents an 8.86% increase in unique metabolites in the non-native individuals (7,961 vs 6,803), particularly pronounced in below-ground tissues. Root exudates showed the most dramatic divergence, with non-native plants producing 2,497 metabolites compared to 1,991 in natives - a 25% increase in metabolic richness (Fig. 5). Similar patterns emerged in root tissues (3,710 vs 3,380 metabolites) and flowers (5,499 vs 4,951), while leaves maintained near-identical profiles (5,384 vs 5,407) (Fig. 5).

Diversity of unannotated metabolites

A comparative analysis of metabolite richness and diversity across different plant organs (root, leaf, and flower) and root exudates from native and non-native *A. cotula* plant samples showed largely similar profiles (Fig. S1). No significant differences were observed in metabolite richness in plant organs or root exudate ($p > 0.05$). Minor variations were observed, such as slightly higher median richness in native flowers and non-native roots; these differences were insignificant. Inverse Simpson diversity index, Shannon diversity index, and Pielou's evenness index were also consistent across flowers, leaves, and roots between these native and non-native groups (Fig. S2-S4). However, significant differences were observed only in root exudates, where native plants showed a significantly higher diversity ($p = 0.0357$), indicating greater diversity and more equitable distribution of metabolites.

Hill diversity of unannotated metabolites

The comparison of Hill diversity and evenness between native and non-native groups showed no significant differences across all diversity orders and indices (Fig. S5). For Hill diversity at $q = 1$, which accounts for both richness and evenness, ($t(34.06) = 0.41$, $p = 0.68$), no differences were found between the samples of native and introduced ranges, similarly at $q = 2$ (emphasising dominant metabolites), ($t(32.88) = 0.4$, $p = 0.69$), and for Hill evenness, ($t(34.37) = 1.12$, $p = 0.27$) no significant differences were found between these two groups.

Multivariate analysis of metabolite composition

Principal Component Analysis (PCA) (Fig. 6) showed a clear separation of the metabolic profile of different plant organs (flower, leaf, root) and root exudates of native and non-native samples *A. cotula*, indicating substantial metabolomic differences among these sample types. The strongest differentiation was observed in root exudates, where native and non-native plants formed well-separated clusters, suggesting significant differences in their secreted metabolite profiles (Table S2).

NMDS showed that flowers and leaves grouped closely together, suggesting their metabolite composition remained relatively unchanged between native and non-native plants (Fig. 7). However, roots showed some degree of separation, with native and non-native groups forming slightly distinct clusters, suggesting metabolite differentiation was likely influenced by environmental factors. The most significant difference was observed in root exudates, where samples from non-native plants formed a distinct cluster that was widely separated from samples of native plants (PERMANOVA $p = 0.001$) (Table 1). This indicated a significant change in metabolite composition in root exudates. These results suggest that invasion-associated metabolic changes are most pronounced in root-secreted compounds, potentially influencing rhizosphere interactions.

Table 1
Results of PERMANOVA based on Bray-Curtis dissimilarity, assessing differences in metabolite composition among native and non-native *A. cotula* plants. pseudo-F value, R², and p-values indicate the significance and proportion of variation explained by groupings.

Term	Df	Sum of Sqs	R ²	F value	P value
Model	7	10.5048	0.72401	11.992	0.001
Residual	32	4.0044	0.27599		
Total	39	14.5093	1.00000		

Richness of identified (library-matched) metabolites

A total of 273 annotated metabolites were identified in both native and non-native *A. cotula* plants (Figs. 8–10). Among these, 141 metabolites were identified in non-native and 132 in native *A. cotula* plants (Fig. 8). Both groups shared 117 metabolites, while 24 were unique to non-native and 15 to native plants. The highest metabolite counts were observed in flowers (non-native: 98, native: 95), followed by leaves (non-native: 82, native: 72) and roots (non-native: 45, native: 41). Root exudates showed the lowest numbers, with non-native and native samples containing 9 and 8 metabolites, respectively (Fig. 9). Flavonoids were the dominant class in flowers and leaves, with a slightly higher proportion in non-native plants (Fig. 10). Amino acids were most abundant in roots, where non-native plants had a slightly higher count. Other metabolite classes showed minimal differences between groups. Root exudates were rich in fatty acids, with amino acids and terpenes found only in non-native plants.

Diversity of identified metabolites

Richness and Hill diversity metrics (evenness, diversity, and functional diversity) showed no significant differences among these identified metabolites between native and non-native *A. cotula* plants ($p > 0.05$) (Fig. S6).

Richness measures the total number of different metabolites identified (Fig. S6). The t-test showed no significant difference between the two groups, suggesting that the overall number of distinct metabolites was similar in native and non-native plants. (T-test, $t(5.99) = -0.17$, $p = 0.87$, $n = 8$). Hill evenness reflects how evenly metabolites are distributed within each group, independent of richness. Native plants appeared to have higher evenness, but this observed difference in metabolite diversity was also insignificant (t-test, $t(3.54) = 1.55$, $p = 0.2$, $n = 8$) (Fig. S6). Hill diversity also did not show significant variation between native and non-native groups (t-test, $t(5.93) = 0.47$, $p = 0.65$, $n = 8$), suggesting similar diversity and abundance of metabolites in both plant groups (Fig. S6).

A comparison of functional Hill diversity (FuncHillDiv) between the metabolites of native and non-native *A. cotula* is presented as a boxplot (Fig. S6). The analysis revealed no significant difference in functional Hill diversity between the two groups (t-test, $t(6) = 0.21$, $p = 0.84$, $n = 8$).

Diversity profile of identified metabolites

Hill diversity profiling across orders $q = 0-3$ (Figs. 11–12) revealed scale-dependent patterns in metabolic organization. Non-native plants showed slightly higher diversity at lower diversity orders ($q = 0$), indicating greater metabolite richness. As q increased, emphasizing dominant metabolites, diversity declined in both groups and became more similar. However, at higher orders ($q \geq 1$, weighting dominant metabolites), native populations exhibited superior evenness, especially in leaves and exudates. Across plant organs, flowers had the highest and most even metabolite diversity in both groups, while root exudates showed characteristically flat, low-diversity curves. These patterns suggest that non-natives may maintain a broader metabolic repertoire while native plants more evenly distribute resources among core compounds - a trade-off that could influence ecological flexibility in novel environments.

Multivariate analysis of metabolite composition

The principal component analysis (PCA) plot (Fig. 13) illustrates the variation in metabolite composition between samples categorized by their range (native vs. non-native) and organ type (flower, leaf, root, and root exudate). Although the first two principal components (PC1 and PC2) explain a very small proportion of the total variance (0.7% each), important patterns can still be discerned. Non-native samples (depicted in cyan) form a relatively tight cluster around the origin, indicating that their metabolite profiles are more homogeneous. In contrast, native samples (in red) are more widely dispersed along the first principal component (PC1), suggesting a greater variability in metabolite composition among native populations. The 95% confidence ellipses drawn around the two groups further emphasize these differences. The ellipse for non-natives is much narrower, consistent with lower within-group variability, whereas the native samples occupy a broader metabolic space. The symbols representing different organs show that although the type of organ contributes some variation, the clustering patterns are primarily driven by the range (native vs. non-native). In other words, differences in metabolite composition are more strongly associated with biogeographical status than with organ identity in this analysis. Overall, the PCA suggests that non-native samples exhibit a more conserved metabolomic profile across organs compared to native individuals, pointing toward either adaptive streamlining of metabolism post-introduction or pre-existing selection for particular metabolomic traits that facilitate successful invasion.

The non-metric multidimensional scaling (NMDS) plot (Fig. S7) provides a complementary view of the variation in composition of identified metabolites between native and non-native samples, while preserving the rank order of dissimilarities. The stress value of 0.001 indicates an excellent two-dimensional representation of the data, suggesting that the ordination very accurately captures the underlying distance relationships. Both native and non-native samples are broadly overlapping in NMDS space, and the 95% confidence ellipses for the two groups largely coincide. This pattern suggests that there is considerable overlap in the overall metabolite composition between native and non-native individuals.

Unlike the PCA plot, the NMDS does not show a pronounced separation or clustering by range. The distribution of points representing different organs indicates that organ identity contributes to some dispersion in the dataset, but no strong organ-specific clustering is observed either. Both range (native vs. non-native) and organ type, therefore, seem to explain only a small part of the total metabolite variation.

To ensure that differences between native and non-native metabolite compositions were not simply due to differences in within-group variability, we conducted a distance-to-centroid analysis. The boxplot (Fig. 14) shows that the dispersion of samples around their group centroid was similar for native and non-native groups, with no significant difference between them ($p > 0.05$). This suggests that the observed patterns in multivariate space are not driven by differences in variability but reflect true compositional differences between native and non-native populations.

The principal coordinates analysis (PCoA) showed clear separation in metabolite composition among different organs and root exudates of native and non-native *A. cotula* (Fig. 15). Root exudates showed the most distinct divergence, with non-native samples forming a separate cluster from native ones. In contrast, roots exhibited moderate overlap, while flowers and leaves from both groups clustered closely together, suggesting minimal differences in their metabolite profiles. This was statistically confirmed through PERMANOVA analysis using Bray-Curtis distances (Table 2), which showed that sample type significantly affected metabolite composition ($p < 0.05$).

Table 2
Results of PERMANOVA based on Bray-Curtis dissimilarity, assessing differences in metabolite composition across native and non-native samples *A. cotula*. pseudo-F value, R^2 , and p-values indicate the significance and proportion of variation explained by groupings.

Term	Df	Sum of Sqs	R^2	F value	Pr (> F)
Model	4	0.7428	0.7549	2.3094	0.003
Residual	3	0.2412	0.2451		
Total	7	0.9841	1.0000		

Molecular networks of identified metabolites

Molecular network analysis revealed fundamental similarities in metabolic infrastructure with population-specific modifications (Fig. S8). Shikimate-derived flavonoids formed dense, highly interconnected clusters in both populations, though non-native plants showed a greater edge density in phenylpropanoid networks. Organ-specific patterns emerged, with flowers exhibiting tight flavonoid clustering, leaves showing hybrid terpenoid-flavonoid networks, and roots displaying dispersed alkaloid-fatty acid modules (Fig. S9). Root exudate networks were notably sparse but contained population-specific hub metabolites, potentially representing key biochemical mediators of invasion success. The

conservation of core metabolic networks alongside divergence in specialized compounds suggests a "toolkit" model of invasion biochemistry.

Discussion

The biochemical strategies underlying plant invasion have long been debated between two dominant paradigms: the Enemy Release Hypothesis, which predicts reduced defence investment in novel environments (Liu and Stiling 2006), and the Novel Weapons Hypothesis, which posits that invaders evolve new chemical arsenals (Callaway and Ridenour 2004). Our metabolomic analysis of *Anthemis cotula* reveals a more nuanced reality—one where invasive success emerges not through the wholesale abandonment or invention of biochemical strategies but through targeted modification of ancestral metabolic networks coupled with remarkable conservation of core chemical defences. This "selective remodelling" strategy, particularly evident in root exudates, challenges conventional wisdom about the metabolic costs of invasion and suggests a sophisticated biochemical recalibration that may explain the global success of *A. cotula*. The absence of morphological differentiation suggests that the invasive success of non-native populations likely stems from biochemical rather than gross phenotypic adaptations when grown in common environments (Bock et al., 2016; Gioria et al., 2023). Interestingly, while non-native plants showed slightly higher mean values for both root and shoot length, these differences were well within the range of phenotypic plasticity observed within each population.

At the heart of our findings lies a striking dichotomy between above- and below-ground metabolic adaptation. While leaves and flowers maintained near-identical metabolite profiles between native and non-native plants—differing by less than 5% in key defensive flavonoids—root exudates underwent dramatic chemical restructuring. The increase in root exudate metabolite richness in non-native populations, accompanied by the appearance of terpenoids and amino acids absent in native exudates, points to rhizosphere engineering as a central invasion strategy. This tissue-specific metabolic re-programming aligns with the studies on *Centaurea stoebe* (Thorpe and Callaway 2011) and also goes further by demonstrating that such below-ground innovation can occur without compromising above-ground defences—a critical insight that resolves previous contradictions between the Novel Weapons and Enemy Release Hypotheses. The root and root exudate metabolite profiles revealed fewer annotated metabolites and more inter-group differences. In particular, non-native root exudates contained unique compounds, supporting the hypothesis that belowground chemical variation plays a role in invasion success, as observed in other species like *Centaurea maculosa* and *Alliaria petiolata*. (Callaway and Vivanco 2006; Inderjit et al. 2021). These compounds are known to function as multifunctional agents in soil ecosystems, suppressing competitor germination through allelopathy (Li et al. 2020; Mauli et al. 2022; Effah and Clavijo McCormick 2024), recruiting beneficial microbes (Ehlers et al. 2020), and potentially disrupting signalling among native plant communities (Clavijo McCormick et al. 2023; Akbar et al. 2024). That these compounds appear only in non-native populations suggests an adaptive response to novel selection pressures in invaded ranges.

The Hill diversity profiles uncovered a fundamental reorganization of metabolic investment strategies across invasion fronts. Non-native plants maintained a broader arsenal of rare metabolites (greater richness at $q = 0$), while native populations showed more balanced investment across dominant compounds (higher evenness at $q \geq 1$). This pattern suggests invaders may adopt a "many-small-bets" strategy—producing numerous specialized metabolites at low concentrations to address diverse, unpredictable challenges in novel environments while maintaining core defences at stable levels (Skubel et al. 2020; Barrett et al. 2024). Such a strategy would be particularly advantageous during range expansion, where encounters with new herbivores, pathogens, and competitors are frequent but unpredictable.

Molecular networking revealed the architectural underpinnings of this adaptive flexibility as the increase in shikimate pathway connectivity within non-native plants, particularly through denser phenylpropanoid networks, implies reinforcement of core metabolic infrastructure ((Nakabayashi et al. 2014; Borda et al. 2022)). Such network densification may confer robustness against environmental fluctuations—a biochemical analogue to the "portfolio effect" seen in diverse ecosystems (Harborne 2013; Zhao et al. 2020; Ramarosan et al. 2022). More remarkably, the emergence of population-specific hub metabolites in root exudate networks suggests the evolution of specialized signalling compounds tailored to new ecological contexts (Zhang et al. 2019; Qu et al. 2021; Yu et al. 2022). These hubs, though few, could disproportionately influence invasion success by mediating critical interactions with soil biota—a possibility supported by the work on *Alliaria petiolata* (Inderjit et al. 2021).

Ecologically, our findings position *A. cotula* as a biochemical "dual strategist." The conservation of flavonoid defences in leaves and flowers allows it to resist generalist herbivores—a critical capacity given that invasive plants often face attacks from generalists as they establish (Borda et al. 2022). Simultaneously, the innovative root exudate chemistry enables below-ground niche construction, potentially explaining its ability to dominate diverse soils worldwide (Ens et al. 2009). This dual strategy contrasts sharply with classic invasion models that predict trade-offs between defence and competitiveness, suggesting that some invaders can circumvent such constraints through tissue-specific metabolic specialization (Heckman et al. 2019; Mesa and Dlugosch 2020).

Several caveats warrant consideration. Our growth chamber conditions, while controlling for environmental noise, may mask field-relevant biotic interactions that shape metabolite production (Gibson et al. 1999; Mitchell et al. 2006). Additionally, the functional roles of most differentially abundant metabolites remain uncharacterized—a knowledge gap that future studies should address through bioactivity assays and rhizosphere manipulation experiments. Nevertheless, this work advances invasion biology by demonstrating that successful invaders need not choose between evolutionary paradigms—they can simultaneously maintain ancestral defences and evolve novel weapons through spatial partitioning of metabolic innovation. The discovery that such sophisticated biochemical strategies can emerge without morphological changes suggests that "invisibility" at the whole-plant level may itself be an adaptive trait, allowing invaders to establish before triggering ecological resistance. Future research should explore whether this pattern holds across other invasive species, potentially revealing a new

class of "cryptic biochemical invaders" that reshape ecosystems through subtle but potent molecular innovations.

Declarations

Acknowledgements We thank Head, Department of Botany, University of Kashmir, Srinagar, J&K, India and Director, Indian Institutes of Science Education and Research (IISER), Pune, India for providing laboratory facilities.

Author contributions SN and ZAR conceived of the study; SN and SP carried metabolomic studies; ZAR analysed the data; SN wrote the manuscript; ZAR and SP revised and edited the manuscript. All authors have approved the final version of this manuscript.

Funding SN was provided Junior Research Fellowship (JRF) by the University Grants Commission, New Delhi, during the course of this study

Competing interests The authors declare that they have no conflict of interest, financial or otherwise.

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Figures

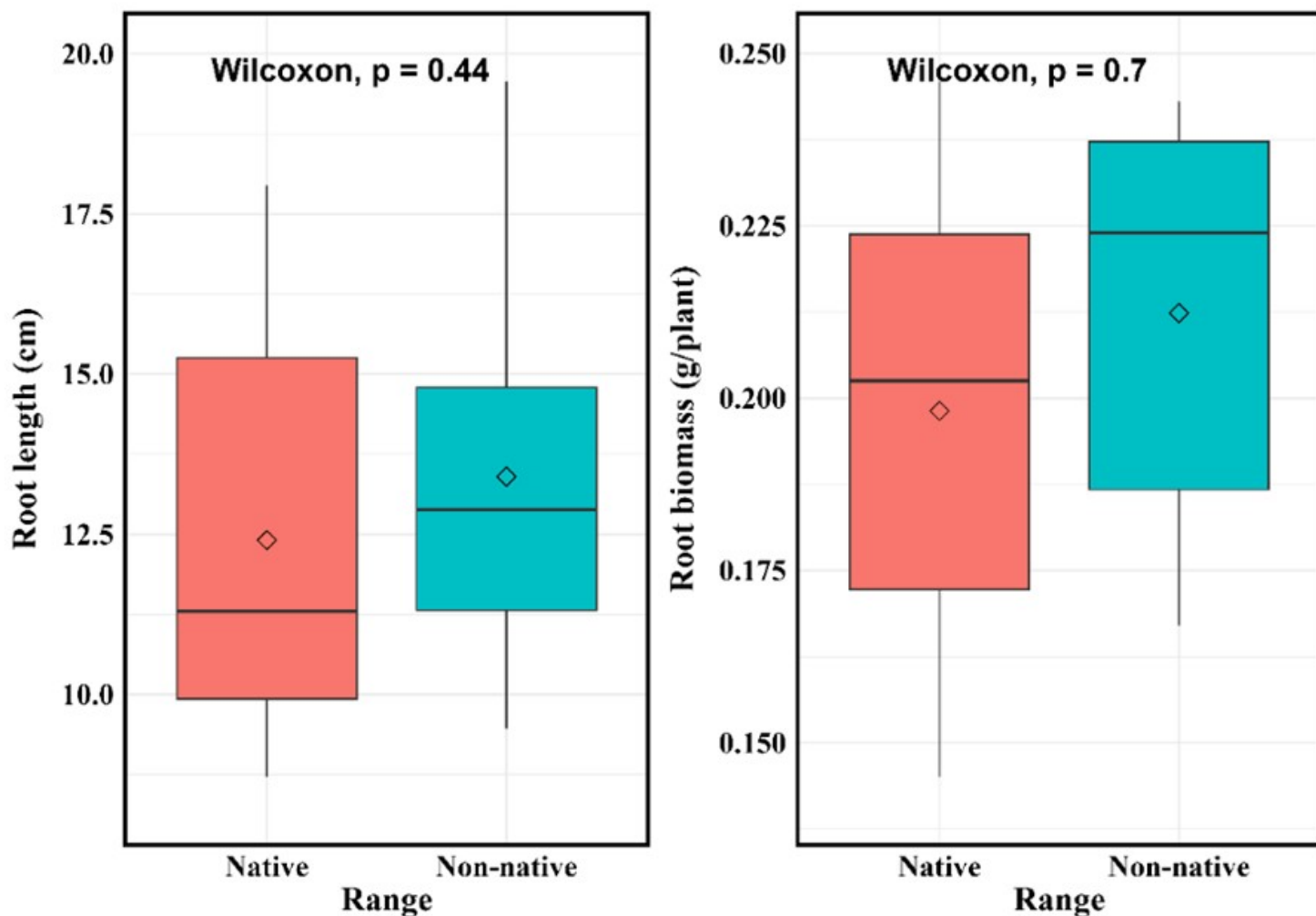


Figure 1

Box plots showing the root length and biomass comparison between native and non-native *A. cotula* plants. Statistical analysis using the Wilcoxon rank-sum test revealed no significant differences between the two groups ($p > 0.05$).

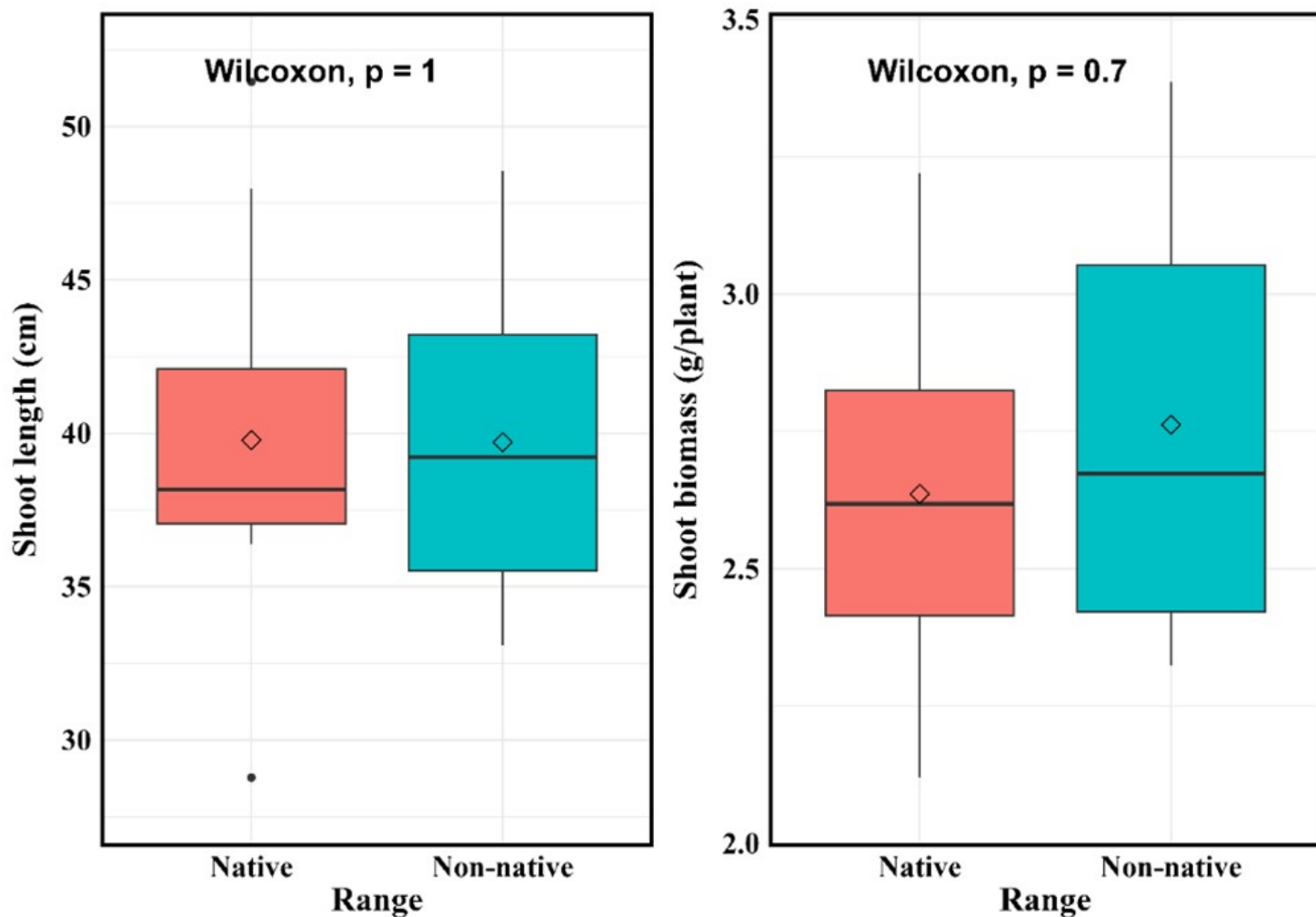


Figure 2

Box plots showing the comparison of shoot length and shoot biomass between native and non-native *A. cotula* plants. Statistical analysis using the Wilcoxon rank-sum test revealed no significant differences between the groups ($p > 0.05$)

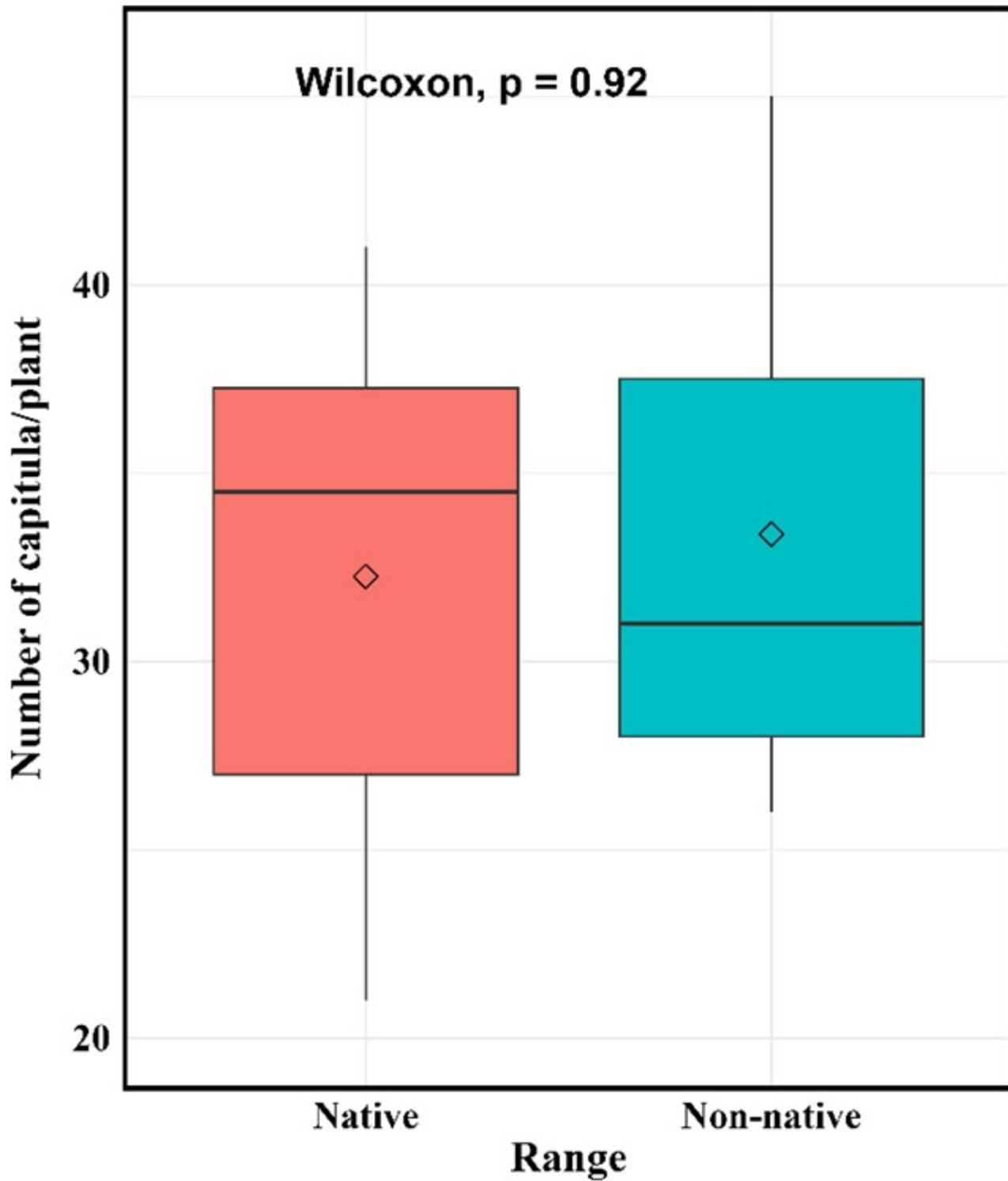


Figure 3

Box plot showing the number of capitula between native and non-native *A. cotula* plants. Statistical analysis using the Wilcoxon rank-sum test revealed no significant differences between the groups ($p > 0.05$)

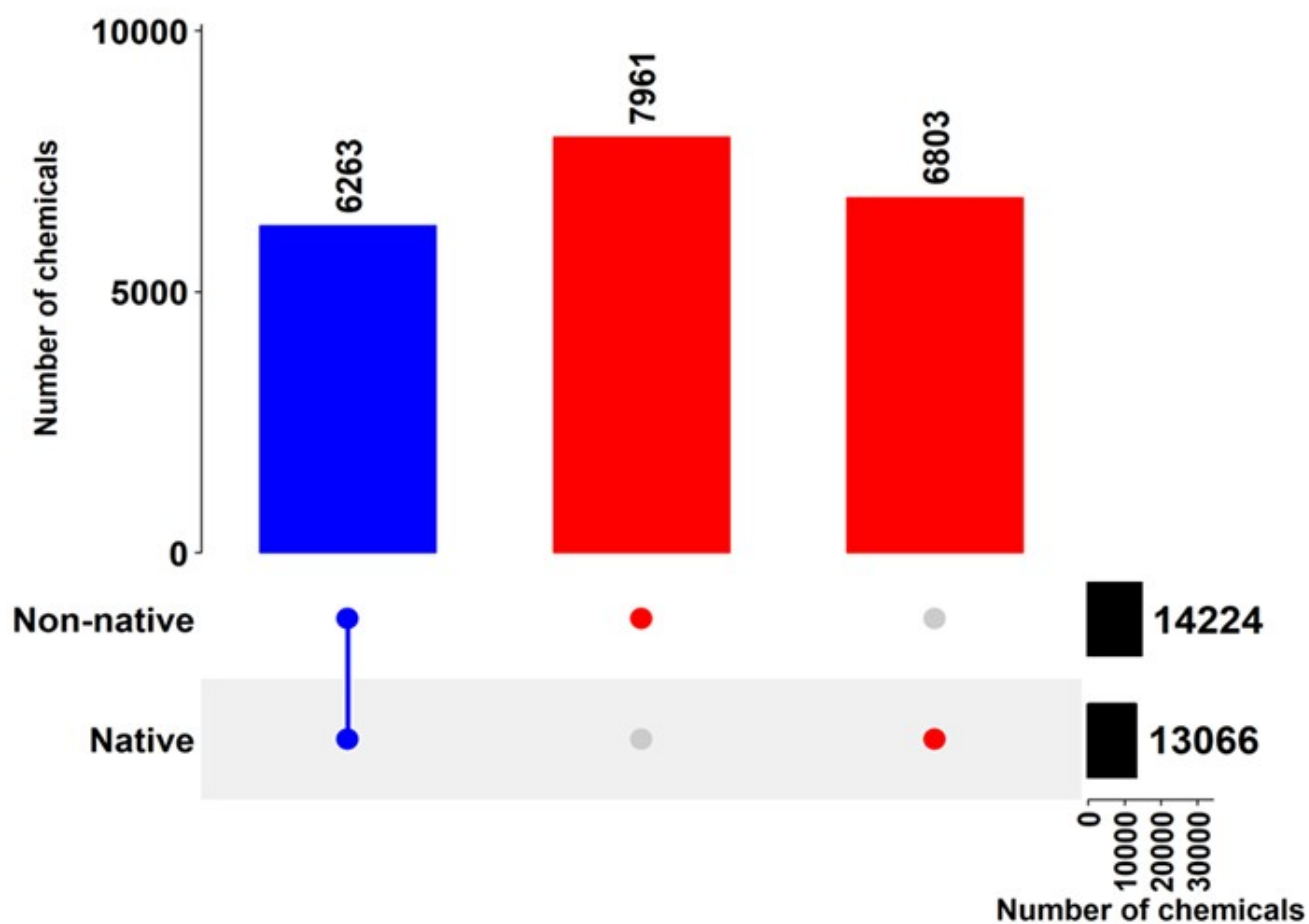


Figure 4

Upset plot showing the number of chemicals or metabolites detected (horizontal bars), shared, and unique (vertical bars) in native and non-native *A. cotula* plants

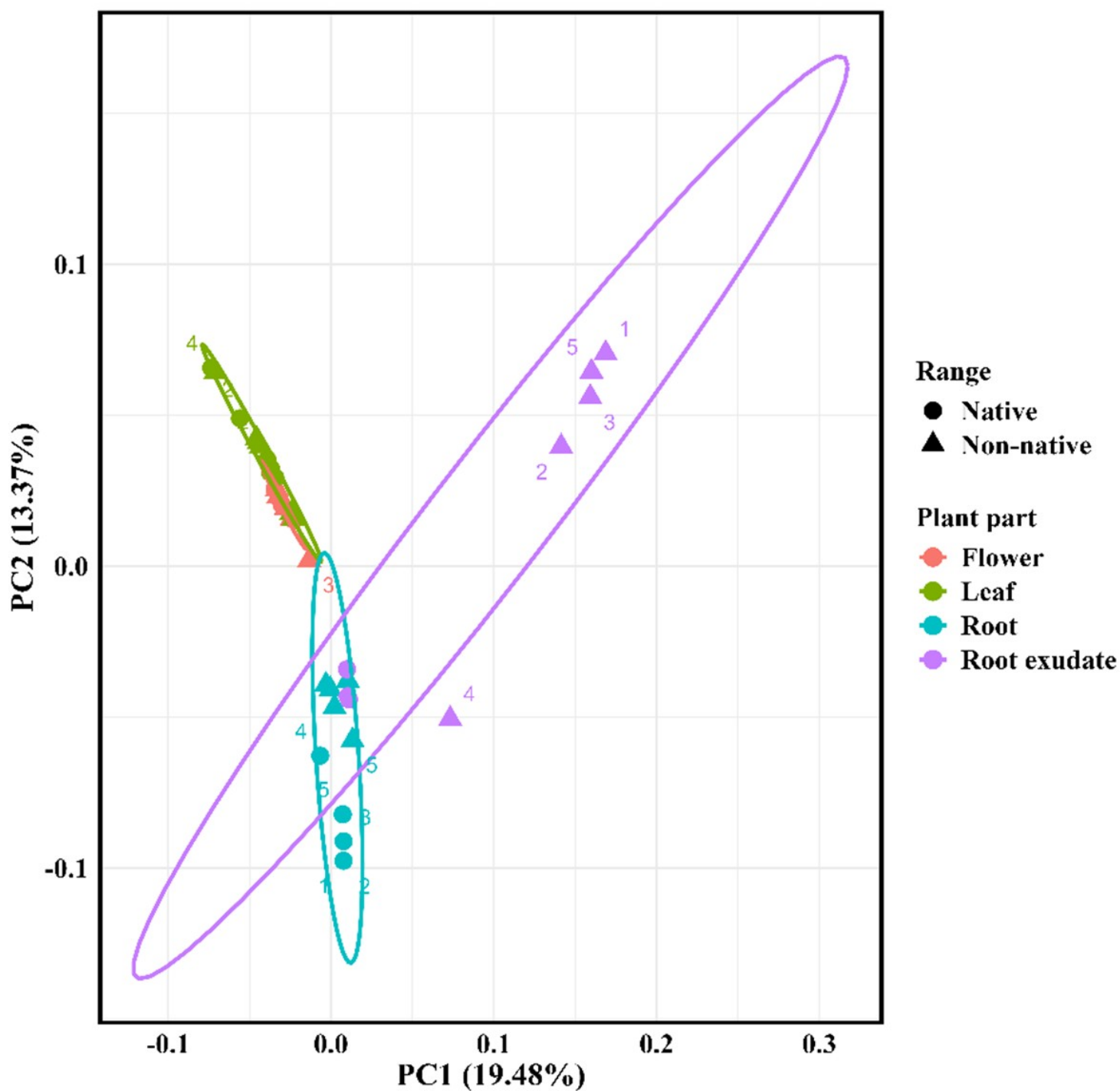


Figure 6

PCA of metabolite profiles from different plant organs and root exudates of *A. cotula*. Each point represents a sample replicate, coloured by plant parts: orange for the flower, green for the leaf, cyan for the root, and purple for root exudates. Circle shapes represent natives, and triangles represent non-natives.

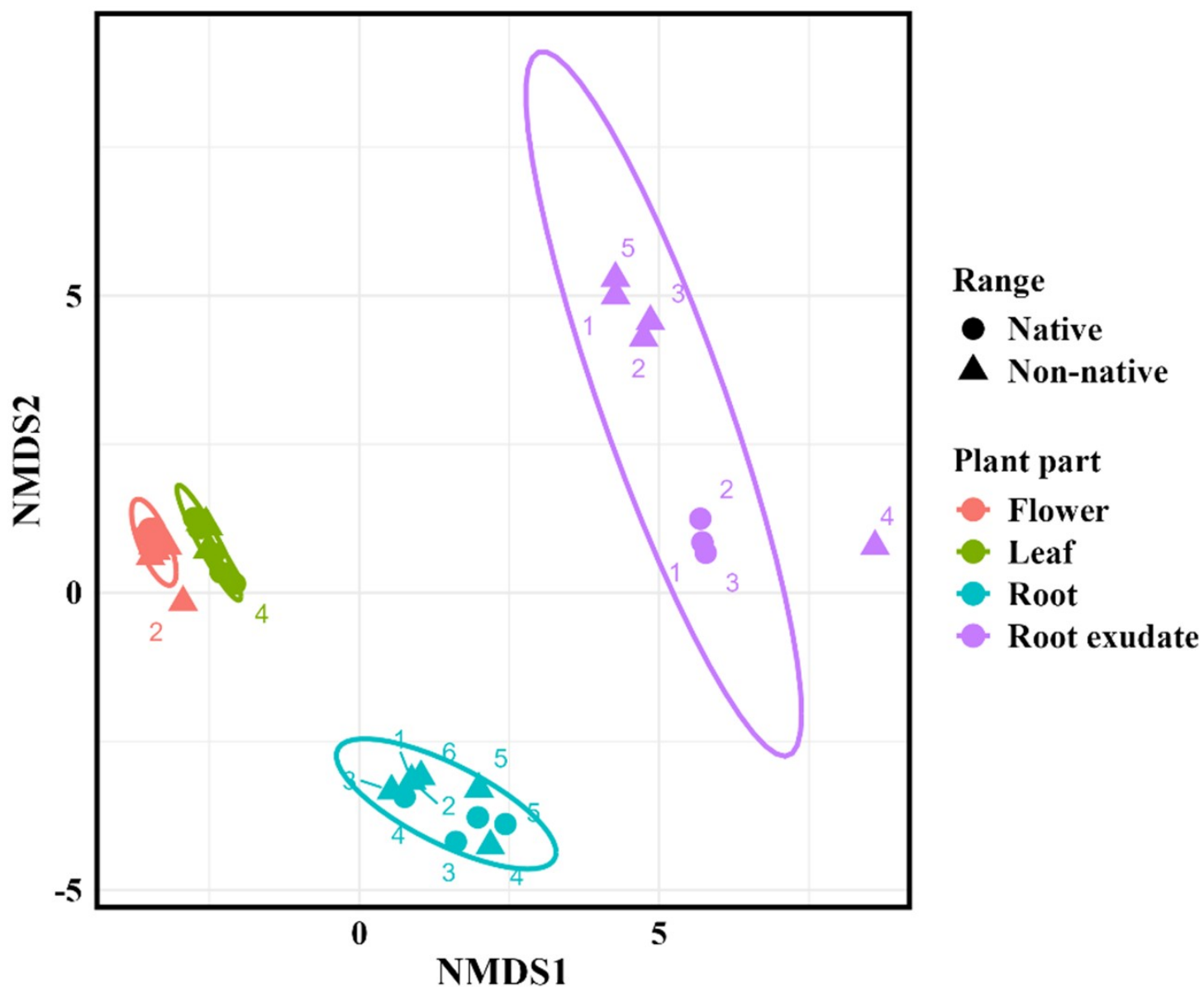


Figure 7

NMDS plot of metabolite profiles from different plant organs and root exudates of *A. cotula*. Each point represents a sample replicate, coloured by plant parts: orange for the flower, green for the leaf, cyan for the root, and purple for root exudates. Circle shapes represent natives, and triangles non-natives.

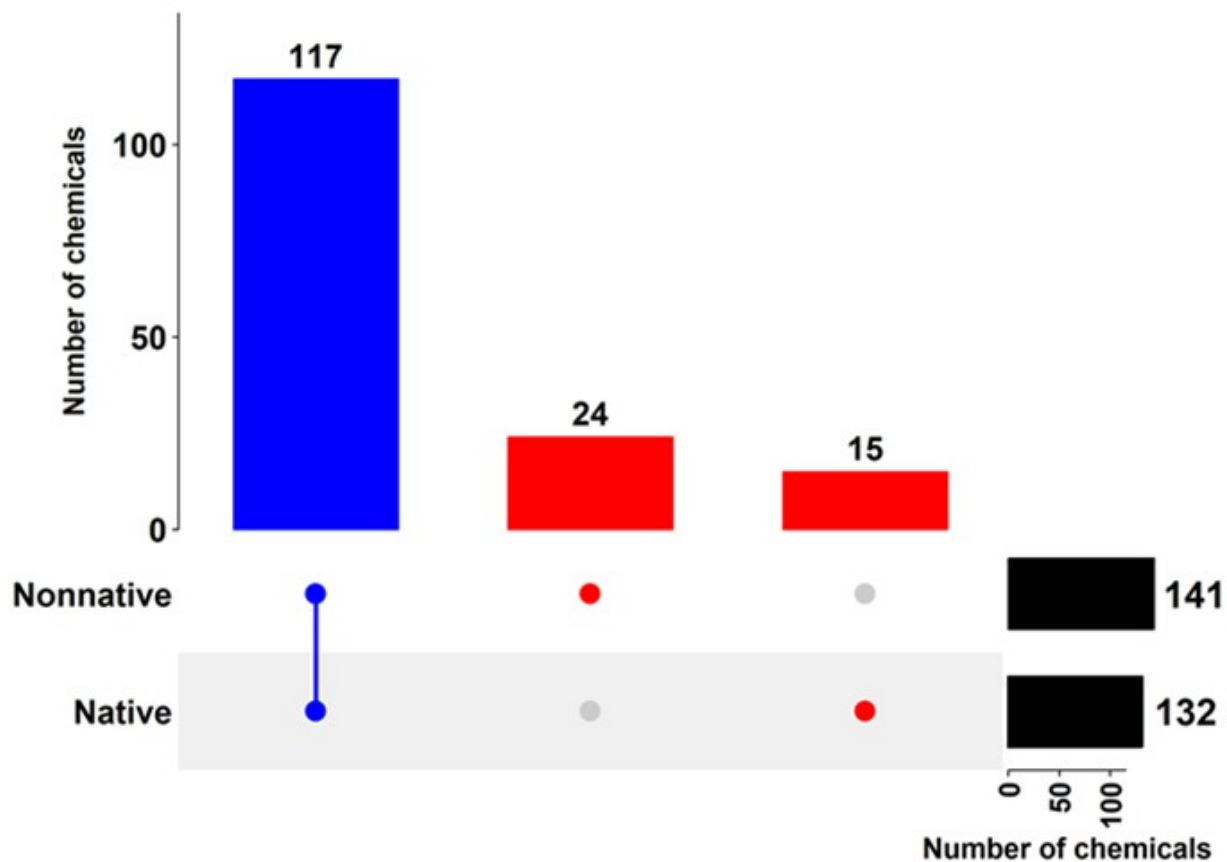


Figure 8

Upset plot with horizontal bars showing the total number of identified or annotated metabolites in native and non-native *A. cotula* plants, the Y axis shows the shared and unique metabolites between the native and non-native *A. cotula* plants.

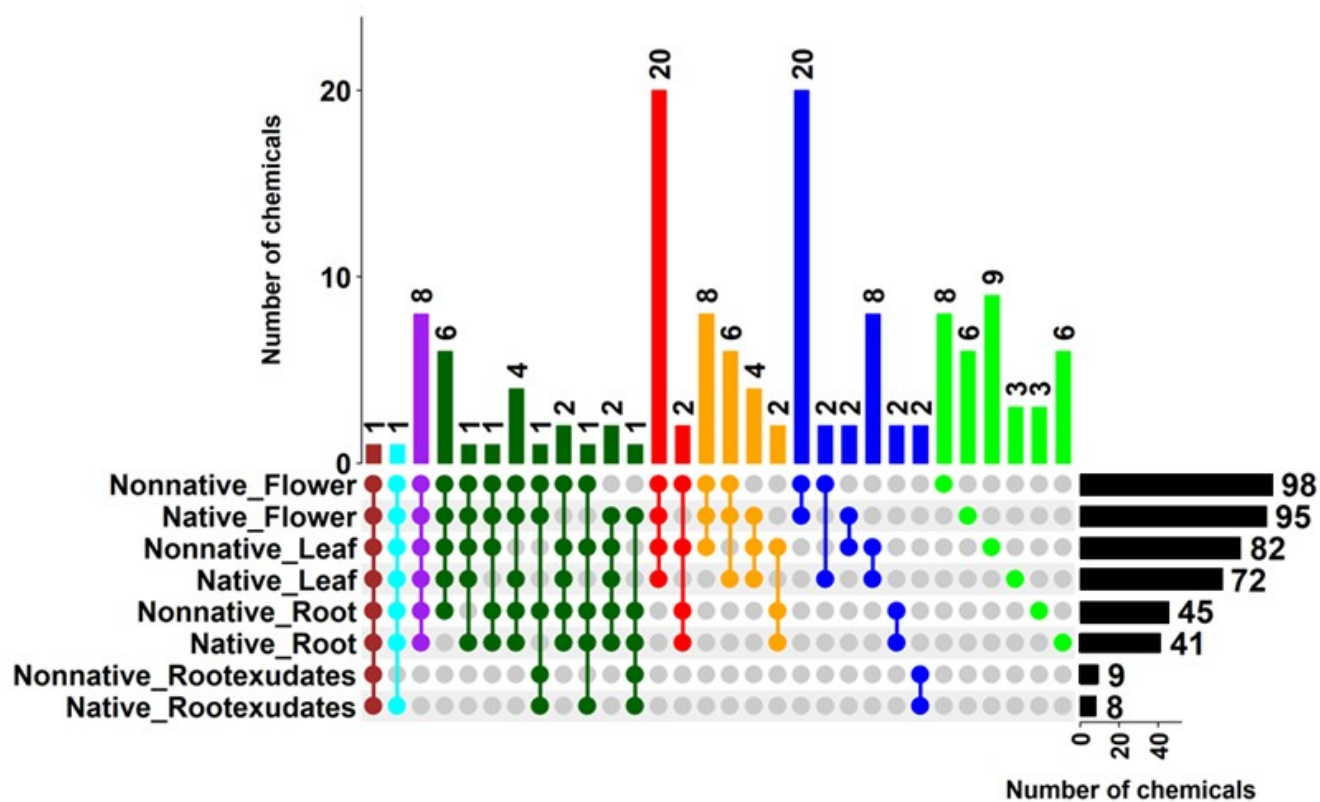


Figure 9

Upset plot showing the total number of metabolites identified (horizontal bars), shared, and unique (vertical bars) across different organs and root exudates of native and non-native samples of *A. cotula*

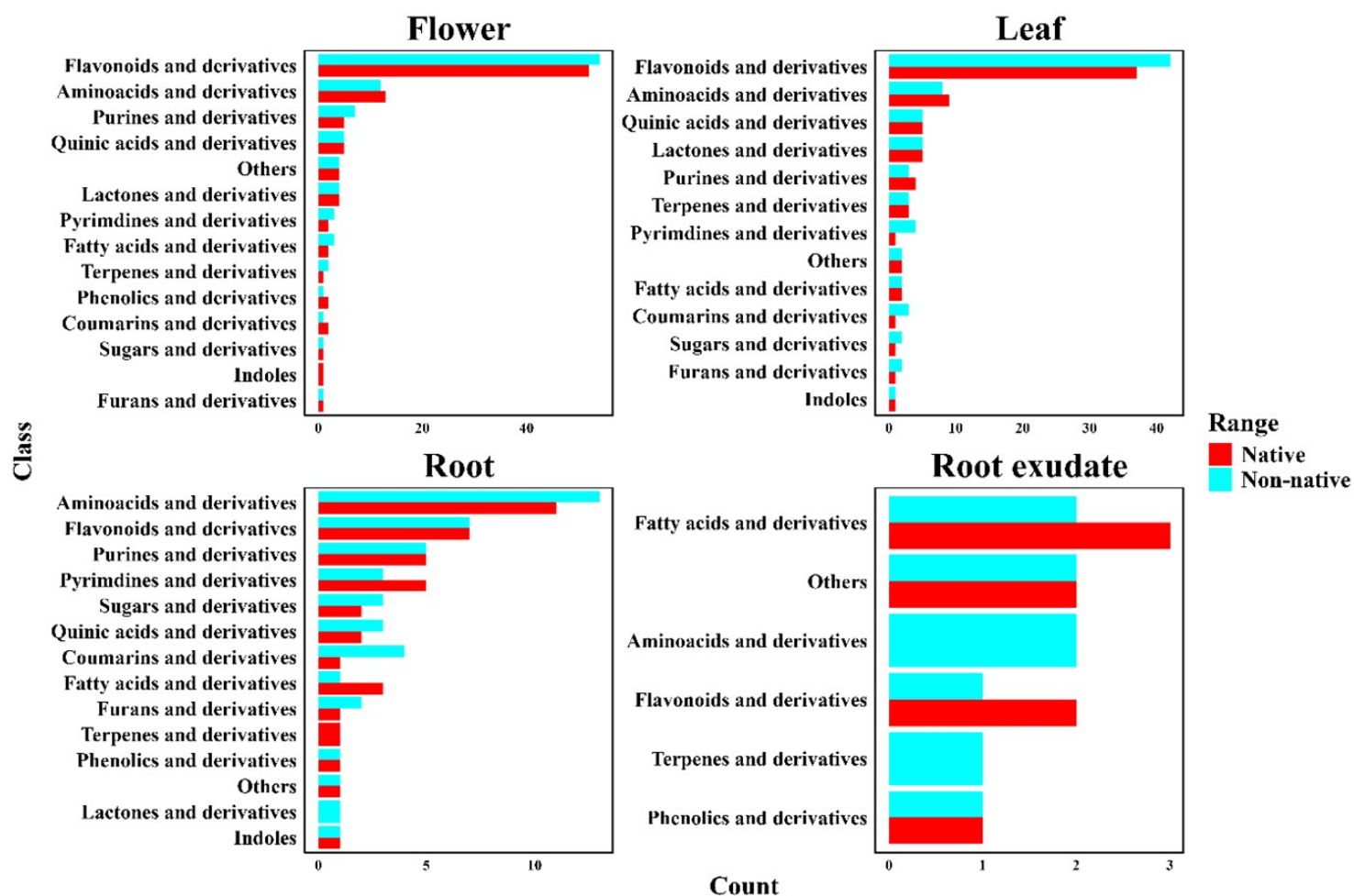


Figure 10

Bar plots showing the different classes of identified metabolites in different organs and root exudates of native and non-native samples of *A. cotula* plants

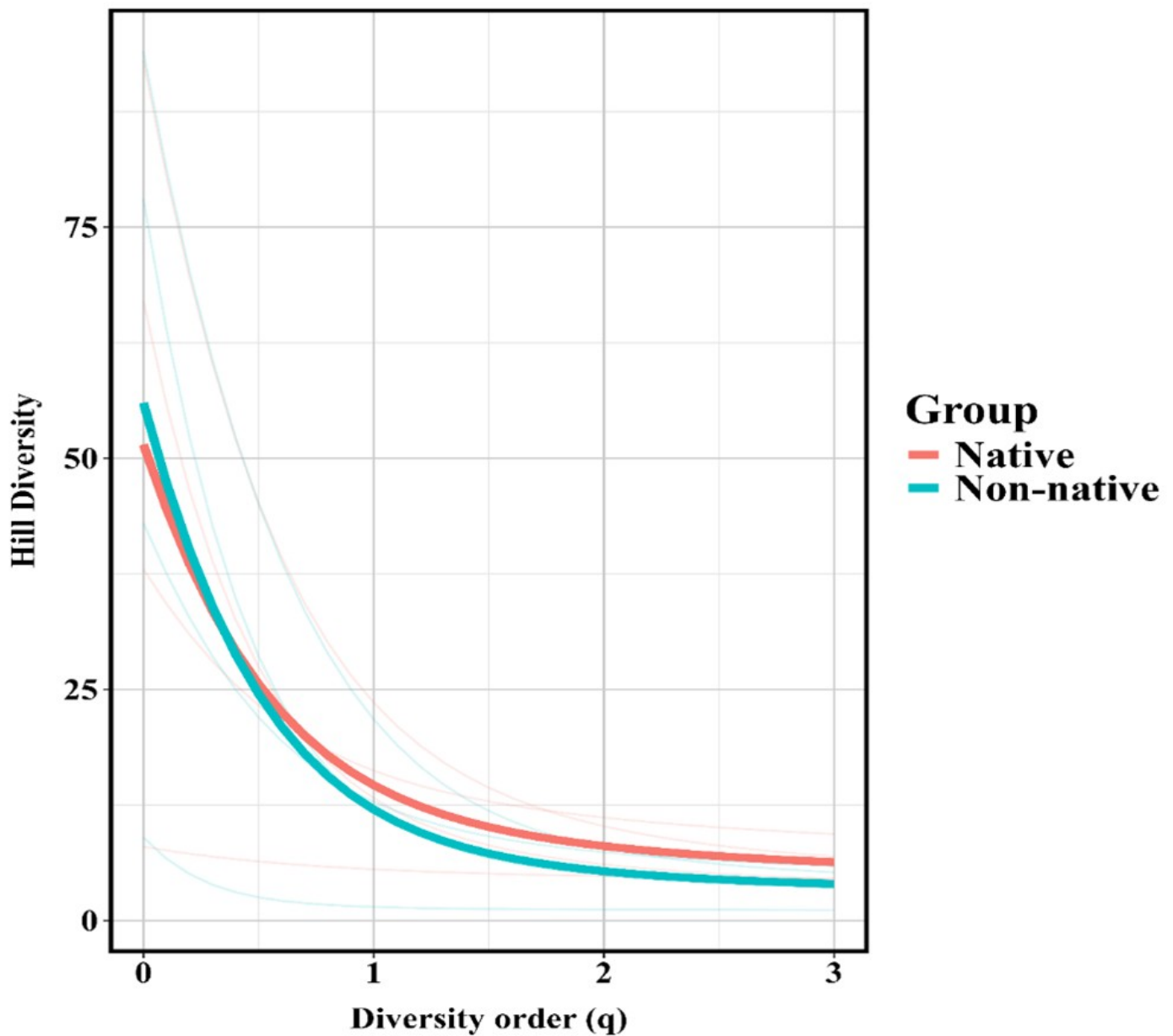


Figure 11

Comparison of Hill diversity of native and non-native *A. cotula* plants across different diversity orders (q). Thick lines represent group means, while thin lines represent individual samples. The x-axis represents the diversity order q, ranging from 0 to 3

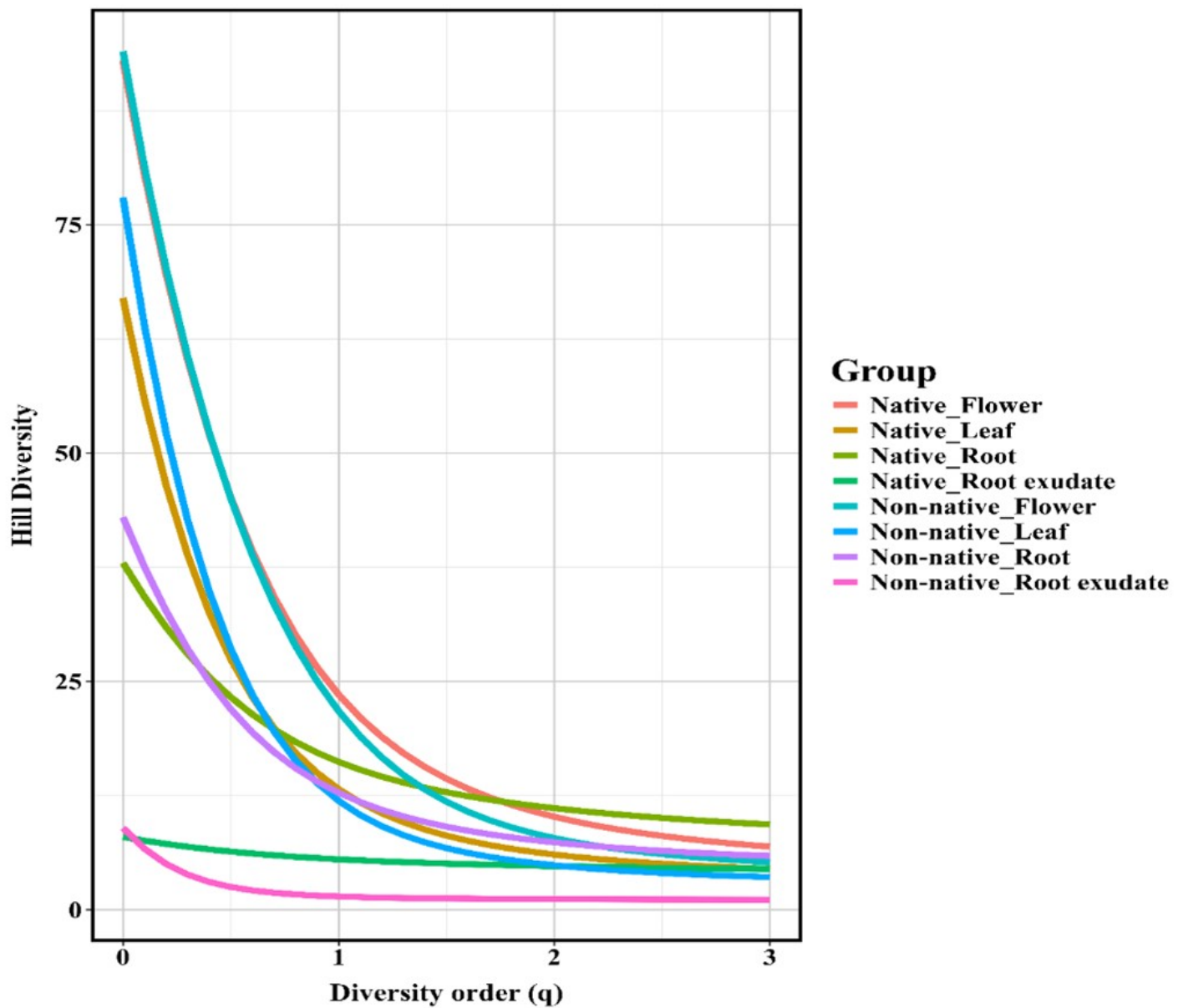


Figure 12

Comparison of Hill diversity of native and non-native flowers, native and non-native leaves, native and non-native roots, and native and non-native root exudates of *A. cotula* plants across different diversity orders (q). The x-axis represents the diversity order q , ranging from 0 to 3.

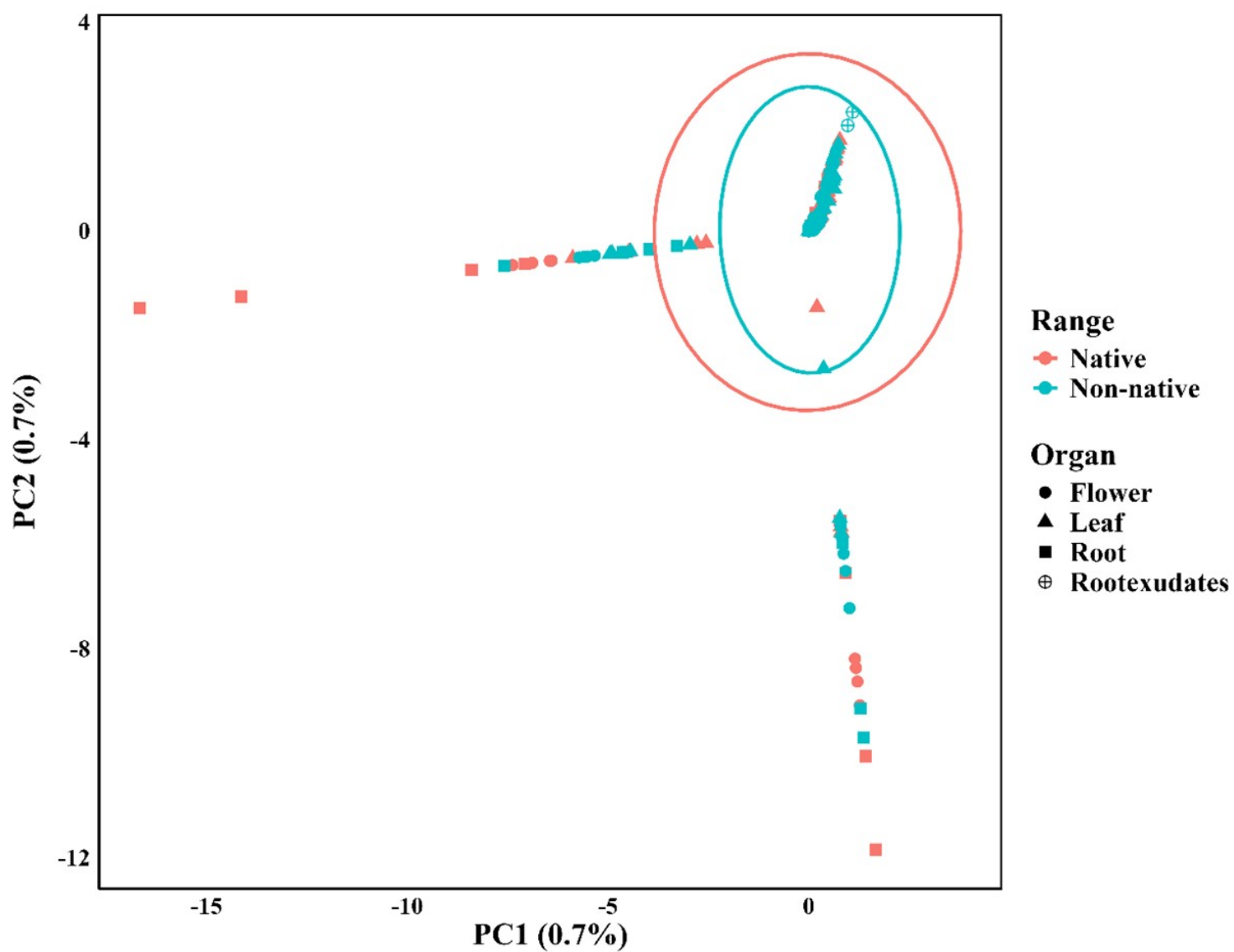


Figure 13

Principal Component Analysis (PCA) of metabolite composition across samples. Symbols represent different plant organs (flower, leaf, root, root exudates), and colours differentiate native (red) and non-native (blue) populations.

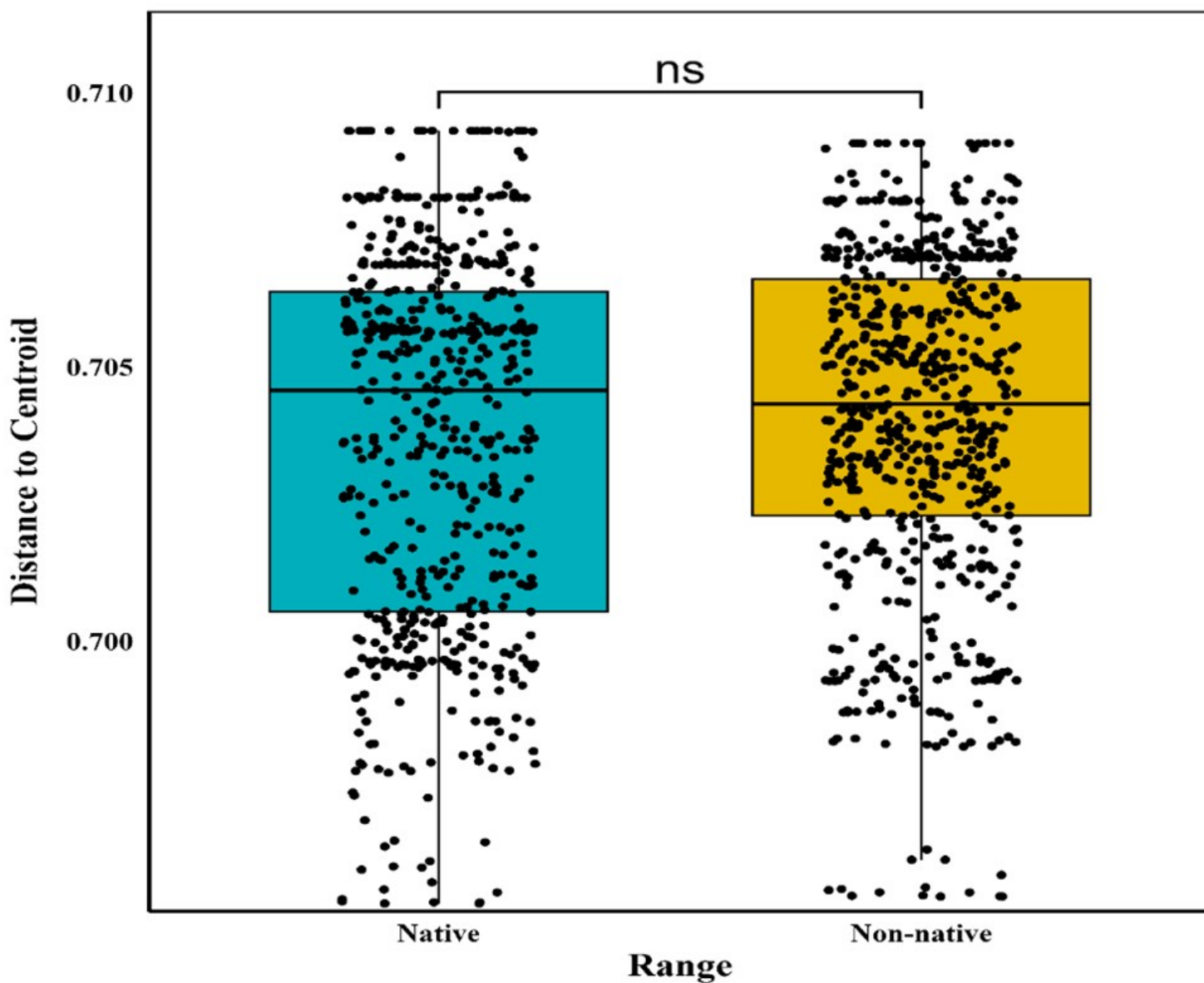


Figure 14

Distance-to-centroid analysis comparing within-group dispersion of metabolite composition between native and non-native samples. Boxplots show the distribution of sample distances to their group centroid. No significant difference (ns) was detected, suggesting comparable variability within each group.

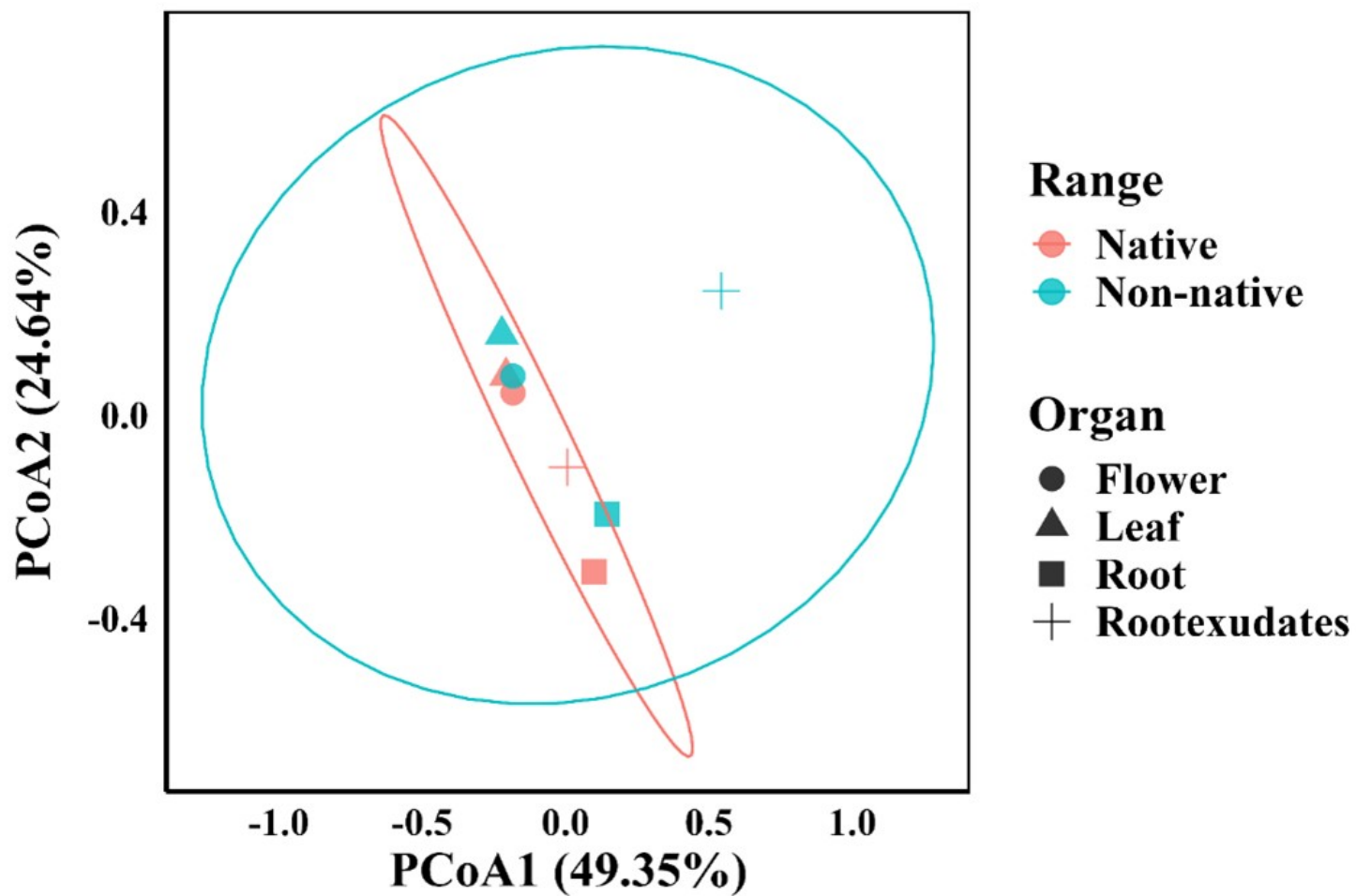


Figure 15

PCoA plot showing the differences in metabolite composition among different plant organs (flowers, leaves, and roots) and root exudates of native and non-native *A. cotula* plants. Circle shapes denote flowers, triangle leaves, squares denote roots, and crosses denote root exudates. Red colour denotes native and cyan non-native *A. cotula*.