Integrated Analysis of Rhizosphere Metabolome and Hormones Reveals Regulatory Mechanisms in Intercropped Asparagus cochinchinensis

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Research Article

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Abstract

Background

The increasing demand for high-quality medicinal herbs necessitates efficient land-use strategies. Understory intercropping offers a sustainable solution, yet the biochemical mechanisms governing plant adaptation and quality formation remain unclear.

Methods

This study investigated the intercropping of Asparagus cochinchinensis (*A. cochinchinensis*) under Pinus massoniana (*P. massoniana*) plantations. We compared agronomic traits, endogenous phytohormones, and rhizosphere metabolites among three groups, namely *P. massoniana* forest soil (Soil-A), *A. cochinchinensis* monoculture soil (Soil-B), and intercropping soil (Soil-C), using LC-MS-based metabolomics and targeted hormone profiling.

Results

Intercropping did not alter key agronomic traits but significantly increased total saponin content ($P < 0.05$) while decreasing chlorophyll levels ($P < 0.01$). Phytohormone analysis revealed a distinct hormonal signature in intercropped plants, characterized by decreased abscisic acid (ABA) and jasmonic acid (JA), alongside elevated salicylic acid (SA), cis-12-oxophytodienoic acid (cis-OPDA), and bioactive cytokinins. Rhizosphere metabolomics identified 25 differential metabolites, with notable enrichment of trans-cinnamic acid and gibberellin A_4 (GA_4) in Soil-C. KEGG analysis highlighted the significant activation of plant hormone signal transduction and aminobenzoate degradation pathways.

Conclusion

A. cochinchinensis exhibits morphological-metabolic decoupling under intercropping, prioritizing secondary metabolism over structural growth. The accumulation of trans-cinnamic acid and GA_4 in the rhizosphere appears to interface with endogenous hormone signaling, specifically involving the SA pathway and *cis*-OPDA-mediated regulation, to enhance saponin biosynthesis. This study provides a theoretical basis for optimizing *P. massoniana/A. cochinchinensis* agroforestry systems to achieve stable yields and improved medicinal quality.

1. Introduction

As the global demand for herbal medicines continues to rise while arable land resources dwindle, there is an urgent need to explore sustainable cultivation models. Understory intercropping, which integrates

forestry with the cultivation of herbaceous medicinal plants, offers a viable strategy for optimizing land-use efficiency[1]. *Pinus massoniana* (*P. massoniana*), a major afforestation species in southwestern China, is characterized by rapid growth, strong adaptability, and extensive canopy coverage. Although previous studies have demonstrated that understory cultivation in *P. massoniana* plantations can improve soil physicochemical properties and microbial activity, research on integrating high-value medicinal plants within such ecosystems remains insufficient[2].

Asparagus cochinchinensis (*A. cochinchinensis*) is a shade-tolerant perennial herb whose dried tuberous roots constitute an important component of traditional Chinese medicine. These roots are rich in steroidal saponins and exhibit antiviral properties[3,4]. Importantly, its preference for shaded environments makes it an ideal candidate for understory cultivation. However, existing studies on the understory cultivation of *A. cochinchinensis*, whether beneath *Camellia oleifera* or other tree species, have primarily focused on changes in soil nutrients and enzyme activities[5]. The complex biochemical interactions underlying changes in plant quality, particularly the metabolic dialogue involving soil metabolites and endogenous plant hormones, remain poorly understood[6].

Plants dynamically reshape their rhizosphere environment through the secretion of metabolites, which may trigger allelopathic effects or modulate plant hormone signaling[7,8]. Phytohormones such as jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), and gibberellins (GA) not only serve as master regulators of plant stress responses but also play a critical role in the biosynthesis of secondary metabolites, including saponins[9,10]. Given that soil metabolites represent the direct interface of plant-soil interactions, a comprehensive analysis of the rhizosphere metabolome is essential for elucidating the mechanisms underlying the benefits of understory cultivation[11,12].

This study hypothesizes that intercropping *P. massoniana* with *A. cochinchinensis* enhances the medicinal quality of the latter by reprogramming the rhizosphere metabolome and endogenous hormone profiles. To test this, we employed LC-MS-based metabolomics to compare the metabolic profiles of soil from a pure *P. massoniana* plantation (Soil-A), soil from monocultured *A. cochinchinensis* (Soil-B), and soil from the intercropping system (Soil-C). The findings untangle the metabolic synergies within this agroforestry system and provide a theoretical basis for optimizing the cultivation of high-quality medicinal plants under forest canopies.

2. Methods

2.1 Experimental site and soil collection

Sampling was conducted at a site located in Danzhai County, Guizhou Province, China (26°31'27.97"N, 107°48'44.81"E). Three treatments were established in the field experiment: Soil-A, representing soil from a pure *Pinus massoniana* (*P. massoniana*) forest; Soil-B, representing soil from a monoculture of *Asparagus cochinchinensis* (*A. cochinchinensis*); and Soil-C, representing soil from an intercropping system of *P. massoniana* and *A. cochinchinensis*. Rhizosphere soils from treatments Soil-A, Soil-B, and

Soil-C were collected using the ring knife method[13]. Three biological replicates were established for each group. Immediately after collection, the samples were flash-frozen in liquid nitrogen and subsequently stored at -80°C for metabolomic analysis.

2.2 Agronomic traits and quality parameters

During the *A. cochinchinensis* harvest period, whole-plant sampling was conducted using a combination of the five-point sampling method and random sampling. Plant height, stem count, projected area, basal stem diameter, fresh weight of aboveground and belowground parts, fresh and dry weight of tuberous roots, number of tuberous roots, as well as root length and diameter were measured. The samples were oven-dried at 60°C to a constant weight, after which the aboveground and belowground biomass of the plants was recorded.

Chlorophyll content in *A. cochinchinensis* was determined by referring to the method described by Rahimi-Rizi et al.[14]. Using saponins from *Songguo saponin* as the reference standard, a standard curve was plotted with the mass of the standard on the horizontal axis and the absorbance value at 310 nm on the vertical axis. The absorbance values of the test samples were measured according to the colorimetric method, and the total saponin content in *A. cochinchinensis*was calculated based on the standard curve[15]. Using aspartic acid as the amino acid reference standard and distilled water as the running blank control, the amino acid content was determined with reference to the method described by Starzyńska-Janiszewska et al[16].

2.3 Determination of hormone assay

Healthy and mature *A. cochinchinensis* plants were selected from each cropping system. Three samples of young leaves were collected from each system, and 50 mg of each sample was weighed. The samples were immediately ground after rapid freezing in liquid nitrogen or stored in a -80°C freezer for later use. The determination of hormone content was performed using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)[17].

2.4 Rhizosphere soil Metabolites

Soil samples were slowly thawed at 4°C. Aliquots were mixed with pre-cooled methanol/acetonitrile/water (2:2:1, v/v/v), vortexed, and sonicated at low temperature for 30 min, followed by standing at -20°C for 10 min. Supernatants were centrifuged at 14,000 × g, 4°C for 20 min, and the supernatants were vacuum-dried. Prior to MS analysis, dried residues were reconstituted in 100 µL acetonitrile/water (1:1, v/v), vortexed, centrifuged at 14,000 × g, 4℃ for 15 min, and final supernatants were transferred for injection.

Chromatographic separation was performed on a Vanquish LC UHPLC system equipped with a HILIC column. Column temperature: 25°C; flow rate: 0.3 mL/min; injection volume: 2 µL. Mobile phase A: water with 25 mM ammonium acetate and 25 mM ammonia; Mobile phase B: acetonitrile. Mass spectrometry was conducted using a Q Exactive series mass spectrometer (Thermo Fisher Scientific) in both ESI + and

ESI - modes[18]. Raw data were processed for peak alignment, retention time correction, and peak area extraction, followed by metabolite annotation, data preprocessing, quality control, and statistical analysis[18].

2.5 Statistical analysis

Agronomic traits and quality parameters were analyzed by one-way ANOVA with Duncan's multiple comparison test using SPSS 26.0 (IBM SPSS Inc., USA)[19]. Significance was set at $P < 0.05$. Multivariate analysis included Pareto-scaled PCA and partial least squares discriminant analysis (PLS-DA) performed in R. Model robustness was evaluated via 7 - fold cross-validation and response permutation testing. Variable importance in projection (VIP) values were calculated to assess metabolite contribution to group separation. Student's *t*-test was used for pairwise comparisons between two independent groups. Significant metabolites were defined as VIP > 1 and $P < 0.05$. Pearson correlation coefficients were computed to assess relationships between variables[20].

3. Results

3.1 Agronomic traits of *A. cochinchinensis* remained stable under intercropping, while saponin accumulation was significantly enhanced

To assess the growth adaptability of *A. cochinchinensis* intercropped under *P. massoniana* forest, we first compared a series of agronomic traits and quality indicators between intercropping and field monoculture conditions (Fig. 1, 2). The results indicated no significant differences between the two cultivation modes in terms of plant height, stem number, basal stem diameter, projected area, root tuber number, root tuber size, or aboveground and belowground biomass (Fig. 2), suggesting that understory intercropping did not compromise the overall morphological development of *A. cochinchinensis*. Regarding quality assessment, total amino acid content showed no statistical difference between the two groups (Fig. 3A). Notably, however, chlorophyll content in intercropped *A. cochinchinensis* was significantly lower ($P < 0.01$; Fig. 3B), while the total saponin content, the primary bioactive component, was markedly elevated ($P < 0.05$; Fig. 3C). These results suggest that by maintaining structural stability, *A. cochinchinensis* shifted its metabolic focus toward the biosynthesis of secondary metabolites, such as saponins, in response to the intercropping environment.

3.2 Phytohormone reprogramming coordinately regulates stress resistance and growth vitality

To elucidate the physiological mechanisms underlying the observed phenotypic stability, targeted phytohormone profiling was performed on leaf tissues of *A. cochinchinensis*. Among the 21 phytohormones detected in this study, the correlation coefficient for all analytes was greater than 0.99, and the QC RSD was less than 0.3%, ensuring high data reliability (Fig. 4). Heatmap analysis revealed a

distinct hormonal signature in intercropped plants (LB-b1-3) compared to the field monoculture control group (LB-s1-3). Specifically, salicylic acid (SA), cis-12-oxo-phytodienoic acid (cis-OPDA), isopentenyladenosine (iPR), N^6-Δ^2-isopentenyladenine (iP), indole-3-acetic acid (IAA), *cis*-zeatin riboside (cZR), and 1-aminocyclopropane-1-carboxylic acid (ACC) were consistently upregulated; conversely, jasmonoyl-isoleucine (JA-Ile), abscisic acid (ABA), jasmonic acid (JA), trans-zeatin (tZ), and cis-zeatin (cZ) were downregulated.

The decreased ABA level suggested that intercropped *A. cochinchinensis* experienced only mild, rather than severe, abiotic stress. The accumulation of *cis*-OPDA, coupled with the suppression of JA, indicated that *cis*-OPDA primarily functions as an independent signaling molecule rather than merely as a precursor for JA biosynthesis. The increased SA content further implied enhanced disease resistance. Meanwhile, elevated levels of IAA and active cytokinins (iPR) reflected active cell division and elongation, supporting the maintenance of vigorous growth in plants under understory shading conditions.

3.3 Soil metabolomics revealed extensive remodeling of the rhizosphere environment

To bridge plant physiological responses and rhizosphere conditions, untargeted LC-MS metabolomics was conducted on the three soil types (Soil-A, Soil-B, and Soil-C). A total of 356 metabolites were identified. Among these, lipids and lipid-like molecules constituted the highest proportion of the metabolite library (37.64%), followed by organic acids and derivatives (13.20%), phenylpropanoids (12.36%), and heterocyclic organic compounds (10.11%) (Fig. 5). Principal component analysis (PCA) showed a clear separation of Soil-C from Soil-B along the PC1 axis (Fig. S1), with biological replicates within each group clustering closely together, indicating good intra-group reproducibility. Partial least squares discriminant analysis (PLS-DA) further confirmed this differentiation (Fig. S2). These results collectively confirmed that understory intercropping induced significant metabolic reprogramming in the rhizosphere environment.

3.4 Differential soil metabolites highlighted enrichment of phenolic compounds and phytohormones

To identify the specific metabolic drivers of rhizosphere differentiation, differential metabolites between Soil-C and Soil-B were screened. Volcano plot and heatmap analyses (Fig. S3 and Fig. 6) revealed that 25 metabolites met the selection criteria (VIP > 1 and $P < 0.05$). Among these, 23 metabolites were upregulated in the intercropped soil, primarily including *trans*-cinnamic acid, gibberellin A_4 (GA_4), histidine, and a series of phenolic compounds, alcohols, esters, and base-related metabolites. Only two metabolites, N-carboxyethyl-γ-aminobutyric acid and lysine, were found to be downregulated. The significant accumulation of *trans*-cinnamic acid (a core precursor of phenylpropanoid metabolism) and GA_4 in Soil-C suggests that intercropping not only reshapes the rhizosphere chemical composition but also enriches signaling molecules that may feedback to regulate plant growth and defense.

3.5 Plant hormone signal transduction and aminobenzoate degradation pathways were significantly enriched

Finally, to translate the metabolite changes into biological significance, KEGG pathway enrichment analysis was performed on the 25 differential metabolites identified above. A total of 15 metabolic pathways were annotated, among which plant hormone signal transduction and aminobenzoate degradation were the most significantly enriched pathways (Fig. S4). Within the hormone signaling pathway, the salicylic acid (SA) biosynthesis branch, centered on phenylalanine ammonia-lyase (PAL), was significantly activated. PAL catalyzes the conversion of phenylalanine to *trans*-cinnamic acid, which is consistent with the significant upregulation of this metabolite in Soil-C. Concurrently, the gibberellin (GA) biosynthesis pathway was also enhanced, where GA3-oxidase (GA3ox) converts inactive GA_{20} into active GA_4, aligning with the elevated GA_4 levels detected in the intercropped soil. Taken together, these pathway-level changes indicate that intercropping orchestrates a metabolic network spanning the rhizosphere and plant tissues, enhancing the defense capacity of *A. cochinchinensis* while also safeguarding its growth potential.

4.1 Phenotypic Stability and Metabolic Adaptation Under Intercropping Conditions

Similar to the research results of predecessors, intercropping *A. cochinchinensis* under *P. massoniana* induced a distinct decoupling between vegetative growth and secondary metabolism[21, 22]. Although key agronomic traits, including plant height, biomass accumulation, and root tuber morphology, remained statistically unchanged relative to field monoculture, the metabolic profile of the plants shifted markedly (Fig. 2). Notably, chlorophyll content decreased significantly ($P < 0.01$), while the content of total saponins, the primary bioactive constituents, increased significantly ($P < 0.05$) (Fig. 3B, C). This trend parallels observations in tea - soybean intercropping systems, where secondary metabolite profiles in tea plants were similarly altered, as well as in mountain-cultivated *Panax ginseng* (*P. ginseng*), where saponin accumulation was notably enhanced[23, 24]. This phenomenon, termed "morphological-metabolic decoupling," suggests that exposure to the understory microenvironment prompts *A. cochinchinensis* to prioritize resource allocation toward the synthesis of defensive and pharmacologically relevant compounds over structural expansion[25, 26].

Profiling of plant hormones provided mechanistic insights into this adaptive response (Fig. 4). The observed reduction in ABA levels indicates that intercropped plants experienced mild environmental fluctuations rather than severe abiotic stress, thereby conserving metabolic energy[27]. Concurrently, elevated levels of IAA and iPR supported sustained cell division and elongation, which explains the stability of agronomic traits[28]. Remarkably, the accumulation of *cis*-OPDA, accompanied by the suppressed levels of JA, suggests a decoupling of oxylipin signaling[29, 30]. *Cis*-OPDA may function not merely as an intermediate in JA biosynthesis but as an independent signaling molecule that fine-tunes

secondary metabolic pathways, potentially promoting saponin biosynthesis without triggering the high metabolic costs typically associated with JA-mediated defense responses[29, 31].

4.2 Rhizosphere Metabolic Shifts Affecting Medicinal Plant Growth and Quality

In addition to endogenous plant responses, intercropping profoundly reshapes the rhizosphere metabolic environment. Multivariate analysis confirmed a distinct metabolic separation between Soil-B and C (Fig. S1, 2). This finding aligns with the effects observed in the microbial fertilizer derived from *Aspergillus purpureobrunneus* HZ23 on soil properties and metabolites in *Brassica rape* grown on reclaimed land, where lipids and lipid-like molecules dominated the soil metabolome (37.64%), followed by organic acids and benzenoid compounds (Fig. 5)[32]. Previous studies have shown that intercropping tobacco with maize during the seedling stage promotes crop growth by regulating the rhizosphere microenvironment[33]. Thus, we infer that the unique metabolic characteristics of Soil-C relative to Soil-B and Soil-A result from changes in root exudate patterns and microbial turnover triggered by *P. massoniana*.

Among the 25 identified differential metabolites (Fig. 6), trans-cinnamic acid and GA_4 emerged as key nodes linking soil chemistry to plant physiology. The significant accumulation of *trans*-cinnamic acid in Soil-C is particularly noteworthy, as it serves as a central precursor in the phenylpropanoid biosynthetic pathway leading to SA[34]. This finding aligns with the elevated SA levels detected in intercropped plant tissues (Fig. 4), resembles the antagonistic regulation model of SA and JA pathways in rice immunity, and further supports the involvement of the PAL pathway highlighted by KEGG enrichment analysis[35]. Furthermore, the enrichment of GA_4 in the rhizosphere suggests a potential exogenous source, possibly derived from root secretions or microbial transformations, that may supplement the endogenous GA pool to sustain plant vigor[36]. Contrary to concerns regarding allelopathic inhibition, the specific metabolite alterations observed in this study, including the downregulation of certain amino acid derivatives such as N-carboxyethyl-γ-aminobutyric acid, appear to be consistent with a compatible rather than antagonistic plant-plant interaction within this agroforestry system[33].

5. Conclusion

In summary, our findings delineate a synergistic regulatory network spanning the soil-plant continuum. We propose a conceptual model wherein *P. massoniana* induces systemic responses in *A. cochinchinensis* by reshaping the understory microenvironment across both physical dimensions (shading) and chemical dimensions (rhizosphere metabolites). Specifically, the accumulation of trans-cinnamic acid and GA_4 in the rhizosphere interfaces with endogenous hormone signaling in *A. cochinchinensis*, fine-tuning the balance between growth (IAA, iPR) and stress adaptation (SA, cis-OPDA). This optimized hormonal milieu subsequently activates downstream secondary metabolic pathways, most notably driving PAL-mediated phenylpropanoid biosynthesis to enhance saponin accumulation.

Consequently, this intercropping system achieves a strategic trade-off by maintaining structural integrity while prioritizing the production of high-value medicinal compounds. This study underscores the utility of integrating rhizosphere metabolomics with plant hormone profiling to unravel biochemical dialogues in complex agroforestry systems.

Declarations

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable. All the Asparagus plants belong to wild-type materials and were cultivated in Danzhai County, Qiandongnan Miao and Dong Autonomous Prefecture, Guizhou Province, China. They were identified by Professor Ming-sheng, Zhang. The methods involved in this study were carried out in compliance with local and national regulations.

Availability of data and materials

All data sheets and codes to process data are available upon request to the corresponding author, Liu Miao (miaoliu1991@163.com).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Jia-hui Gao performed the field experiments, collected plant and soil samples, and measured agronomic traits and total saponin content. **Gonggu Lv** conducted the LC-MS metabolomic profiling and phytohormone analysis, performed the statistical analyses, prepared all figures and tables, and wrote the original draft. **Ling-li Luo** and **Liu Tang** assisted with sample preparation and data curation during the field investigation. **Ming-sheng Zhang** designed the experimental framework, provided guidance on agroforestry practices, and revised the manuscript critically for important intellectual content. **Liu Miao** conceived and supervised the project, acquired funding, and reviewed and edited the manuscript.

Jia-hui Gao and Gonggu Lv contributed equally to this work.

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Figures

Figure 1

Field planting sites of *A. cochinchinensis and P. massoniana* understory intercropping.(A) Field planting sites of two-year-old *A. cochinchinensis*. (B) P. massonianaunderstory intercropping sites. Plants in the plantations were used for comparison of agronomic traits and soil sample collection.

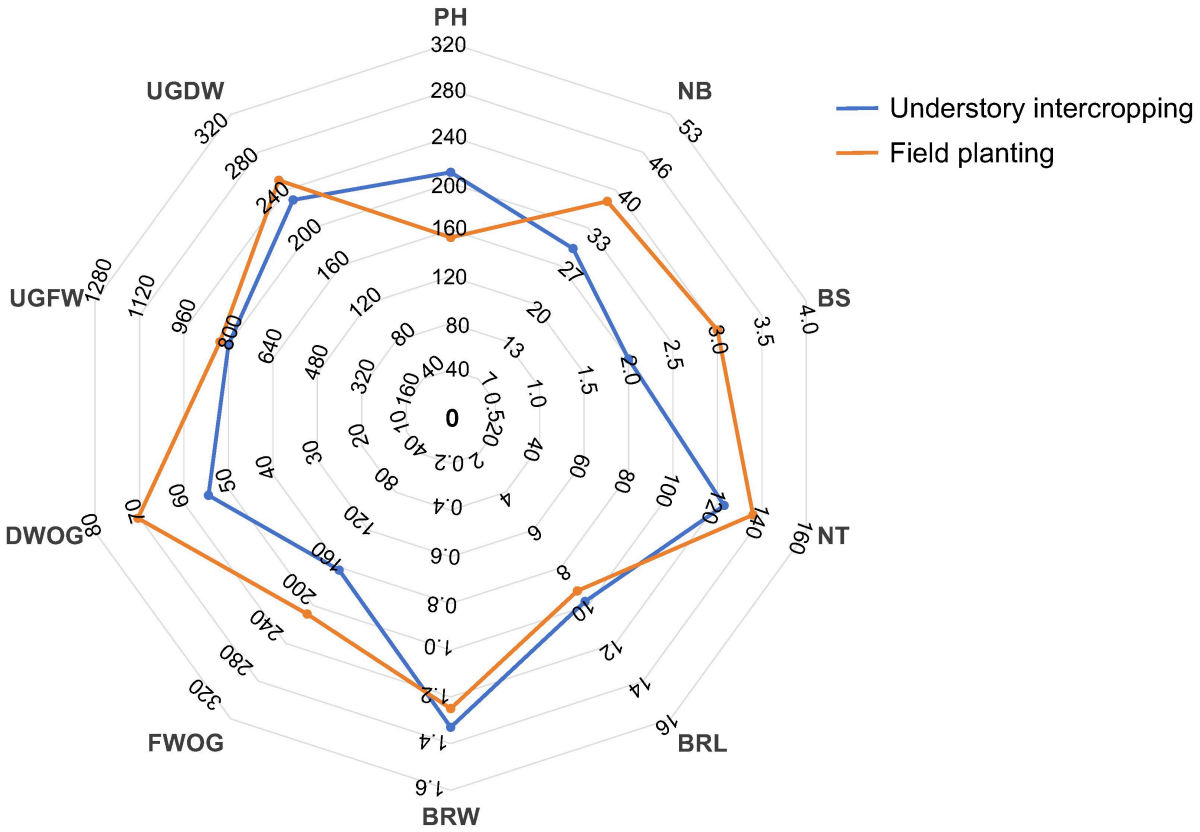

Figure 2

Radar plot comparing ten agronomic traits of *A. cochinchinensis* in understory intercropping vs. field planting.PH: Plant height; NB: Number of branches; BS: Base stem; NT: Number of tubers; BRL: Block root length; BRW: Block root width; FWOG: Fresh weight on ground; DWOG: Dry weight on ground; UGFW: Underground fresh weight; UGDW: Underground dry weight. Trait values are presented as mean ±SD (if applicable). No significant differences were found between the two systems.

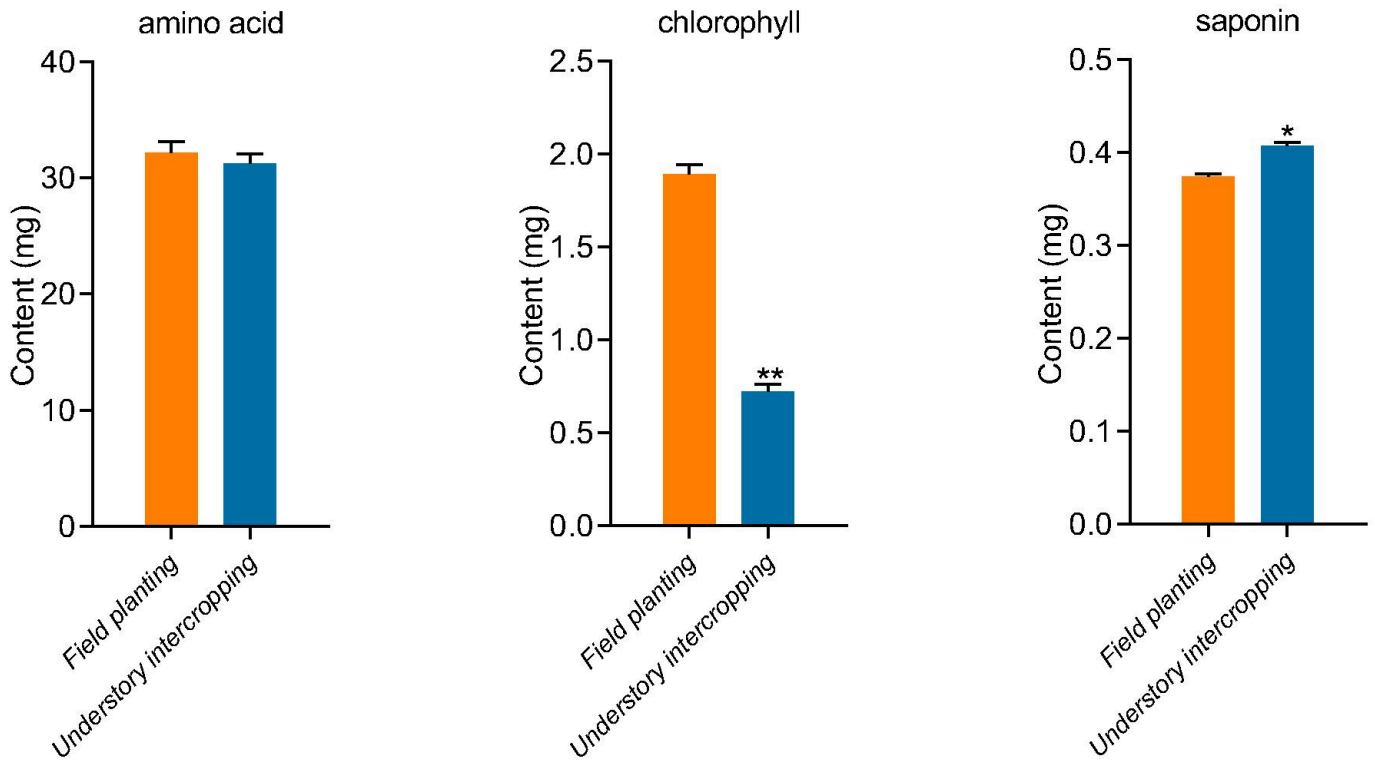

Figure 3

Comparison of total amino acid, chlorophyll, and saponin contents in *A. cochinchinensis* between field planting and understory intercropping. Bars represent mean ± standard deviation (SD, *n* = 3-5 per group). Statistical significance was assessed by Student's *t*-test: ** $P < 0.01$ for chlorophyll, * $P < 0.05$ for saponin. No significant difference was observed for total amino acid content.

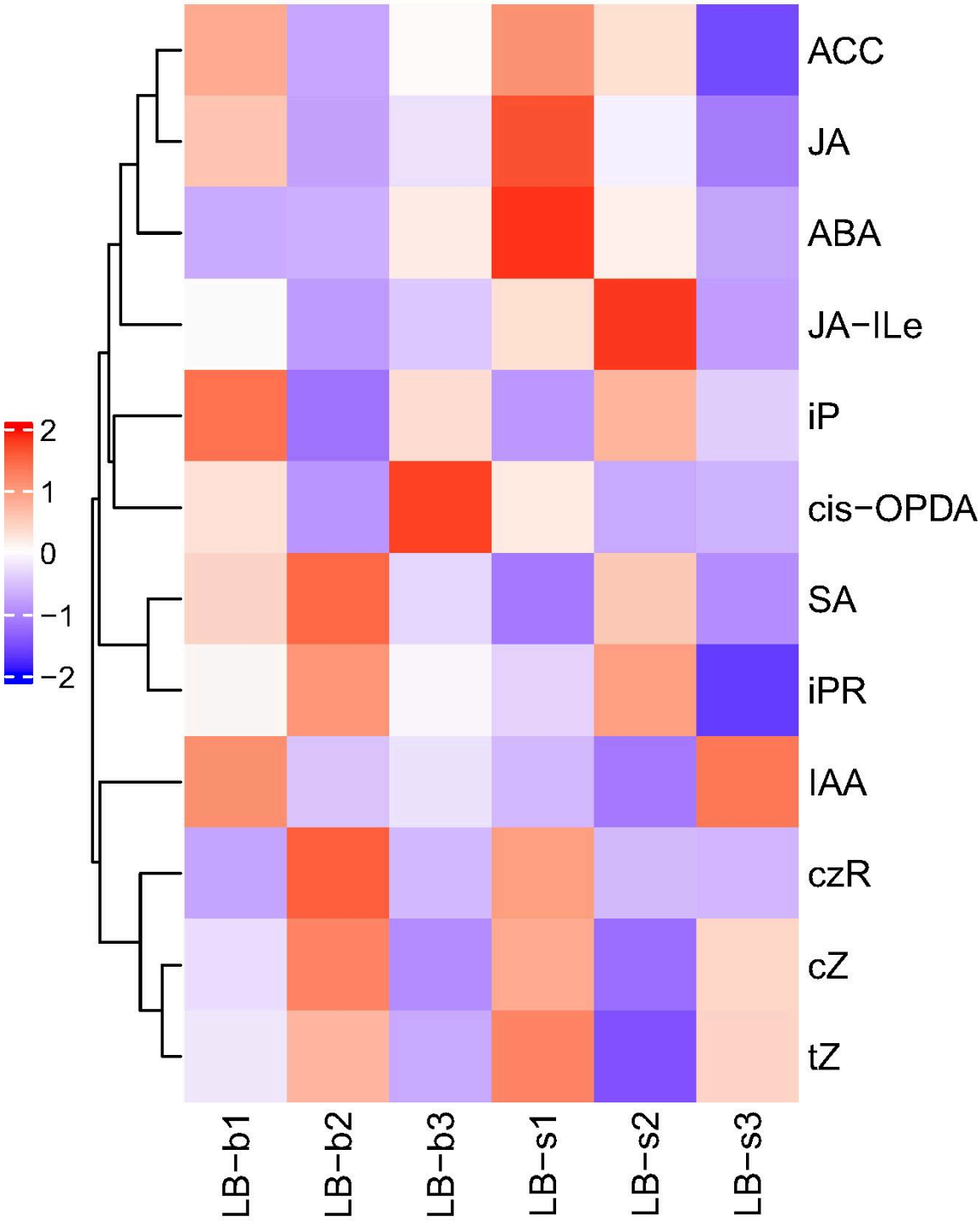

Figure 4

Hormonal differences in *A. cochinchinensis* under different cropping patterns. (LB-b1, b2, b3): Understory intercropping; (LB-s1, s2, s3): Field planting. The heatmap shows relative expression levels (*Z*-score) of 12 phytohormones. Upregulated in understory intercropping: SA, *cis*-OPDA, iPR, iP, IAA, cZR, ACC. Downregulated: JA-ILE, ABA, JA, tZ, cZ. Analytical validation: Correlation coefficients > 0.99, QC RSD < 0.3% for all hormones.

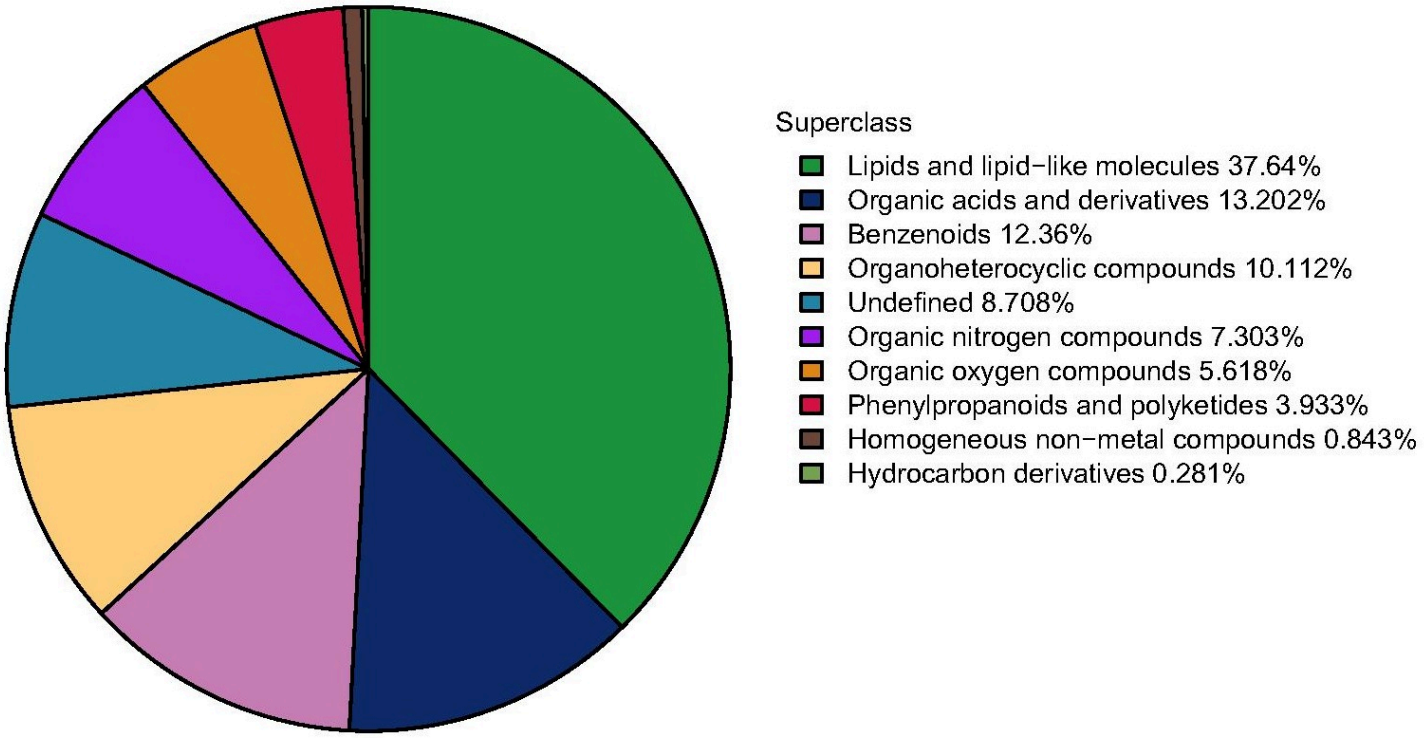

Figure 5

Distribution of soil metabolite chemical superclasses. Lipids and lipid-like molecules were the most abundant (37.64%), followed by organic acids and derivatives (13.202%), benzenoids (12.36%), organoheterocyclic compounds (10.112%), and undefined compounds (8.708%). Other classes, including organic nitrogen, organic oxygen, and phenylpropanoids, accounted for the remaining proportions.

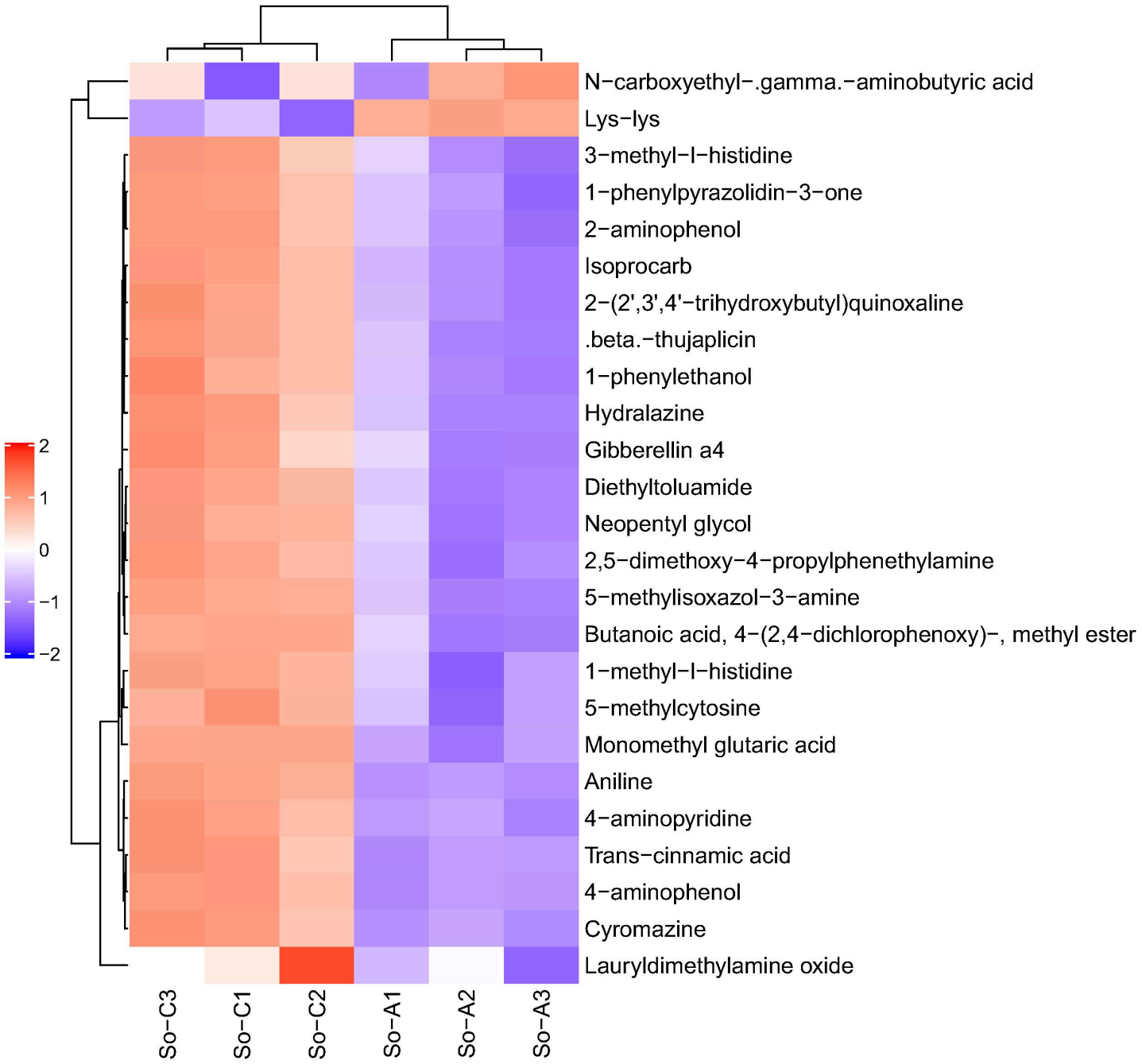

Figure 6

Heatmap of differentially abundant soil metabolites between understory intercropping and field planting. Rows: metabolites; columns: replicate samples (So-C1/2/3 = field planting; So-A1/2/3 = understory intercropping). Color scale indicates relative abundance (red = upregulated in intercropping; blue = downregulated). Metabolites were hierarchically clustered into two groups: 23 upregulated and 2 downregulated (N-carboxyethyl-γ-aminobutyric acid, Lys-Lys) in intercropping compared to field planting.

Supplementary Files

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