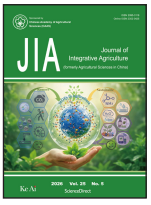
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RESEARCH ARTICLE

Decade-long fertilization and *Bradyrhizobium* inoculation reconfigure soybean rhizosphere microecology through fungal community assembly and metabolic niche partitioning

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Highlights

- Nitrogen fertilization initially reduces key antioxidant metabolite concentrations in soil.
- *Bradyrhizobium* inoculation augments stress-related metabolite profiles at maturity.
- Essential soil metabolites demonstrate correlation with fungal abundance and activity patterns.
- Pathway analysis identifies tryptophan and caffeine metabolism in stress response.

Abstract

Soil microbial-metabolite interactions influence crop productivity, yet their responses to long-term nutrient management in legume systems warrant further investigation. This study examined how fertilization and *Bradyrhizobium* inoculation reshape soybean rhizosphere fungal-metabolite networks to improve soil health. Through a decade-long field trial utilizing internal transcribed spacer (ITS) sequencing and liquid chromatography-mass spectrometry (LC-MS) metabolomics, four treatments were evaluated: no fertilizer application (CK); phosphorus and potassium fertilization (PK); PK chemical fertilizers combined with urea (PK+N); PK fertilization with *Bradyrhizobium japonicum* 5821 inoculation (PK+R). Results indicated that nitrogen fertilization increased fungal diversity at maturity and enhanced co-occurrence network complexity (displaying the highest node and edge counts), while *Bradyrhizobium* inoculation promoted stochastic assembly. Soil fungi exhibited notable correlations with 3-hydroxymethylantipyrine, chrysophanol, 3,7-dihydroxyflavone and triethylamine. Metabolite profiling revealed nitrogen suppression of stress-resistant flavonoids (3-hydroxymethylantipyrine, chrysophanol, 3,7-dihydroxyflavone), whereas *Bradyrhizobium* enhanced these key metabolites. KEGG enrichment identified tryptophan and caffeine metabolism as central during flowering–podding stage, coordinating nitrogen assimilation and defense responses. Additionally, the key metabolites correlated significantly with soil total nitrogen, organic matter, and available nitrogen. These findings reveal that *Bradyrhizobium* acts synergistically with fertilization to activate fungal-driven metabolic pathways, offering a microbiome-based approach to enhance nitrogen efficiency and reduce agrochemical dependency in soybean systems.

Keywords: fungal community, key differential metabolites, *Bradyrhizobium*, soil microecology

1. Introduction

Metabolomics, the comprehensive analysis of small molecules produced during metabolism, represents a crucial approach for elucidating the complex dynamics of soil ecosystems (Kuhlisch and Pohnert 2015; Withers *et al.* 2020). In agricultural systems, long-term fertilization and *Bradyrhizobium* inoculation modify rhizosphere metabolite profiles — altering amino acids, organic acids, and signaling flavonoids that regulate microbial functionality and plant resilience. These metabolites function as ecological mediators of soil health, influencing processes from nitrogen

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fixation to stress adaptation (Chen *et al.* 2019a). Specific metabolites function as sensitive indicators of soil health dynamics, where alterations following fertilization and inoculation directly reflect treatment efficacy (Zhu *et al.* 2016; Withers *et al.* 2020). Monitoring these changes facilitates data-driven optimization of fertilization strategies for improved productivity and environmental sustainability (Lange *et al.* 2008; Hungria *et al.* 2013).

The application of nitrogen fertilizers increases the abundance of amino acids and organic acids in the rhizosphere, which are essential for microbial growth and activity (Wang J *et al.* 2021; Savarese *et al.* 2022). These metabolites enhance nutrient availability by mobilizing essential elements from soil particles and facilitating microbial interactions that promote nutrient uptake in plants (Bardgett and van der Putten 2014; Qiao *et al.* 2024). In contrast, *Bradyrhizobium* inoculation enriches the soil with metabolites that support symbiotic relationships between legumes and nitrogen-fixing bacteria (Wang *et al.* 2022). This process enhances the production of signaling molecules such as flavonoids, which attract and stimulate the growth of beneficial rhizobia (Jiao *et al.* 2018; Wolde-Meskel *et al.* 2018). The metabolites produced in this symbiotic relationship enhance nitrogen availability while contributing to improved root development and resistance against biotic and abiotic stresses.

Furthermore, research indicates that the combined application of fertilizers and *Bradyrhizobium* inoculation generates synergistic effects on soil metabolite profiles. Specifically, integrating these practices produces a more balanced nutrient profile, creating an environment conducive to the proliferation of beneficial microbial communities (He *et al.* 2024). This interaction between fertilization and microbial inoculation is essential for optimizing nutrient cycling and promoting sustainable agricultural practices.

Despite these advances, additional research is required regarding the long-term effects of fixed-position fertilization and *Bradyrhizobium* inoculation on soil metabolites. While previous research (Wei *et al.* 2023) established that these treatments significantly influence bacterial communities, the fungal component, particularly their role in metabolite alterations, remains unexplored. *Bradyrhizobium* inoculation influences rhizosphere fungal community composition in a host genotype dependent manner (Xu *et al.* 2020). Such investigations are crucial for three reasons: First, fungi dominate carbon transformation pathways that directly influence metabolite profiles, complementing bacterial roles in nitrogen cycling (Sokol *et al.* 2022; Xie *et al.* 2022). Second, fertilization-induced fungal restructuring may significantly alter metabolite niches essential for soil health, a mechanism unexplored in bacterial-focused analyses (Yue *et al.* 2023). Third, understanding how *Bradyrhizobium*–fungi–metabolite interactions influence plant resilience provides a comprehensive framework for sustainable agriculture, incorporating more systematic microbial synergies (Jin *et al.* 2024).

This study examines northeastern black soil subjected to long-term fixed-position fertilization and *Rhizobium* inoculation over a 10-year period. Through non-targeted metabolomics, we investigate the effects of these agricultural practices on metabolite profiles in the rhizosphere during two critical soybean growth phases: the flowering–podding stages and maturity. We examine three hypotheses: (1) Nitrogen supplementation restructures fungal networks through metabolite suppression, enhancing community interconnectivity; (2) *Bradyrhizobium* inoculation promotes stochastic assembly by increasing plant-health metabolites, enriching stress-response pathways; (3) Key metabolites mediate fungal–soil feedbacks, where their abundances directly regulate fungal gene expression and correlate with soil physical and chemical factors.

This study demonstrates significant correlations between fertilization practices, *Bradyrhizobium* inoculation, fungal community restructuring, and soil metabolite profiles. These correlations offer valuable insights for enhancing soil health and implementing sustainable agricultural management practices. The identified relationships between organic fertilization and beneficial microbial-metabolite changes indicate potential mechanisms for improving soil carbon storage and nutrient retention, while the associations between inoculation-enhanced fungal communities and disease suppression markers suggest strategies for reducing dependence on agrochemicals. These evidence-based relationships establish a comprehensive framework for developing targeted soil management strategies to enhance agricultural sustainability.

2. Materials and methods

2.1. Sampling site

Soil samples were collected from a long-term experimental station (43°52′88′′N, 124°80′55′′E) situated at the Modern Agricultural Industrial Technology Demonstration Base of the Jilin Academy of Agricultural Sciences in Gongzhuling City, China. The experiment, initiated in 2011, utilizes an annual cropping system with soybean as the primary crop. The soybean variety Jiyu 86 was planted in late April and harvested in mid-October. Soil sampling occurred during two critical growth stages in 2021: the flowering–podding stage (July 16) and the maturity stage (September 29).

The experimental design incorporated three replications per treatment, with each plot measuring 36 m^2 and comprising 12 rows, each 5 m in length and spaced 0.6 m apart. Treatment sites remained fixed throughout the study to maintain consistency. Four treatments were implemented: no fertilizer application (CK); phosphorus and potassium fertilization (PK); PK chemical fertilizers combined with urea (PK+N); PK fertilization with *Bradyrhizobium* inoculation (PK+R). Fertilization rates consisted of 75 kg ha^{-1} of P_2O_5, 75 kg ha^{-1} of K_2O, and 60 kg ha^{-1} of nitrogen as urea. *Bradyrhizobium japonicum* 5821, isolated from soybean nodules of the variety Kenjian 28 at Nenjiang Jiusan Farm, served as the inoculant. The inoculant concentration was 5×10^9 CFU mL^{-1}, with 5 mL of inoculant mixed per kilogram of soybean seeds (seeding rate: 45 kg ha^{-1}). This inoculation protocol has been maintained consistently for the past 10 years (Wei *et al.* 2024).

Fixed-position fertilization involved applying fertilizers at

consistent soil depths and spatial locations within each plot throughout the experimental period. The fertilization sites were determined based on the soybean planting pattern, with fertilizers placed 10–15 cm below the soil surface and 10 cm away from seedling roots to ensure uniform distribution and prevent root damage. The same fertilization locations were maintained across cropping seasons to enable long-term monitoring of soil microecological changes in fixed positions. This approach minimized spatial variation from inconsistent fertilization locations and facilitated accurate treatment effect comparisons over time (Geisseler and Scow 2014).

Rhizosphere soil samples were collected using a five-point sampling method by randomly selecting five soybean plants, removing excess soil, and collecting soil tightly adhering to the roots. Rhizosphere samples were aseptically transferred to sterile cryovials, stored in pre-cooled dry ice containers (–20°C), and transported to the laboratory. Following contaminant removal, soil samples were sieved through a 2 mm mesh sieve for subsequent analysis. One portion underwent air-drying for physical and chemical property analysis, while the remainder was stored at –80°C for molecular biology experiments and metabolomics sequencing.

The soybean root nodule dry weight, soybean yield, and soil physicochemical properties were previously documented in our study (Wei *et al.* 2023; Appendix A). This analysis focuses specifically on examining the correlations between these variables, the fungal community structure, and key metabolites.

2.2. DNA extraction and quantification

DNA was extracted from 0.5 g of fresh soil using the DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Rockwood, TN, USA) and 1% (w/v) agarose gel electrophoresis. The fungal internal transcribed spacer (ITS) gene abundance was determined using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) on an ABI7500 real-time quantitative PCR system (Applied Biosystems, USA). The PCR mixture contained 12.5 μL SYBR, 0.5 μL RoX-II (TaKaRa, Japan), 1 μL of each primer, 9 μL sterile water, and 1 μL DNA template. The annealing temperature was maintained at 50°C. A standard curve was generated through serial dilution of plasmids containing the target gene, and gene copy numbers were calculated based on the threshold cycle (Ct) values from the melting curve (Zhou *et al.* 2022).

2.3. Amplicon sequencing

After DNA purification and quality assessment, fungal amplicons were sequenced on the PE300 platform at Meiji Biotech (Shanghai, China) using primers ITS1F and ITS2R (Illumina, San Diego, CA, USA). Raw sequences underwent quality control and filtering with FASTP (v. 0.19.6). Overlapping paired-end reads were merged using FLASH (v. 1.2.7) to generate high-quality sequences. DADA2 was employed to denoise the sequences, resulting in amplicon sequence variants (ASVs). Non-redundant sequences were clustered into ASVs with 70% similarity using the classify-sklearn Bayesian algorithm. Chimeric sequences were eliminated, and representative sequences for each ASV were identified. Sequence alignment was performed using species annotation databases (its_fungi for fungi), and a taxonomy abundance table was generated in QIIME2 (v. 2022.2). The ASV representative sequences and their abundance data were subsequently utilized for species classification, community diversity analysis, and differential species identification (Pang *et al.* 2023). The original data were deposited in the National Center for Biotechnology Information database under the accession number PRJNA1195624.

2.4. Non-targeted metabolomics

In this study, a liquid chromatograph-mass spectrometer (LC-MS) was utilized for comprehensive metabolite profiling due to its high sensitivity and accuracy in detecting a wide range of metabolites in complex soil matrices. Prior to LC-MS analysis, soil samples were pre-treated with HPLC-grade methanol, acetonitrile, formic acid, and propanol to enhance metabolite solubility and extraction efficiency while minimizing enzymatic degradation. The resulting extracts were ionized in a high-vacuum mass spectrometer, where metabolites were separated by their mass-to-charge ratio (m/z), generating a detailed mass spectrum essential for subsequent analyses (Kanehisa *et al.* 2016).

Rigorous preprocessing of raw data included filtering, imputation of missing values, normalization, and logarithmic transformation to ensure consistency and comparability across samples. Metabolites were annotated by matching their spectral data with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to facilitate pathway mapping and functional analysis (Xiao *et al.* 2012). Differential metabolites across treatments were identified using univariate (*T*-test) and multivariate (partial least squares discriminant analysis (PLS-DA)) statistical approaches, with significance thresholds set at $P<0.05$ and a variable importance in projection (VIP) score>1, following established analytical standards (Qian *et al.* 2022). Additionally, KEGG pathway enrichment analysis provided insights into the biological pathways modulated by different treatments, based on established methodologies.

2.5. Statistical analyses

Statistical analyses were conducted using various software tools. One-way analysis of variance (ANOVA) with Duncan's test was performed in SPSS 24 (Chicago, USA). The "*ggplot2*" package in R (v. 4.2.3) generated boxplots and regression analyses, while non-metric multidimensional scaling (NMDS) was implemented through the "*vegan*" package to visualize ASV distribution across treatments and growth stages. The neutral community model (NCM) assessed microbial community ecological stochasticity using the "Hmisc," "minpack.lm," and "stats4" packages in R (Chen *et al.* 2019b). Co-occurrence network analysis

at the genus level utilized Gephi 0.9.2 for microbial communities with relative abundances>0.1% and significant Spearman correlation coefficients (P<0.01, $|r|$>0.80) (Bi *et al.* 2020). The "randomForest" package assessed indicator species importance, while Mantel tests using "linkET", "ggplot2", and "dplyr" packages analyzed correlations between soil physicochemical properties and fungal community structures (Hu *et al.* 2023).

Advanced statistical analyses were performed using R packages. Principal component analysis (PCA) and PLS-DA evaluated sample similarities and variations using "ggbiplot", "ropls", "ggforce", "ggplot2", and "ggprism". The "VennDiagram" package created Venn diagrams to illustrate relationships among treatment groups (Love *et al.* 2014). Volcano plots depicting differential metabolite expression were generated using multiple R packages including "ggplot2", "RColorBrewer", "ggrepel", "RUnit", "ggforce", "tidyverse", "ggpubr", "ggprism", and "paletteer". The "ComplexHeatmap" package generated heatmaps, while "ggplot2" created boxplots and bubble charts. Structural equation modeling (SEM) was employed to decipher the integrated effects of fertilization and *Bradyrhizobium* inoculation on fungal community restructuring and metabolite shifts. Mantel test correlations and SEM standardized effects were visualized using Origin 2021 (OriginLab Corp., USA).

3. Results

3.1. Community structure of fungi

Fig. 1-A presents the fungal gene abundance and alpha diversity results. During the soybean flowering–podding stage, the PK treatment demonstrated significantly higher ITS gene copy numbers (21.72×10^8 copies g^{-1}) compared to other treatments, while the PK+N treatment showed significantly lower numbers (10.56×10^8 copies g^{-1}). At the maturity stage, fertilization significantly decreased the ITS

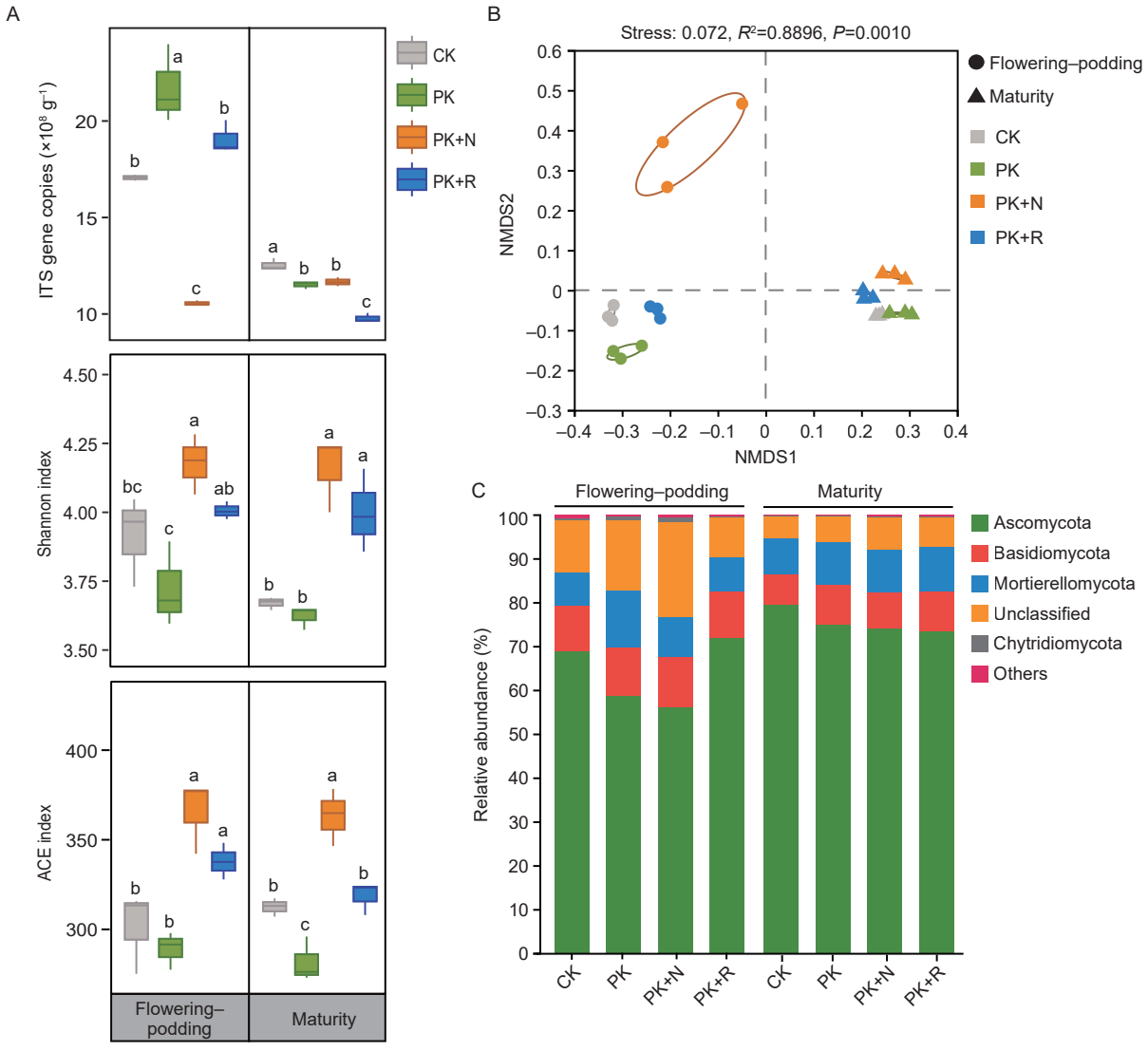


Fig. 1 Gene abundance, alpha diversity (A), beta diversity (B), and phylum composition (C) of fungal communities were assessed during the flowering–podding and maturity stages under different treatments. CK, no fertilizer application; PK, phosphorus and potassium fertilization; PK+N, PK chemical fertilizers combined with urea; PK+R, PK fertilization with *Bradyrhizobium* inoculation. Different lowercase letters indicate statistical differences among treatments at each stage in bulk or rhizosphere soil (P<0.05).

gene copy number, with the lowest values observed in the *Bradyrhizobium* inoculation treatment. The PK treatment exhibited significantly lower Shannon and ACE indices compared to other fertilization treatments during both growth stages.

Appendix B demonstrates the linear relationships between soybean nodule dry weight, yield, and fungal indicators. The fungal gene copy number in the rhizosphere showed a significant positive correlation with nodule dry weight at the flowering–podding stage (R^2=0.552; P=0.006), while displaying a significant negative correlation with soybean yield at maturity (R^2=0.579; P=0.004). Neither fungal diversity index (Shannon or ACE) demonstrated significant correlations with soybean nodule dry weight or yield.

The NMDS analysis revealed distinct separations in fungal community structures across treatments at different growth stages along the first and second NMDS axes (stress=0.072, R^2=0.8896, P=0.0010; Fig. 1-B), demonstrating that growth stage significantly influences soil fungal communities. During the soybean flowering–podding stage, fungal ASVs in the PK+N treatment exhibited distinct separation from other treatments, indicating that nitrogen fertilization substantially modified the fungal community composition in the rhizosphere. The predominant fungal phyla identified in the soil comprised Ascomycota (42.19–79.62%), Basidiomycota (6.76–33.59%), and Mortierellomycota (7.54–20.06%) (Fig. 1-C).

At the genus level, analysis of relative fungal community abundance showed that in the PK treatment, *Didymella* and *Trichoderma* exhibited significantly lower abundance at the mature stage compared to other treatments (Appendix C). Nitrogen fertilization enhanced *Gibberella* and *Cephalotrichum* during the flowering–podding stage, while at maturity, it increased *Neonectria* and *Pseudombrophila*, notably decreasing the relative abundance of *Neocosmospora* in both stages. *Rhizobium* inoculation during the flowering–podding stage enhanced *Tausonia* and *Trichoderma*, decreased *Minimedusa*, and at maturity, increased *Gibberella*.

Linear discriminant analysis effect size (LEfSe) analysis identified differentially enriched fungal genera across treatment groups, with statistical significance established through Kruskal-Wallis/Wilcoxon testing and discriminative features (LDA>2) illustrated in Appendix D. Biomarker analysis indicated that at the flowering stage, *Monocillium*, *Trichoderma*, *Pyxidiophora*, *Parasola*, *Paracylindrocarpon*, *Chloridium*, *Pseudogymnoascus*, *Eucasphaeria*, and *Curvularia* were characteristic of PK+R, while at the maturity stage, *Exophiala*, *Coniochaeta*, *Holtermanniella*, and *Scytalidium* were distinctive for PK+R.

3.2. Neutral community model and network structure of soil fungi

The NCM model was employed to assess the contribution of stochastic processes to the assembly of fungal communities by analyzing the relationship between the occurrence frequency and relative abundance of taxa (Fig. 2-A). Among fungal taxa, the R^2 and Nm values across treatments followed this pattern: PK+R (R^2=0.85, Nm=2,097.46)>PK (R^2=0.81, Nm=1,517.78)>CK (R^2=0.77, Nm=1,476.56)>PK+N (R^2=0.74, Nm=1,272.33). The PK+R treatment showed the highest m value (0.08), indicating greater stochastic process contribution to fungal community assembly in the *Bradyrhizobium* inoculation treatment. This treatment also showed the broadest fungal distribution and highest dynamic migration capacity, while nitrogen fertilization yielded the lowest values.

The correlation network diagram illustrated associations between fungal community compositions at the ASV level (Fig. 2-B). The most prevalent fungal phyla across treatments included Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Mortierellomycota. The PK+N treatment exhibited the highest number of nodes (264) and edges (978), while PK+R treatment showed the lowest nodes (215) and edges (621). Network topological analysis revealed distinct patterns: PK+N treatment demonstrated the highest node degree, while CK treatment showed the maximum clustering coefficient (P<0.05; Fig. 2-C).

3.3. Expression profiles of the differential metabolites

A total of 4,959 (positive ion mode) and 5,123 (negative ion mode) metabolites were detected in soybean rhizosphere soil. Based on the KEGG database, 546 metabolites were identified and annotated. PCA and PLS-DA analyses revealed distinct metabolite profiles in the rhizosphere soil during the flowering–podding and maturity stages of soybean, under different fertilization levels and *Bradyrhizobium* inoculation (Fig. 3-A and B). Both PCA (PC1=22.40%, PC2=14.50%) and PLS-DA plots (Component 1=36.90%, Component 2=13.50%) showed distinct treatment separation at the flowering–podding stage. However, treatments clustered together at the maturity stage.

The top 20 differential metabolites between treatments during the soybean flowering–podding and maturity stages are illustrated in Fig. 3-C. During the flowering–podding stage, the PK treatment enhanced the abundance of metabolites including 3-hydroxymethylantipyrine, chrysophanol, 3,7-dihydroxyflavone, (+/–)12,13-dihome, pinolenic acid, and P-chlorophenylalanine. The PK+N treatment demonstrated reduced expression levels of kojibiose and (+/–)12,13-dihome, while the PK+R treatment showed elevated expression levels of cassiamin C and 3-O-acetylepisamarcandin. During the maturity stage, the PK+N treatment exhibited elevated expression levels of pinolenic acid, (4E,8E,10E-d18:3) sphingosine, farnesyl acetone, and 9,12,15-octadecatrien-1-ol. In contrast, the PK+R treatment showed increased expression levels of cassiamin C, while geranylcitronellol, farnesyl acetone, 9,12,15-octadecatrien-1-ol and armillaripin demonstrated reduced expression levels. The findings indicate that nitrogen fertilization promotes the upregulation of specific lipids and nitrogen-containing compounds, whereas *Bradyrhizobium* inoculation exhibits a more pronounced influence on certain organic acids and secondary metabolites, particularly during the maturity stage.

3.4. Differential metabolites and KEGG pathway analysis in rhizosphere soil under different treatments

The volcano plot analysis of differential metabolites between

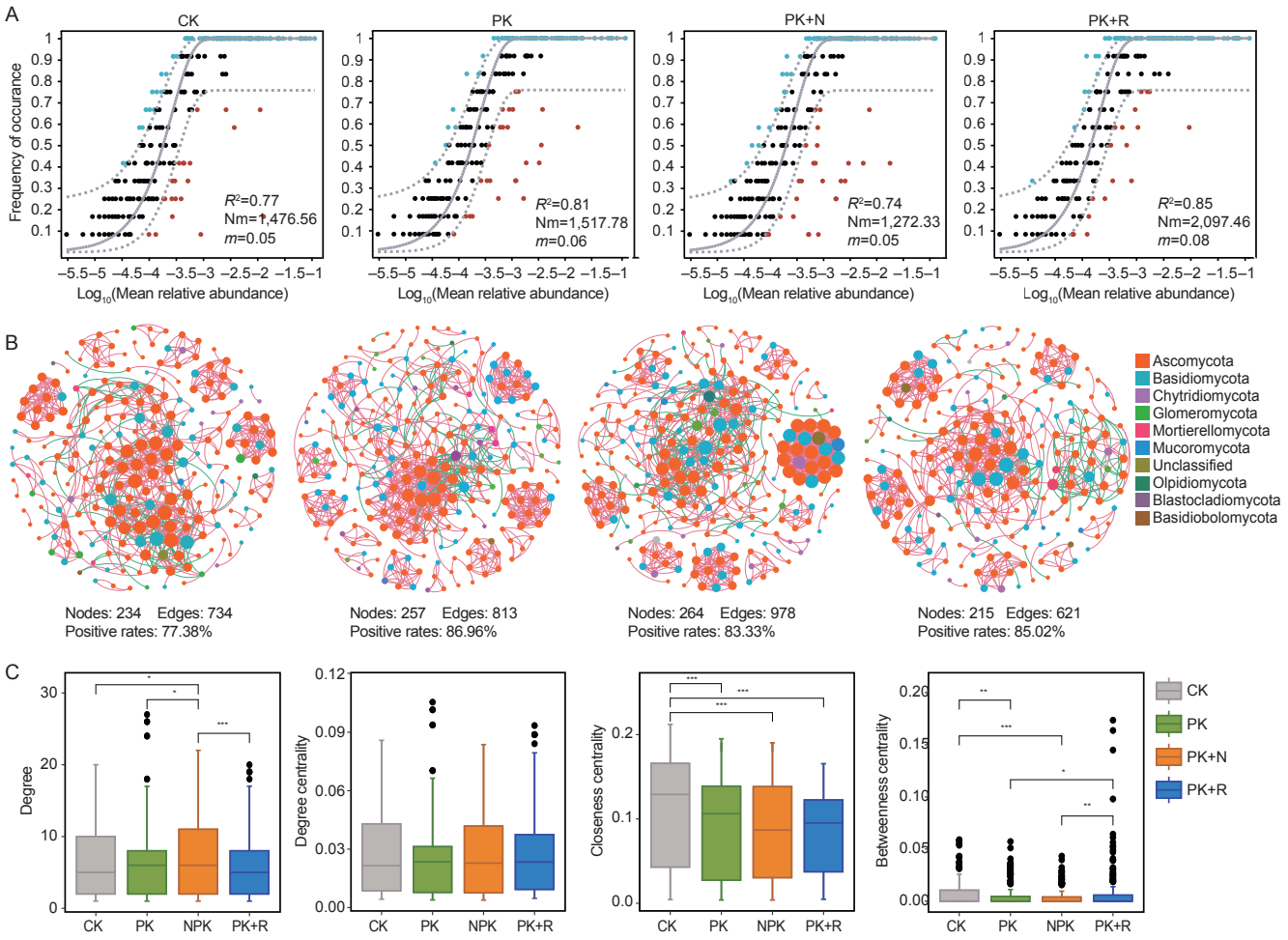


Fig. 2 Neutral processes dominate community assembly while fertilization restructures fungal network complexity. Neutral community models (NCM) are employed to evaluate the role of neutral processes in shaping community assembly at a general level (A). The 95% confidence intervals of the neutral model predictions are denoted by gray dashed lines. The goodness of fit to the models is evaluated using R^2 value. Nm estimates community dispersal by examining the correlation between occurrence frequency and regional relative abundance. The parameter *m* indicates community mobility at the landscape level. CK, no fertilizer application; PK, phosphorus and potassium fertilization; PK+N, PK chemical fertilizers combined with urea; PK+R, PK fertilization with *Bradyrhizobium* inoculation. The co-occurrence patterns of dominant fungal genera, with relative abundances greater than 0.1%, were analyzed across the CK, PK, PK+N, and PK+R treatments (B). Significant (P<0.01) and strong (Spearman's $|r|$>0.8) correlations are indicated by connections between nodes. Each node represents a fungal phylum, where node size corresponds to connectivity degree and color indicates the specific phylum. The degrees and clustering coefficient values of fungal co-occurrence networks under different treatments are presented (C). *, P<0.05; **, P<0.01; ***, P<0.001.

various treatments during the flowering–podding and maturity stages of soybean (VIP>1, P<0.05) is presented in Fig. 4. During the flowering–podding stage, compared to CK, 214, 123, and 204 metabolites were significantly upregulated in the PK, PK+N, and PK+R treatments, respectively, while 32, 76, and 23 metabolites were significantly downregulated. The organic oxide 3-methyl-3-buten-1-ol emerged as a consistently downregulated metabolite across all treatments. When comparing PK to PK+N and PK+R, 31 and 102 metabolites were significantly upregulated, while 169 and 77 metabolites were significantly downregulated. Several metabolites, including (+/–)12,13-dihome, (+/–)9-hpode, porphobilinogen, 9(R)-hode, 12-hydroxyheptadecanoic acid, and 13(S)-hode, exhibited consistent downregulation across treatments. Porphobilinogen, an organic nitrogen compound, participates in metabolic pathways including secondary metabolite biosynthesis, porphyrin and chlorophyll metabolism, and microbial metabolism in diverse environments. The comparison between PK+N and PK+R revealed 199 significantly upregulated metabolites and 29 significantly downregulated metabolites. Lathodoratin, achieving the highest VIP score, emerged as the most significantly upregulated metabolite, while the organic heterocyclic compound 2-hydroxydesipramine showed the most significant downregulation (Fig. 4-A).

During the soybean maturity stage, 174, 109, and 133 metabolites demonstrated significant upregulation in the PK, PK+N, and PK+R treatments, respectively, compared to CK, while 33, 64, and 56 metabolites showed significant downregulation. Secoeremopetasitolide A emerged as a consistently downregulated metabolite across all treatments, while 7(14)-farnesene-9,12-

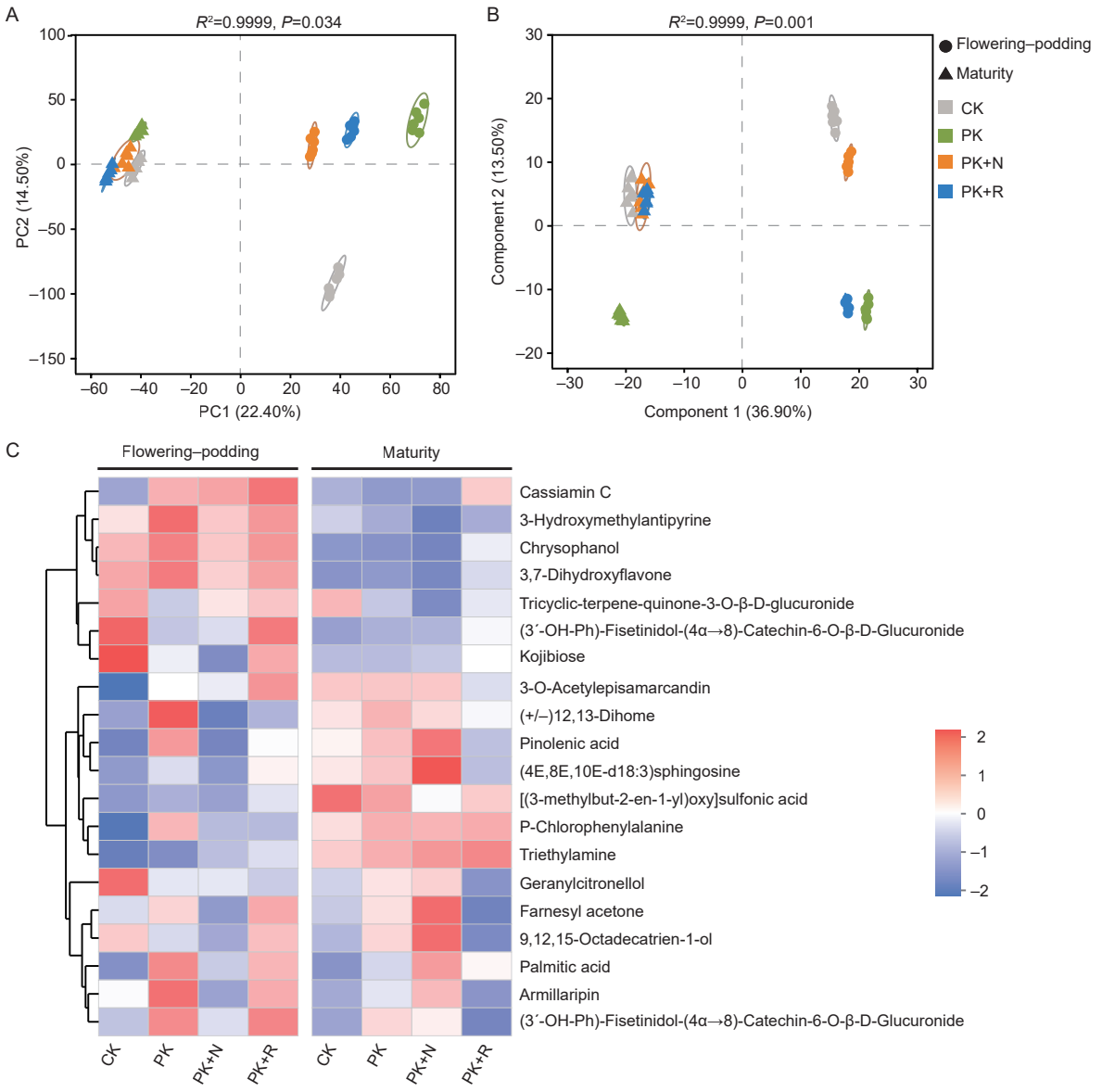


Fig. 3 Multivariate analysis reveals treatment-driven and growth-stage-specific metabolomic shifts in soybean rhizosphere. Principal component analysis (PCA) of metabolites is shown with data points color-coded by treatment and shaped according to growth stage (flowering-podding or maturity) (A). CK, no fertilizer application; PK, phosphorus and potassium fertilization; PK+N, PK chemical fertilizers combined with urea; PK+R, PK fertilization with *Bradyrhizobium* inoculation. Partial least squares discriminant analysis (PLS-DA) illustrates significant variations among different treatments (B). Multivariate analysis (PCA/PLS-DA) was integrated with Adonis for statistical inference. Essential differential metabolites (top 20) among treatments across growth stages (C).

diol and hexadecanedioic acid maintained consistent upregulation. These three metabolites belong to the lipids and lipid-like molecules classification. In comparing PK to PK+N and PK+R, 58 and 68 metabolites showed significant upregulation, while 137 and 121 metabolites exhibited downregulation, respectively. Common downregulated metabolites in these treatments included 1-pentadecanecarboxylic acid, fraxetin, glyyunnansapogenin B (a lipid-like molecule), and porphobilinogen (an organic nitrogen compound). The PK+N versus PK+R comparison revealed 103 significantly upregulated metabolites and 85 downregulated metabolites. The compound 6-[(E)-2-(4-hydroxyphenyl) ethenyl]-4-methoxy-5,6-dihydro-2H-pyran-2-one achieved the highest VIP score among upregulated metabolites, while cucurbitacin C, a lipid-like molecule, demonstrated the highest VIP score among downregulated metabolites (Fig. 4-B).

The topological analysis of KEGG pathway enrichment is presented in Fig. 5. During the flowering–podding stage, significant pathways include tryptophan metabolism, caffeine metabolism, and pyrimidine metabolism (Fig. 5-A). Notable differences are seen in pathways such as sphingolipid metabolism and taurine and hypotaurine metabolism across different comparisons. PK treatments demonstrate significant enrichment in tryptophan metabolism, particularly in the PK *vs*. PK+N and PK+R comparisons. In the maturity stage, similar patterns are

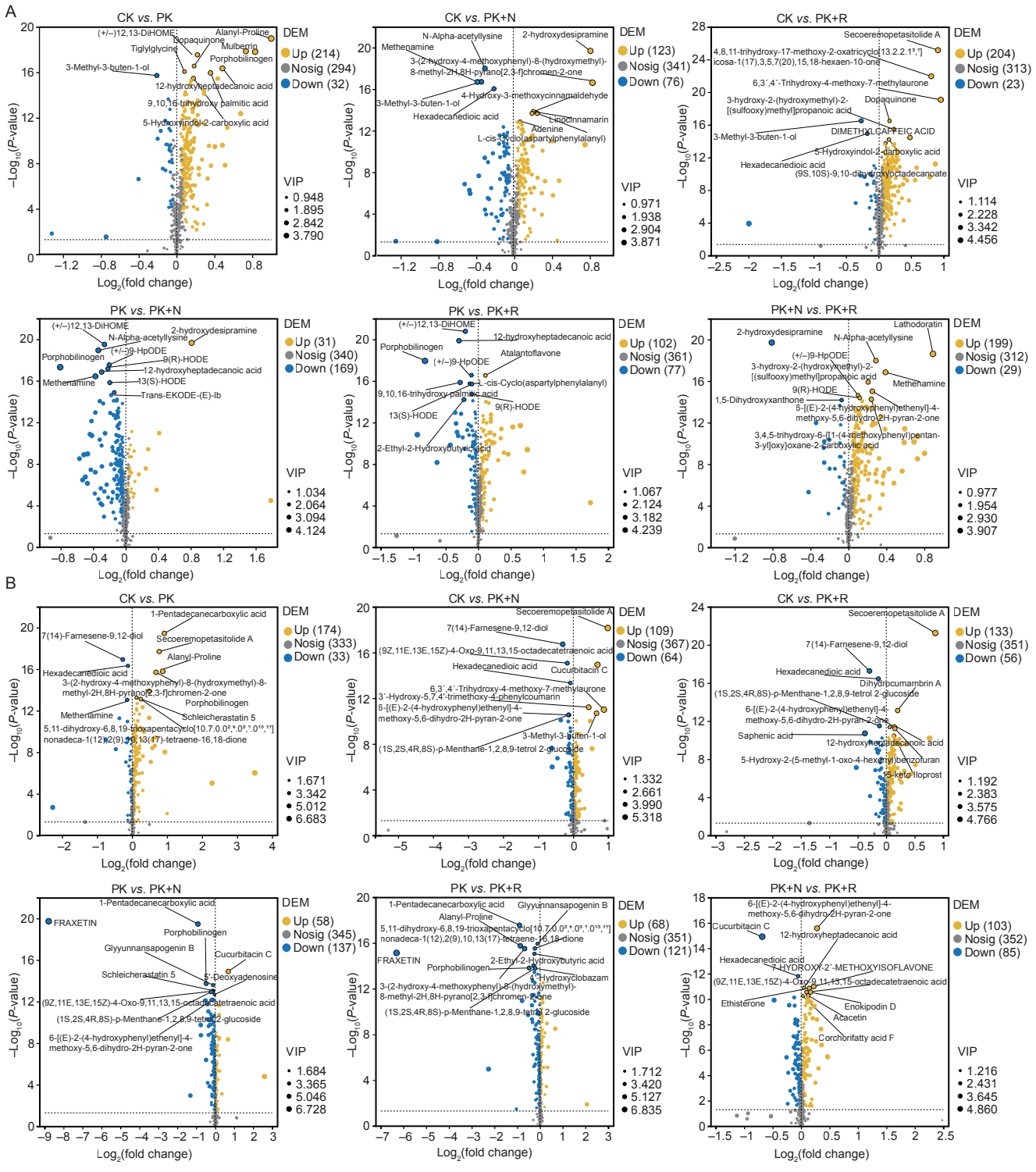


Fig. 4 The volcano plots illustrate differential metabolites across treatments during the soybean flowering–podding stage (A) and maturity stage (B). CK, no fertilizer application; PK, phosphorus and potassium fertilization; PK+N, PK chemical fertilizers combined with urea; PK+R, PK fertilization with *Bradyrhizobium* inoculation. DEM, different expressed metabolites. The variable importance in the projection (VIP) value indicates the magnitude of each metabolite's inter-group differences on the classification and discrimination of samples in each group within the model. Each subplot highlights metabolites that are significantly upregulated (yellow) or downregulated (blue), with the top 10 VIP-value metabolites labeled. Gray dots represent nonsignificant differential metabolites.

observed, with a focus on tryptophan metabolism, caffeine metabolism, and taurine and hypotaurine metabolism (Fig. 5-B). The PK *vs*. PK+N comparison emphasizes caprolactam degradation and sphingolipid metabolism, while caffeine metabolism remains consistently prominent across all treatments. These pathways represent critical metabolic routes among the differential metabolites and may be linked to metabolic processes and biological

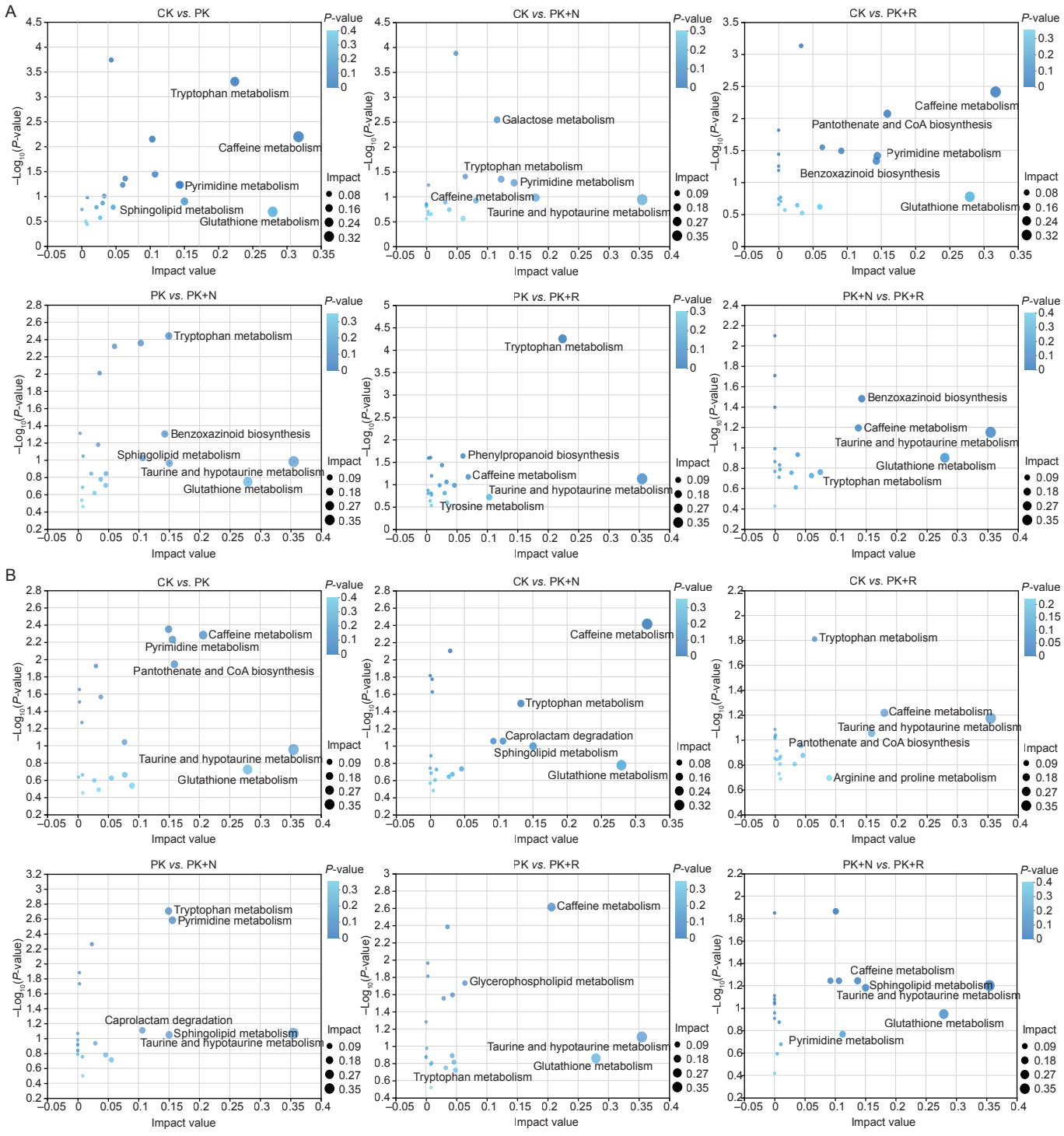


Fig. 5 KEGG pathway enrichment analysis of differential metabolites between treatments during the flowering–podding (A) and maturity (B) stages of soybean. The five pathways with the highest impact values are indicated. CK, no fertilizer application; PK, phosphorus and potassium fertilization; PK+N, PK chemical fertilizers combined with urea; PK+R, PK fertilization with *Bradyrhizobium* inoculation.

activities under various treatment conditions.

3.5. Correlation between key differential metabolites and dominant fungal communities and the effects of physicochemical factors on fungal structure and metabolite profiles

The Spearman correlations between key differential metabolites and bacterial and fungal communities are depicted in Fig. 6-A. The fungal genera *Neonectria*, *Cephalotrichum*, *Tausonia*, *Solicoccozyma*, *Cadophora*, and *Clonostachys* demonstrate significant negative correlations with 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone, while exhibiting significantly

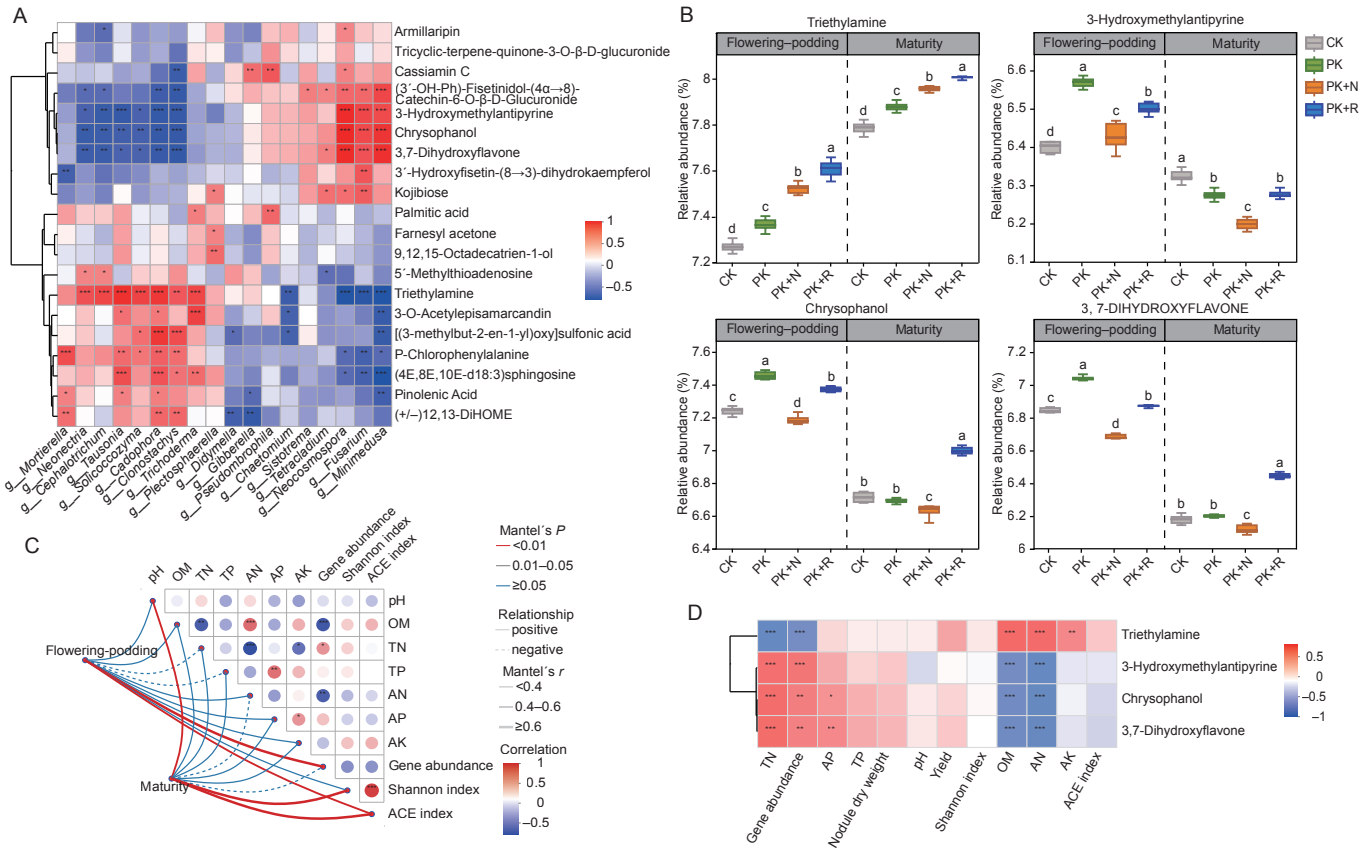


Fig. 6 Integrated analysis of metabolite-fungal-physicochemical interactions in the soybean rhizosphere. Spearman correlation analysis between key differential metabolites and fungal genera (A). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. The abundance of four key metabolites in soybean rhizosphere soil during the flowering-podding and maturity stages across different treatments (B). Lowercase letters indicate significant differences between treatments within the same growth stage. CK, no fertilizer application; PK, phosphorus and potassium fertilization; PK+N, PK chemical fertilizers combined with urea; PK+R, PK fertilization with *Bradyrhizobium* inoculation. The Mantel test evaluated the influence of physicochemical factors on the fungal community structure at different growth stages (C). The physicochemical factors comprised pH, OM, TN, TP, available nitrogen (AN), available phosphorus (AP), and available potassium (AK). The heatmap illustrates the impact of these physicochemical factors on key metabolites (D).

positive correlations with triethylamine. In contrast, the fungal genera *Neocosmospora*, *Fusarium*, and *Minimedusa* display significant positive correlations with 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone, while showing significantly negative correlations with triethylamine. These results indicate that these four metabolites serve a crucial role in soil microbial metabolic activities. The findings suggest a complex interaction between fungal communities and specific metabolites, potentially indicating niche adaptations or metabolic dependencies.

The relative abundance of four key metabolites — triethylamine, 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone — in soybean rhizosphere soil at the flowering–podding and maturity stages across different treatments varied significantly (Fig. 6-B). The relative abundance of triethylamine followed a distinct pattern: PK+R>PK+N>PK>CK in both stages. Nitrogen fertilization markedly reduced the abundance of 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone across both growth stages. These metabolites exhibited peak abundance under PK treatment during the flowering–podding stage and under PK+R at maturity. These findings indicate that nitrogen fertilization and *Bradyrhizobium* inoculation decreased the levels of these metabolites during the flowering–podding stage, while *Bradyrhizobium* inoculation increased them during the maturity stage.

The Mantel test revealed the influence of physicochemical factors on the soil fungal community structure (Fig. 6-C). During the soybean flowering–podding stage, the fungal community structure exhibited significant positive correlations with fungal gene abundance and the ACE index, with no significant impact from other physicochemical factors. At the maturity stage, the structure demonstrated strong correlations with soil pH, Shannon index, and ACE index. The heatmap analysis identified significant correlations between four key metabolites and soil TN, OM, available nitrogen (AN), and fungal gene abundance (Fig. 6-D). Triethylamine showed negative correlations with both TN and fungal gene abundance, while 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone displayed positive correlations with these factors. Triethylamine demonstrated positive correlations with OM and AN, whereas the latter three

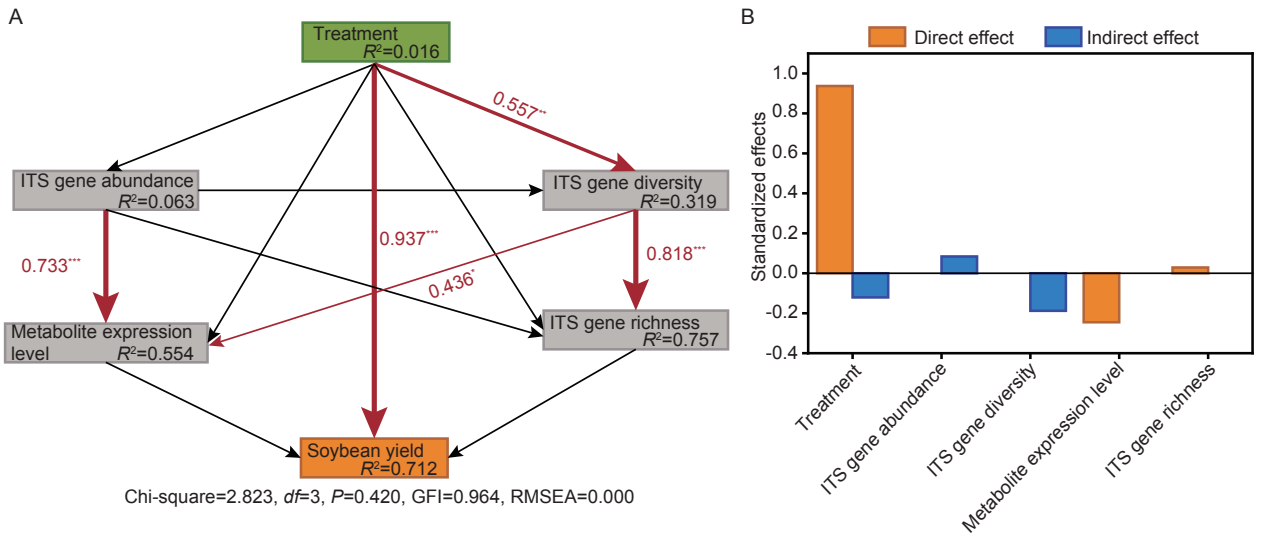


Fig. 7 Structural equation modeling (SEM) analysis of the interactions between treatment, microbiome, and metabolites underlying soybean yield. The SEM demonstrates relationships among treatment, ITS gene abundance, ITS gene diversity (Shannon index), metabolite expression level (component 1 of PLS-DA), ITS gene richness (ACE index), and soybean yield (A). Red arrows indicate significant positive paths (P<0.05). The black line represents non-significant paths. Arrow thickness corresponds to the magnitude of significance, with thicker arrows denoting higher significance. Numbers adjacent to arrows represent path coefficients, indicating the strength and direction of relationships between variables. The R^2 values indicate the proportion of variance explained by each endogenous variable. *, P<0.05; **, P<0.01; ***, P<0.001. The standardized direct and indirect effects of different indicators on soybean yield are illustrated in bar plots (B).

metabolites showed negative correlations with OM and AN.

In the SEM analysis (Fig. 7-A), treatment demonstrated strong positive effects on fungal diversity (path coefficient=0.557, P<0.01) and soybean yield (path coefficient=0.937, P<0.001). Fungal abundance (path coefficient=0.733, P<0.001) and diversity (path coefficient=0.436, P<0.05) showed positive associations with metabolite expression levels. Notably, fungal diversity directly enhanced taxonomic richness (path coefficient=0.818, P<0.001). Standardized direct/indirect effects are quantified in Fig. 7-B.

4. Discussion

4.1. Response of fungal community structure to long-term fertilization and *Bradyrhizobium* inoculation

Our results reveal significant findings regarding fungal population dynamics and their implications for soil health and plant performance. The PK treatment yielded the highest fungal gene copy number during the soybean flowering–podding stage but demonstrated significantly lower alpha diversity indices compared to the PK+N and PK+R treatments (Fig. 1-A), indicating a complex relationship between nutrient availability and microbial community structure in the rhizosphere (Philippot *et al.* 2013). The elevated fungal biomass in the PK treatment may indicate dominance by phosphorus-specialized taxa rather than greater diversity. This reduced diversity could restrict the community's functional capacity, as specialized communities may lack the ecological resilience characteristic of diverse systems. Conversely, the PK+N and PK+R treatments maintained a wider range of fungal taxa, fostering a more balanced, functionally diverse microbial ecosystem. The diversity patterns suggest that nitrogen fertilization and *Bradyrhizobium* inoculation significantly promote microbial diversity. Nitrogen, crucial for plant and microbial growth, supports diverse fungal taxa, while *Bradyrhizobium* inoculation enhances fungal diversity through mutualistic plant interactions (Mus *et al.* 2016).

The stage-specific reduction in ITS gene copy numbers under PK+N during flowering–podding, compared with that during maturity, reflects nitrogen-mediated fungal suppression followed by compensatory shifts. Elevated mineral nitrogen in flowering–podding likely intensified bacterial competition for labile carbon and induced fungal C/N/P stoichiometric imbalance, suppressing biomass (Das *et al.* 2022; Wang and Kuzyakov 2024). By maturity, soybean's high nitrogen demand during pod filling depleted soil mineral N, alleviating fungal inhibition, while phenolic acid-enriched root exudates stimulated stress-tolerant fungal taxa (Branco *et al.* 2022), restoring community abundance. This dynamic highlights how phenology-driven resource reallocation modulates fungal responses to fertilization.

This divergence in community structure between the phosphorus-only (PK) treatment and the nitrogen or *Bradyrhizobium*-inoculated treatments raises important questions about the trade-offs between nutrient inputs and microbial community dynamics. Although phosphorus may enhance fungal growth and activity, its effects on microbial diversity might prove limiting over time (Lekberg *et al.* 2021). The reduced diversity in the PK treatment may compromise the community's adaptability to environmental changes, potentially increasing ecosystem vulnerability. Conversely, the enhanced diversity in the PK+N and PK+R treatments

suggests greater community resilience and functional capacity. These observations emphasize the importance of considering both microbial abundance and diversity when developing fertilization strategies for sustainable agriculture. Optimizing nutrient inputs to balance fungal abundance and diversity appears crucial for maintaining healthy soil ecosystems.

The relationship between fungal community indicators and soybean physiological traits, including nodule dry weight and yield, further elucidated these trends (Appendix B). At the flowering–podding stage, a significant positive correlation between fungal gene copy number and nodule dry weight suggests that fungal populations may play a supportive role in nitrogen fixation or other processes beneficial to plant growth. In contrast, at maturity, a significant negative correlation was found between fungal gene abundance and soybean yield, indicating that fungal communities might become less beneficial or even inhibitory to plant productivity as the crop matures (van der Heijden *et al.* 2008).

At the genus level, distinct shifts in fungal composition were observed in response to fertilization treatments (Appendix C). Specifically, nitrogen fertilization during the flowering–podding stage enriched fungal genera such as *Gibberella* and *Cephalotrichum*, while significantly reducing *Neocosmospora* in both growth stages. These findings suggest that nitrogen fertilization may alter the competitive balance of fungal genera, favoring those that are better adapted to the enriched nitrogen environment (Cao *et al.* 2024). On the other hand, *Bradyrhizobium* inoculation during the flowering–podding stage enriched *Tausonia* and *Trichoderma*, potentially enhancing beneficial fungal communities that could support plant health and growth, while reducing the abundance of *Minimedusa*. At maturity, *Gibberella* was particularly enriched in the *Rhizobium* treatment, suggesting a possible interaction between this genus and the soybean's symbiotic nitrogen-fixing processes (Bender *et al.* 2016).

The application of the NCM model in this study provides valuable insights into the relative contributions of deterministic and stochastic processes to fungal community assembly. The analysis of the relationship between taxa occurrence frequency and relative abundance revealed significant differences across treatments, with the PK+R treatment exhibiting the highest R^2 and Nm values, indicating a stronger influence of stochastic processes in this treatment. In contrast, the PK+N treatment showed the lowest R^2 and Nm values, suggesting a more deterministic assembly influenced by nitrogen fertilization.

The observation that the PK+R treatment also exhibited the widest distribution of fungal taxa and the highest dynamic migration capacity further supports the role of stochastic processes in shaping fungal communities in the presence of *Bradyrhizobium* inoculation. Stochastic processes, including dispersal and drift, promote increased community turnover and diversity (Evans *et al.* 2017), potentially explaining the enhanced fungal community variability in the PK+R treatment. This contrasts with the more structured and predictable community assembly observed in the nitrogen-fertilized treatment (PK+N), where deterministic processes appear to exert primary influence (Zhou and Ning 2017).

The correlation network analysis reinforces these findings, offering additional insight into fungal interactions across treatments. The PK+N treatment exhibited the highest number of nodes and edges, indicating a more interconnected and intricate fungal community structure, potentially attributable to the diverse microbial interactions promoted by nitrogen fertilization (Levy-Booth *et al.* 2014). In contrast, the PK+R treatment, characterized by fewer nodes and edges, indicates a less interconnected fungal network, presumably influenced by the more stochastic nature of community assembly under *Bradyrhizobium* inoculation (Burghardt *et al.* 2022).

4.2. Metabolic profiling and differential metabolite distribution in the soybean rhizosphere

This investigation reveals the complex relationship between fertilization strategies and metabolic profiles within the soybean rhizosphere during key growth stages. The examination of the top 20 differential metabolites across treatments during soybean flowering–podding and maturity stages demonstrates how specific fertilization approaches distinctly influence the rhizosphere's metabolite composition, reflecting targeted biochemical responses to potassium-phosphorus, nitrogen, and *Bradyrhizobium* inoculation (Fig. 3-C).

During the flowering–podding stage, the PK treatment enhances metabolites including 3-hydroxymethylantipyrine and chrysophanol, which are linked to stress response and antioxidant pathways (Wu *et al.* 2020). The elevated presence of 3-hydroxymethylantipyrine indicates its significant role in strengthening plant resistance against environmental stressors such as drought and pathogen attacks (Cheng *et al.* 2022). Through enhancement of antioxidant activity, this metabolite aids in reducing oxidative stress in plants, thus supporting overall health and growth. Similarly, chrysophanol, known for its anti-inflammatory properties, demonstrates significant correlation with beneficial microbial genera, suggesting its capacity to promote advantageous plant–microbe interactions. This enhances nutrient availability and uptake while potentially improving soil health through increased microbial diversity and activity, essential factors for effective nutrient cycling (Cheng *et al.* 2018).

Conversely, the PK+N treatment shows reduced levels of certain lipid compounds, including kojibiose and (+/–)12,13-dihome. This finding suggests a potential reallocation of resources toward nitrogen assimilation, potentially altering the rhizosphere's metabolic profile (Balazadeh *et al.* 2014). Furthermore, triethylamine demonstrates positive correlation with specific nitrogen-fixing bacterial genera, emphasizing its function in nitrogen metabolism. However, its negative correlation with antioxidant metabolites suggests that increased triethylamine levels may interfere with stress response metabolic pathways, potentially increasing plant vulnerability to environmental stressors.

As plants progress to maturity, nitrogen fertilization (PK+N) consistently increases lipids and nitrogenous metabolites, including pinolenic acid and sphingosine. This corresponds

with the plant's increased energy and nitrogen requirements approaching senescence (Thomas 2013). Simultaneously, the PK+R treatment promotes higher levels of secondary metabolites such as cassiamin C, likely resulting from enhanced microbial interactions and signaling. This indicates that while nitrogen promotes growth-related pathways, *Bradyrhizobium* inoculation serves a crucial function in enhancing secondary metabolite production that supports microbial defense mechanisms (Zia *et al.* 2021).

The volcano plot analysis of differential metabolites across treatments during the flowering–podding and maturity stages of soybean reveals how fertilization strategies and *Bradyrhizobium* inoculation affect rhizosphere metabolic profiles (Fig. 4). Each treatment generated a distinct pattern of metabolite upregulation and downregulation, highlighting the specific metabolic changes associated with each intervention.

During the flowering–podding stage, significant differences emerge when comparing the PK, PK+N, and PK+R treatments to the CK. The PK treatment exhibited the most substantial upregulation (214 metabolites) and fewer downregulated metabolites (32), indicating that potassium and phosphorus amendments alone may enhance a wider spectrum of metabolic activities compared to treatments incorporating additional nitrogen (PK+N and PK+R) (Han *et al.* 2020). The uniform downregulation of 3-methyl-3-buten-1-ol across all treatments suggests a shared metabolic response, potentially representing a common adaptation to fertilization (Zhang *et al.* 2022). Interestingly, metabolites associated with nitrogen metabolism, such as porphobilinogen, were downregulated in comparison between PK and PK+N or PK+R. This finding suggests that additional nitrogen from either fertilization or *Bradyrhizobium* inoculation may alter nitrogenous compound dynamics, potentially overshadowing the effects of potassium and phosphorus alone.

At the maturity stage, a significant transition occurs, with each treatment maintaining distinct metabolic profiles but exhibiting fewer differentially expressed metabolites compared to the flowering–podding stage. This decrease indicates a potential convergence of metabolic processes as the soybean reaches maturity, wherein primary metabolic pathways may stabilize (Huang *et al.* 2020). The PK treatment maintains a distinctive profile, with marked upregulation of metabolites such as 7(14)-farnesene-9,12-diol and hexadecanedioic acid, both lipid-like molecules, demonstrating the sustained influence of potassium and phosphorus on lipid metabolism (Wang *et al.* 2021). Conversely, nitrogen-related metabolites such as porphobilinogen and fraxetin showed consistent downregulation when comparing PK to PK+N and PK+R, reinforcing the concept that nitrogen-containing treatments establish a distinct nitrogen-driven metabolic environment in the rhizosphere (Yue *et al.* 2023). This nitrogen-mediated metabolic shift likely affects microbial communities and nitrogen cycling processes, distinguishing the metabolic environment from those primarily influenced by potassium and phosphorus (Baker *et al.* 2024).

The upregulation of specific metabolites in the PK+N *vs*. PK+R comparison, such as 6-[(E)-2-(4-hydroxyphenyl) ethenyl]-4-methoxy-5,6-dihydro-2H-pyran-2-one, alongside the downregulation of cucurbitacin C, demonstrates how nitrogen fertilization and *Bradyrhizobium* inoculation distinctly influence secondary metabolite pathways. These differential expressions may indicate the interactive effects of *Bradyrhizobium* inoculation, which introduces nitrogen-fixing bacteria that can influence microbial composition and alter metabolic pathways differently from nitrogen fertilization alone (Wei *et al.* 2025).

The KEGG pathway enrichment analysis reveals additional insights into the metabolic pathways affected by various fertilization strategies across soybean growth stages (Fig. 5). During the flowering–podding stage, pathways including tryptophan metabolism, caffeine metabolism, and pyrimidine metabolism demonstrate significant enrichment, indicating an early metabolic emphasis on amino acid biosynthesis, nucleotide metabolism, and plant–microbe interactions (Bhadwal *et al.* 2024). Variations in sphingolipid metabolism and taurine and hypotaurine metabolism across treatments suggest that lipid and sulfur-containing compounds serve crucial functions at this growth stage, potentially enhancing stress resilience and signaling (Liu *et al.* 2024).

Significantly, tryptophan metabolism shows enrichment in the PK treatment, particularly when compared to PK+N and PK+R, indicating that potassium and phosphorus may enhance auxin-related pathways connected to tryptophan, which are essential for root and rhizosphere development, nutrient uptake, and stress response (Yang *et al.* 2024). At maturity, similar patterns of pathway enrichment continue, with tryptophan, caffeine, and taurine and hypotaurine metabolism remaining prominent. This persistence suggests these pathways maintain important roles in adaptation and growth. The comparison of PK *vs*. PK+N at maturity also reveals significant enrichment in caprolactam degradation and sphingolipid metabolism, while caffeine metabolism remains notable across treatments. These findings suggest an ongoing requirement for nitrogen-containing compounds, lipid-based signaling molecules, and caffeine-related compounds, potentially supporting defense and energy regulation (Nehlig 2018).

4.3. Abundance of key differential metabolites across treatments and essential metabolic pathways

The Spearman correlation analysis of key differential metabolites with fungal communities (Fig. 6-A) demonstrates significant relationships that indicate complex metabolic and ecological interactions within the soil microbial community. The fungal genera *Neonectria*, *Cephalotrichum*, *Tausonia*, *Solicoccozyma*, *Cadophora*, and *Clonostachys* display significant negative correlations with the metabolites 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone, while exhibiting positive correlations with triethylamine. This pattern indicates that these metabolites may function as inhibitors or byproducts that restrict the growth or metabolic activities of specific fungal taxa, thus influencing community composition. The positive correlation with triethylamine suggests these genera may be metabolically adapted to utilize or tolerate this compound,

indicating functional specialization (Pii *et al.* 2015).

Conversely, *Neocosmospora*, *Fusarium*, and *Minimedusa* demonstrate positive correlations with 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone, while exhibiting negative correlations with triethylamine. This inverse relationship suggests these fungal genera may be better adapted to specific metabolic pathways associated with the aforementioned metabolites, potentially utilizing them as carbon or nitrogen sources. The negative correlation with triethylamine indicates these taxa may be sensitive to this compound or lack efficient metabolic pathways for its utilization, thus determining their ecological niche within the soil microbial community. These observations emphasize the essential role of these four metabolites in determining fungal community structure and function in the soil (Farrar *et al*. 2014).

The analysis of four key metabolites illustrates the dynamic response of soybean rhizosphere composition to various fertilization strategies across growth stages (Fig. 6-B). Triethylamine exhibited a consistent pattern across both growth stages, with the highest relative abundance in the PK+R treatment, followed by PK+N, PK, and CK. This distribution highlights the significant impact of *Bradyrhizobium* inoculation in increasing nitrogen-containing organic compounds, likely due to enhanced nitrogen fixation and microbial interactions within the rhizosphere (Hernández *et al.* 2009). Furthermore, nitrogen fertilization decreased the abundance of antioxidant and stress-related metabolites, including 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone, across both stages, with peak concentrations observed in PK treatment during flowering–podding and in PK+R at maturity. This suggests nitrogen addition may suppress specific metabolic pathways associated with stress tolerance, while *Bradyrhizobium* inoculation enhances these pathways, particularly at maturity when microbial interactions intensify (Dunn 2015; Frantzeskakis *et al.* 2020). These findings indicate that nitrogen fertilization and *Bradyrhizobium* inoculation exert stage-specific effects on rhizosphere metabolite profiles. Nitrogen initially reduces certain metabolite levels but correlates with increased abundance during maturity, while *Bradyrhizobium* inoculation enhances key metabolites later in development.

The Mantel test indicated that physicochemical factors significantly influence fungal community structure, with distinct patterns at different growth stages. During soybean flowering–podding, fungal community composition correlated strongly with fungal gene abundance and the ACE index, while at maturity, soil pH and the Shannon and ACE indices exhibited stronger influences (Siciliano *et al.* 2014). Heatmap analysis demonstrated that key metabolites maintained close associations with soil physicochemical parameters. Triethylamine displayed negative correlations with TN and fungal gene abundance, suggesting potential fungal growth inhibition. Conversely, 3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone showed positive correlations with these factors, indicating their potential role in supporting fungal activity. The positive correlation of triethylamine with OM and available nitrogen (AN), and the negative correlation of the latter metabolites (3-hydroxymethylantipyrine, chrysophanol, and 3,7-dihydroxyflavone) with OM and AN, suggests divergent metabolic pathways influencing fungal communities (Liu *et al.* 2010). These results demonstrate complex interactions between soil nutrients, metabolites, and fungal communities, indicating that specific metabolites may either enhance or inhibit fungal activity, thereby influencing microbial community dynamics in the rhizosphere (Trivedi *et al.* 2016; Zheng *et al.* 2019).

Based on the negative correlation between metabolites and fungi (for example, triethylamine inhibits *Neocosmospora*), reducing nitrogen fertilizer application during the flowering stage is recommended to maintain antioxidant metabolite levels, while integrating *Bradyrhizobium* inoculation with secondary metabolite enhancement during the maturation stage to optimize soil health and yield. Although correlation analysis has revealed associations between metabolites and fungal communities, subsequent sterile pot experiments are necessary to verify the direct inhibitory effects of the four key metabolites on dominant fungi. Furthermore, metagenomic sequencing will be utilized to investigate the underlying molecular mechanisms.

Furthermore, our results demonstrated that fertilization and *Bradyrhizobium* inoculation directly shaped fungal communities and enhanced soybean yield, aligning with prior research showing fertilization and inoculation restructure rhizosphere microbiomes to influence plant growth (Xun *et al.* 2015) (Fig. 7). The positive correlations between fungal traits and metabolite expression levels suggest a microbe-driven metabolic regulation mechanism. Enhanced fungal diversity promoted taxonomic richness, consistent with the ecological principle of niche complementarity (Brakhage 2013).

5. Conclusion

This study illuminates the complex relationship between fertilization strategies, soil fungal communities, and metabolic profiles within the soybean rhizosphere during critical growth stages. While PK treatment supports fungal biomass, it results in reduced fungal diversity, potentially compromising community resilience. In contrast, nitrogen fertilization (PK+N) and *Bradyrhizobium* inoculation (PK+R) promote broader fungal diversity, potentially enhancing ecosystem stability and functional capacity. These treatments foster more interconnected and resilient fungal communities, particularly nitrogen fertilization, which increases community complexity and modifies microbial interactions. Metabolic profiling reveals that specific metabolites, including 3-hydroxymethylantipyrine, chrysophanol, and triethylamine, serve crucial roles in shaping fungal community structure and supporting plant growth. Nitrogen fertilization and *Bradyrhizobium* inoculation distinctly alter rhizosphere metabolite profiles, with nitrogen-driven shifts in lipid and nitrogenous metabolites supporting plant growth during maturity, while *Bradyrhizobium* inoculation enhances secondary metabolite production, promoting beneficial plant–microbe interactions. Additionally, correlation analyses highlight the influence of soil physicochemical factors, such as total nitrogen and organic matter, on fungal community

structure, further demonstrating the intricate relationship between microbial activity and soil nutrient availability. These findings emphasize the importance of considering both microbial diversity and metabolic pathways in developing sustainable fertilization strategies that optimize plant health and soil resilience. Future research should investigate the mechanistic links between specific metabolites and microbial interactions to refine these strategies for long-term agricultural sustainability.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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