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Research paper

Temporal shifts in root exudates driven by vegetation restoration alter rhizosphere microbiota in *Robinia pseudoacacia* plantations

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Plant–soil–microbiota interactions mediated by root exudates regulate plant growth and drive rhizosphere microbial feedbacks. It remains unknown how root exudates affect rhizosphere microbiota and soil functions in the course of forest plantation restoration. The metabolic profiles of tree root exudates are expected to shift with stand age, leading to variation in rhizosphere microbiota structure, and in turn, potentially altering soil functions. To unravel the effects of root exudates, a multi-omics study was conducted using untargeted metabonomic profiling, high-throughput microbiome sequencing and functional gene array. The interactions among root exudates, rhizosphere microbiota and nutrient cycling-related functional genes were explored under 15- to 45-year-old *Robinia pseudoacacia* plantations in the Loess Plateau region of China. Root exudate metabolic profiles, rather than chemodiversity, markedly changed with an increase in stand age. A total of 138 age-related metabolites were extracted from a key module of root exudates. The relative contents of six biomarker metabolites, such as glucose-1-phosphate, gluconic acid and N-acetylneuraminic acid, increased distinctly over time. The biomarker taxa (16 classes) of rhizosphere microbiota varied in a time-sensitive manner, which played potential roles in nutrient cycling and plant health. *Nitrospira*, *Alphaproteobacteria* and *Acidobacteria* were enriched in the rhizosphere of older stands. Key root exudates influenced functional gene abundances in the rhizosphere via direct effects or indirectly through biomarker microbial taxa (e.g., *Nitrososphaeria*). Overall, root exudates and rhizosphere microbiota are essential for soil function maintenance in *R. pseudoacacia* plantation restoration.

Keywords: black locust, chemical composition, chemodiversity, Loess Plateau, rhizosphere microbial community.

Introduction

Vegetation restoration is essential for environmental protection, soil erosion control, land quality improvement and terrestrial ecosystem remediation (Wang et al. 2018, Zhang et al. 2019). In restored ecosystems, the role of soil microbiota in nutrient cycling has been demonstrated (Jiao et al. 2018, Li et al. 2022), but complex and hardly tracked rhizosphere processes are still poorly understood. The rhizosphere is a critical, densely populated narrow zone of soil surrounding plant roots, with active biogeochemical transformation and complex signaling processes (Zhalnina et al. 2018, Li et al. 2020). Root exudates provide nutrients that could stimulate microbial activity or suppress microbiome function in the rhizosphere, thereby contributing the biogeochemical cycling of carbon (C) and nitrogen (N) (Li et al. 2020, Meier et al. 2017, Panchal et al. 2022, Shen et al. 2020).

Rhizosphere microbiota perceive root exudates and release signals to modulate the host plant, establishing a feedback loop (Bai et al. 2022). Despite evidence of microbial functions indirectly controlling plant growth (Hou et al. 2021, Ling et al. 2022), current understanding of plant–soil–microbiota interactions induced by root exudates, especially in the course of vegetation restoration, is still limited. Under long-term N deposition, soil microbial communities shift depending on resource availability, rather than plant community composition (Brigham et al. 2022). In addition, maize plants regulate rhizosphere

bacterial interactions by altering root exudate composition in response to N deficiency (Hao et al. 2022). Hence, releasing root exudates may be a strategy for plants to interact with rhizosphere microbiota based on specific nutrient requirements (Zhao et al. 2021).

Plant roots compete for space, water and nutrients to facilitate survival, whereas rhizosphere microbiota exploit nutrient sources surrounding the roots (Sasse et al. 2018). Plant–soil–microbiota interactions are recognized as plant feedbacks to abiotic and biotic factors (Zhang et al. 2017). The biotic interactions regulated by root exudates are either beneficial to plants, for example, in the form of disease resistance enhancement (Yuan et al. 2018), or detrimental to plants, in the form of chemical interference or allelopathy (Bais et al. 2006). Empirical evidence suggests that biotic interactions in the rhizosphere can buffer or amplify environmental pressure on plants (Lambers et al. 2009). However, the previous study was carried out on annual plants with relatively short-term growth. It is still unclear how the composition and chemodiversity of root exudates shift, and what patterns of shifts would be expected over time under long-term forest plantation restoration activities. Additionally, the interactions between root exudates and rhizosphere microbiota in restored forest ecosystems remain largely unclear.

Black locust (*Robinia pseudoacacia*) is a popular tree species used for ecological restoration in the Loess Plateau region of China when returning farmland to forest. The diversity of arbuscular mycorrhiza fungi in the rhizosphere of *R. pseudoacacia* has been reported, with *Funneliformis* being the dominant genus (He et al. 2016). To the best of our knowledge, no studies have systematically analyzed root exudates, rhizosphere microbiota and functional genes in the course of *R. pseudoacacia* plantation restoration. Therefore, it is imperative to identify the key root metabolites and microbiome biomarkers in the rhizosphere of restored *R. pseudoacacia* plantations, and clarify how they interact to modulate soil functions over the long term.

Herein, metabolomic profiling, high-throughput microbiome sequencing and functional gene array were used to explore the complex interactions among root exudates, rhizosphere microbiota and nutrient cycling-related functional genes under *R. pseudoacacia* plantation stands of different ages. It was hypothesized that: (i) the metabolic profiles of root exudates shift over time but their chemodiversity remains stable with stand age, (ii) the shifts in root exudate profiles lead to variation in rhizosphere microbiota structure and (iii) root exudates and rhizosphere microbiota modulate potential soil functions. The hypotheses were tested based on the three following objectives: (i) to decipher the metabolic profiles of root exudates, (ii) to identify biomarker microbial taxa in the rhizosphere and (iii) to identify the relationships among key root exudates, rhizosphere microbiome biomarkers and nutrient cycling-related functional genes. The results of the present study could provide novel perspectives on the aboveground and belowground interactions under long-term *R. pseudoacacia* plantation restoration.

Materials and methods

Study site and sampling

Four *R. pseudoacacia* plantation stands at different ages (15, 25, 35 and 45 years old) were selected in Yongshou County (34°12′–34°50′ N, 108°5′6″–108°5′11″ E), Shaanxi Province, China. The study site has a semi-arid climate, and the main soil type is laterite. Detailed information on the study site is provided in Table S1 available as Supplementary data 2 at *Tree Physiology* Online. Six 20 × 20 m plots were selected in each stand, with six 2 × 2 m quadrats in each plot.

The root zone has three compartments: the endosphere (root tissue), the rhizoplane (root surface with epidermis) and the rhizosphere (soil directly surrounding the root) (Edwards et al. 2018). In the present study, rhizosphere soil samples surrounding the fine roots (diameter <2 mm) were collected as described in previous studies (Chaparro et al. 2014, Liu et al. 2018). The soil samples were designated as RS15, RS25, RS35 and RS45, according to stand age. All samples were kept in a sterile cryogenic tube on dry ice and transported to the laboratory. A portion of each soil sample was stored at 4° C for nutrient analysis (see Methods S1 available as Supplementary data 1 at *Tree Physiology* Online). Another portion was stored at −80° C for subsequent DNA and root exudate extractions.

Root exudate extraction and metabolomic profiling

Root exudates of all samples were extracted with 80% methanol solution through vortex shocking, an ice bath and centrifugation (Chaparro et al. 2013). Liquid chromatography–mass spectrometry (LC–MS) grade water was added to the sample extracts to dilute the methanol content to 53%. After centrifugation, the supernatants were obtained for high-performance liquid chromatography coupled to high-resolution mass spectrometry using a Vanquish UHPLC system (Beijing, China). The raw data files generated by the UHPLC–MS/MS analysis were processed using Compound Discoverer v3.1 (CD3.1, ThermoFisher, Wilmington, DE, USA), for peak alignment, peak picking and quantitation of chemical components. Details of sample preparation, reference standards, analytical instrumentation and metabolomic profiling are provided in Methods S2 available as Supplementary data 1 at *Tree Physiology* Online.

Metabolomics analysis of root exudates was conducted using R v4.0.4 (R Foundation for Statistical Computing, Vienna, Austria). Orthogonal partial least square discriminant analysis (OPLS-DA) and principal component analysis (PCA) were performed using the *ropls* package in R (Etienne et al. 2015). R^2 and Q^2 values close to 1 indicate optimal model performance in OPLS-DA. The *P*-values in both the OPLS-DA and PCA plots were calculated by permutational multivariate analysis

of variance (PERMANOVA) using Euclidean distance matrices (999 permutations) using the *vegan* package in R (Oksanen et al. 2020). The chemodiversity of root exudates was analyzed using the method of Li et al. (2022). A metabolite co-expression network was constructed using the WGCNA package in R (Langfelder and Horvath 2008), with a soft-thresholding power β of 16 for the metabolite co-expression correlation matrix. Network modules were established using the merged dynamic algorithm and assigned to different colors. After calculation of the topological overlap measure (F. Fan et al. 2021), the modules of root exudates were merged ($r < 0.5$). The key modules of root exudates were used to identify biomarkers of root exudates.

Microbiome data acquisition and functional gene analysis

After DNA extraction and PCR amplification, high-throughput sequencing was performed on a Nova Seq600 platform (Illumina Inc., San Diego, CA, USA) by the Novogene Bioinformatics Institute (Beijing, China). The primer pair 515F/907R was used to amplify the V4–V5 region of bacterial 16S rRNA genes (Li et al. 2022). The QIIME2 platform (https://qiime2.org/) was used for noise reduction in high-quality effective tags, in addition to filtering out sequences to obtain the final amplicon sequence variants (ASVs) (Callahan et al. 2016). The ASVs were taxonomically assigned against the Silva database using the class-sklearn module in QIIME2. The data for all samples were homogenized with the sample containing the least amount of data as the standard. After filtering chimeras, on average, 49,094 bacterial effective tags were obtained per sample, which were clustered into 26,643 bacterial ASVs. Principle coordinate analysis (PCoA) was conducted to assess variation in microbial community composition based on Bray–Curtis distances using the *vegan* packages in R v4.0.4 (Oksanen et al. 2020). The Adonis function was used to verify significant differences in microbial community structure across stand age groups and between any pair of samples.

The qPCR primers designed for 71 functional genes related to the cycling of soil nutrients (i.e., C, N, phosphorus [P] and sulfur [S]) were packaged into thin-layer metal alloy nanopore arrays to obtain high-throughput qPCR arrays. The SmartChip real-time PCR System was used to detect and quantify target genes in multiple samples based on the methods described by K. Fan et al. (2021).

Biomarker identification

The potential biomarkers that tracked vegetation restoration were identified using the mothed of Edwards et al. (2018). The relative abundances of microbial taxa (class level) and the relative contents of root exudates were regressed against stand age using randomforest (RF) models (ntree = 500). Seventy percent of the samples from each age group were sampled randomly for the training set. All identified biomarkers were then verified by 10-fold cross-validation, and the number of biomarkers with stabilized cross-validation error was considered. Subsequently, the correlation between key metabolites or taxa, as biomarkers, and stand age, was investigated. The relationships between biomarker metabolites and taxa were analyzed using Pearson correlation coefficient (r), and biomarkers with significant correlations ($r \geq 0.3$ and $P < 0.05$) were selected for regression against stand age.

Statistical analyses

Significant differences in soil nutrient concentrations across stand age groups were tested by one-way analysis of variance. A heatmap was plotted using the score function based on the standardized values of the microbiome dataset. Variance partitioning analysis (VPA) was used to determine the relative contribution of soil nutrients and root exudates to changes in the distribution of rhizosphere microbiota. The co-occurrence network of root exudates was constructed and visualized using R v4.0.4 (https://www.R-project.org/). Partial least squares path modeling (PLS-PM) was used to evaluate the relationships among soil environmental factors, root exudates, rhizosphere microbiota and functional genes. After variable selection (Tian et al. 2019), two soil factors (available potassium [K; AK] and ammonium N [NH_4^+–N]), one key module of root exudates (blue) and three biomarker microbial taxa (*Gammaproteobacteria*, *Nitrososphaeria* and *Thermoanaerobaculia*) were used as explanatory variables, whereas three functional genes (C fixation, S cycling and P cycling) were used as the response variables.

Results

Soil nutrient availability and root exudate profiles under vegetation restoration

Robinia pseudoacacia plantation restoration influenced soil nutrient availability in the rhizosphere in a time-dependent manner (see Table S2 available as Supplementary data 2 at *Tree Physiology* Online). For example, with an increase in stand age, soil NH_4^+–N and available P (AP) concentrations increased by 45.7 and 57.0%, respectively, from the RS15 to RS45 samples. The nitrate N (NO_3^-–N) concentration peaked in the RS35 samples, whereas AK, dissolved organic C (DOC) and dissolved organic N (DON) concentrations all decreased sharply in the RS35 samples, followed by an increase in the RS45 samples.

A total of 1236 chemical compounds were annotated in the root exudates of plantation stands based on matching retention time and mass spectra to an in-house metabolite database. These compounds were classified into 12 categories, including lipids and lipid-like molecules (194), organic acids and derivatives (126), organoheterocyclic compounds (94), benzenoids (88) and phenylpropanoids and polyketides (80; Figure 1a). The chemodiversity of root exudates did not vary remarkably among stand age groups (Figure S1a available as

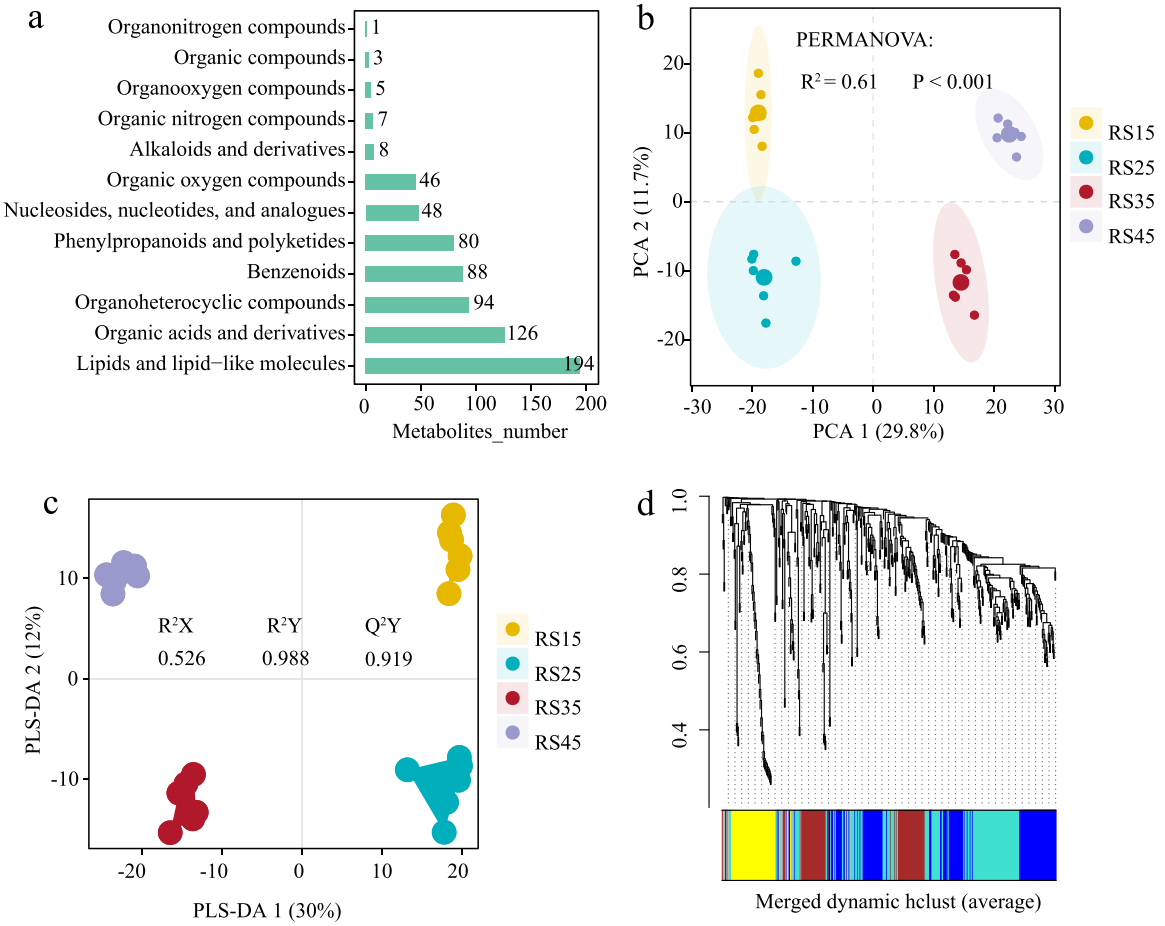

Figure 1. Metabolic profiles of root exudates from *R. pseudoacacia* plantation stands at different ages. (a) Major categories of root exudate metabolites. (b) PCA and (c) OPLS-DA of root exudate profiles. RS15, RS25, RS35 and RS45 denote 15-, 25-, 35- and 45-year-old stands, respectively. (d) Clustering dendrogram of average network adjacency for identification of metabolite co-expression modules.

Supplementary data at *Tree Physiology* Online). Nevertheless, PCA and OPLS-DA results showed significant differences in the composition of root exudates with stand age (PERMANOVA, P = 0.001; Figure 1b and c), indicating that root exudate composition shifted in the course of vegetation restoration.

To explore relationships between root exudate metabolic profiles and soil environmental factors, co-expression network analysis was conducted. Five metabolite co-expression modules were obtained in the network (Figure 1d). Subsequent correlation analysis identified two potentially important modules (blue and turquoise). The blue module was positively correlated with AK, NH_4^+–N and stand age, whereas the turquoise module was negatively correlated with the three factors (Figure 2a). The metabolites of the two modules were mainly divided into nine categories. Among them, fatty acyls, carboxylic acid, benzene and substituted derivatives, steroids and steroid derivatives, and prenol lipids were found in each module. In addition, the blue module contained glycerolipid, isoflavonoids and organonitrogen compounds, whereas the turquoise module consisted of organooxygen compounds, phenols, cinnamic acid derivatives, and pyridines and derivatives (Figure 2b).

Rhizosphere microbial dynamics and potential drivers in restored R. pseudoacacia plantations

The distribution of rhizosphere microbiota in the course of plantation restoration was investigated based on β-diversity. According to the PCoA and PERMANOVA results, the microbial communities differed among stand age groups (P = 0.001; R^2 = 0.448; Figure S1b available as Supplementary data at *Tree Physiology* Online). *Proteobacteria* (28%), *Acidobacteriota* (22%) and *Actinobacteriota* (7.6%) were the predominant phyla across all groups (Figure S1c available as Supplementary data at *Tree Physiology* Online). Overall, microbial community structure at the phylum/class level was significantly positively correlated with soil NH_4^+–N, NO_3^-–N, AK and DOC concentrations, based on the Mantel test and Pearson correlation analysis (Figure S1d available as Supplementary data at Tree Physiology Online).

To identify key features of rhizosphere microbiota at the class level, an RF model was used to determine the correlation between microbial community composition and stand age. The RF model explained 89% of the total variance in rhizosphere microbiota across different stand ages (Figure 2c). To select key

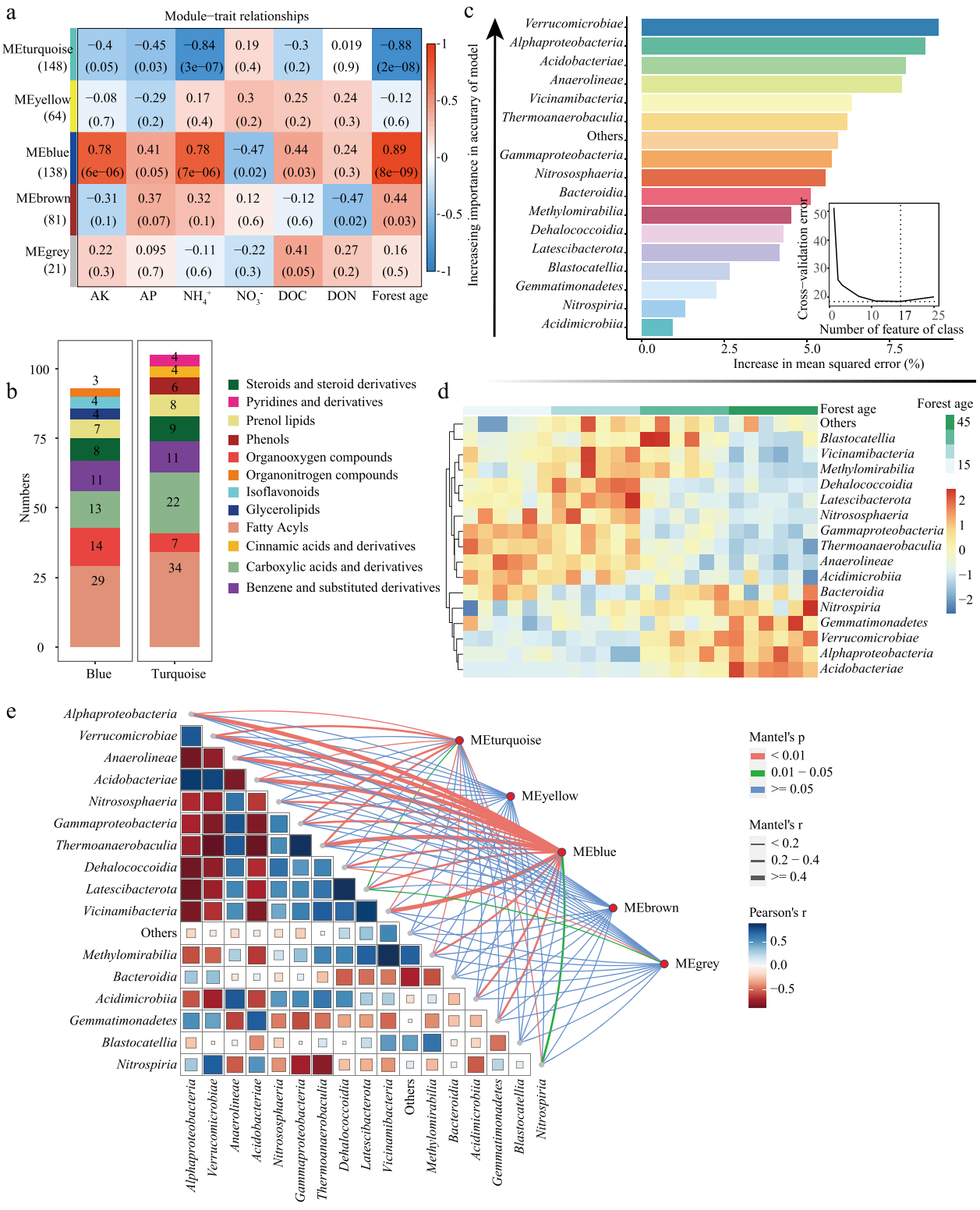

Figure 2. Relationship between root exudates and rhizosphere microbiota under *R. pseudoacacia* plantation restoration. (a) Module–trait relationships based on WGCNA. AK, available potassium; AP, available phosphorus; NH_4^+, ammonium nitrogen; NO_3^-, nitrate nitrogen; and (b) distribution of root exudate categories in two potentially important modules. (c) The top 16 biomarker microbial taxa (class level) identified by random forests regression of taxa relative abundances against stand age. (d) A heatmap shows the relative abundances of 16 age-predictive biomarker taxa against stand age. (e) Correlations between metabolite co-expression modules and biomarker microbial taxa.

microbial taxa (classes) as biomarkers of vegetation restoration, 10-fold cross-validation was used to evaluate the importance of taxa. The number of classes against the cross-validation error stabilized when 16 classes were included. Therefore, the 16 classes were defined as biomarker taxa in the model and ranked in the order of time-discriminatory importance across different stand age groups (Figure 2c). The relative abundances of most biomarker taxa were significantly correlated with stand age. For example, *Alphaproteobacteria*, *Verrucomicrobiae*, *Acidobacteriae* and *Nitrospira* were enriched in the rhizosphere of older stands when compared with younger stands ($P < 0.001$). On the contrary, *Anaerolineae*, *Nitrososphaeria*, *Gammaproteobacteria*, *Dehalococcoidia* and *Latescibacterota* were depleted in the rhizosphere of older stands ($P < 0.001$; Figures 2d and 3a).

The relationships between biomarker microbial taxa and root exudate modules were determined using the Mantel test. The relative abundances of specific microbial taxa, such as *Alphaproteobacteria* and *Vicinamibacteria*, were strongly correlated with the matrix of blue module ($P < 0.01$; Figure 2e). Therefore, the blue module of root exudates was selected as a key module for extracting the biomarker metabolites based on an RF model (Figure S1d available as Supplementary data at *Tree Physiology* Online). The model explained 93.34% of the variance in the composition of root exudates among the stand age groups. Six key metabolites were selected as potential biomarkers in the model (Figure S2b and Table S3 available as Supplementary data at *Tree Physiology* Online). Among them, glucose-1-phosphate, gluconic acid and N-acetylneuraminic acid are assigned to organooxygen compounds, 2-dexyuridine is assigned to pyrimidine nucleosides, glycerol-3-phospate belongs to glycerophospholipids and terbutaline is assigned to phenols (Figure S2b available as Supplementary data at *Tree Physiology* Online). The relative contents of all six biomarker metabolites were significantly positively correlated with stand age ($P < 0.001$; Figure 3b). Furthermore, complex connections were detected between age-sensitive biomarker microbial taxa and root metabolites, based on an RF model (Figure S3 available as Supplementary data at *Tree Physiology* Online). For example, variation in the relative abundances of *Alphaproteobacteria* and *Vicinamibacteria* was mainly explained by N-acetylneuraminic acid, 2-deoxyuridine and gluconic acid ($P < 0.05$).

Relationships among root exudates, rhizosphere microbiota and functional genes under plantation restoration

The influence of plantation restoration on functional gene abundances was minimal (Figure S4 and Table S4 available as Supplementary data at *Tree Physiology* Online). The relative abundance of C fixation-related genes increased toward older stands and significantly differed between the RS15 and RS25 samples, as well as between the RS25 and RS35 samples. However, the relative abundances of C degradation and N cycling-related genes did not change significantly with stand age. The relative abundances of methane metabolism and P cycling-related genes were highest in the RS25 samples, despite no significant differences compared with those of the RS15 and RS35 samples. Furthermore, the biomarker root metabolites exhibited a close relationship with functional genes (Figure S5 available as Supplementary data at *Tree Physiology* Online). In particular, the relative contents of glucose-1-phosphate, gluconic acid, N-acetylneuraminic acid, 2-deoxyuridine, glycerol-3-phosphate and terbutaline were significantly positively correlated with the relative abundances of C fixation, C degradation and S cycling-related genes ($P < 0.05$).

Mantel test results showed that rhizosphere microbiota dissimilarity significantly increased with an increase in root exudate dissimilarity ($R^2 = 0.69$, $P < 0.001$; Figure 4a). The VPA model revealed that soil available nutrients and root exudate metabolites explained 9.6 and 12.3% of unique variance in rhizosphere microbiota, respectively, in addition to 39.8% of overlapping variance (Figure 4b). In summary, the distribution of rhizosphere microbiota was regulated by a combination of soil available nutrients and root exudate metabolites (explained variance = 61.7%).

PLS-PM was used to determine the potential relationships among soil available nutrients, key root exudates, biomarker microbial taxa and functional genes in the rhizosphere. PLS-PM explained 55% of functional gene abundances and provided the best fit to the data (goodness-of-fit = 0.73; Figure 4c). The blue module of root exudates influenced *Gammaproteobacteria*, *Nitrososphaeria* and *Thermoanaerobaculia* via direct negative effects (path coefficient = −0.91, $P < 0.01$), and affected soil AK and NH_4^+–N nutrients via direct positive effects (path coefficient = 0.92, $P < 0.01$). In addition, the key root exudates and biomarker microbial taxa affected C fixation, S cycling and P cycling-related functional genes via direct positive and negative effects, respectively (path coefficient = 0.84 and −0.24, respectively; Figure 4d). The results indicate that root exudates were key factors shaping rhizosphere microbiota, and hence, driving potential soil functions.

Discussion

Metabolic profiles of root exudates shift in the course of vegetation restoration

Plant disease, drought stress and nutrient availability influence root exudate patterns and profiles (Yuan et al. 2018, Hao et al. 2022). Herein, untargeted metabolomics analysis was used to characterize the root exudates of *R. pseudoacacia* plantation stands at different stages. Root exudate metabolic profiles (Figure 1), rather than chemodiversity (Figure S1a available as Supplementary data at *Tree Physiology* Online), markedly changed with an increase in stand age, which supports the first hypothesis of the present study. Temporal shifts

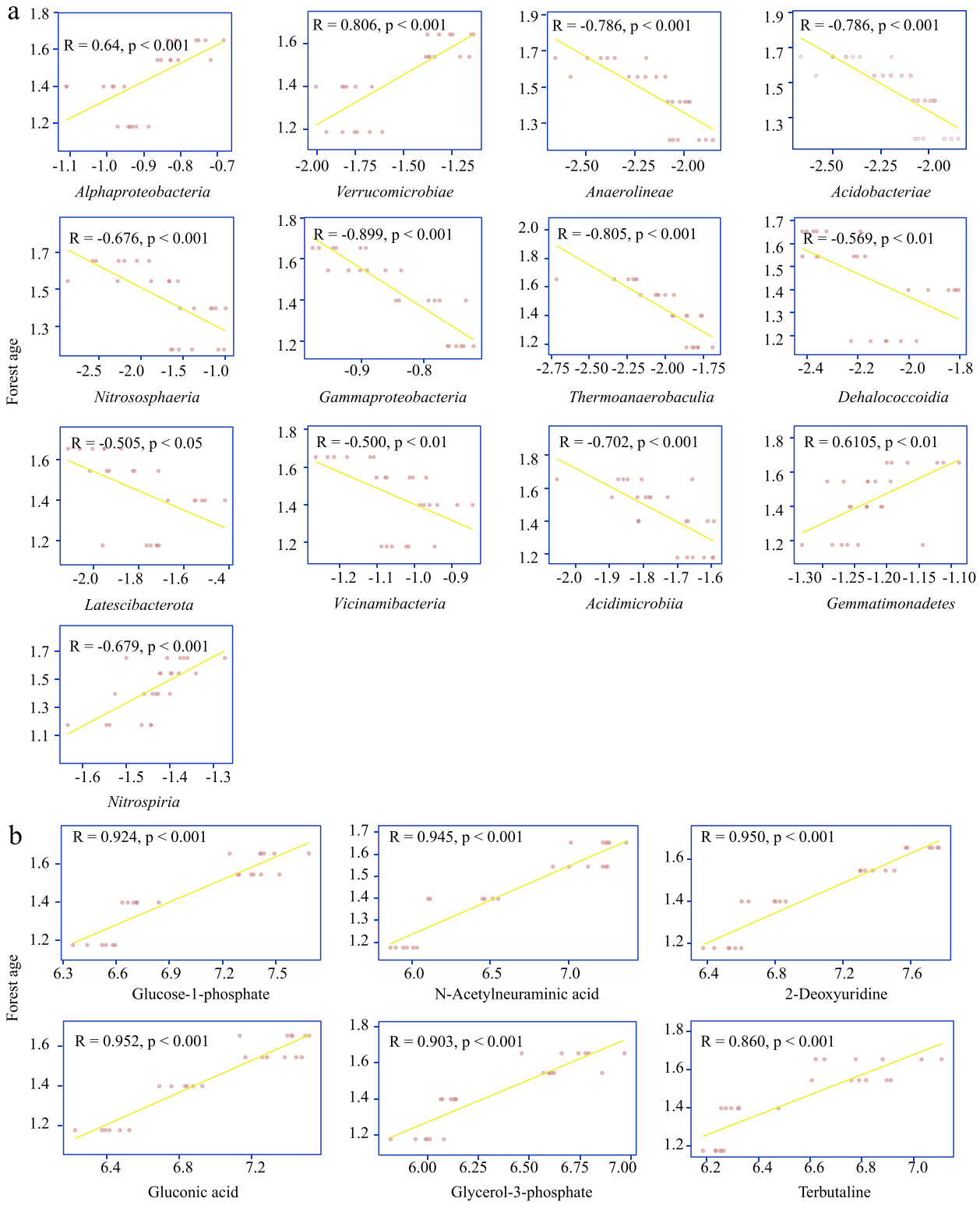

Figure 3. Relationship between potential biomarkers of vegetation restoration and stand age. (a) Sixteen rhizosphere microbial taxa and (b) six root exudate metabolites with significant Pearson correlations ($r \geq 0.3$, $P < 0.05$) were regressed against stand age. The *y*-coordinate is log-transformed.

in root exudate profiles may reflect the long-term responses of *R. pseudoacacia* plantations to the changing rhizosphere environment.

The co-expression network of root exudates consisted of five modules, and one key module containing 138 metabolites was positively correlated with soil nutrients and stand age

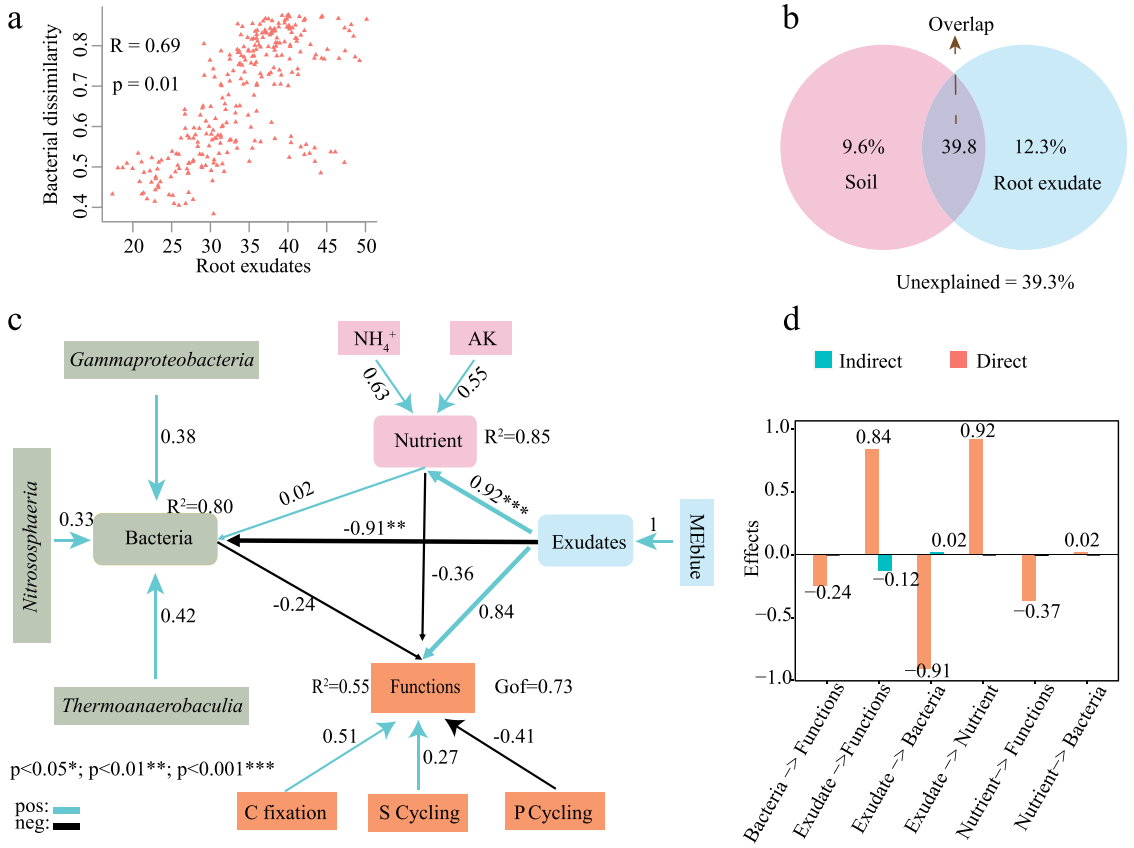

Figure 4. Plant–soil–microbiota interactions in the restored ecosystem of *R. pseudoacacia* plantations. (a) The relationship between rhizosphere microbiota dissimilarity (Bray–Curtis distance) and root exudates (Euclidean distance) based on Mantel test. (b) The influence of soil nutrients and root exudates on rhizosphere microbiota distribution based on variance partitioning. (c) PLS-PM for multidimensional omics data analysis. NH_4^+, ammonium nitrogen; AK, available potassium; Gof, goodness-of-fit; pos, positive correlation; neg, negative correlation. (d) The direct and indirect effects of abiotic and biotic factors on the response variable.

(Figure 2a and e). Among them, six biomarker metabolites showed an increasing trend from the 15-year-old stand to the 45-year-old stand (Figure 3). Based on the PLS-PM results, key root exudates strongly affected soil available nutrients, AK and NH_4^+–N (Figure 4). This effect was also observed in the module–trait relationships, based on WGCNA (Figure 2a and e). Soil factors may have a close relationship with age-sensitive changes in root exudate metabolic profiles. For example, root exudates can enhance plant growth by stimulating nutrient mineralization in the fast-growing stage of *Arabidopsis thaliana* (Zhao et al. 2021). Furthermore, rice roots release biological nitrification inhibitors, such as 1,9-decandiol, which can block the ammonia monooxygenase pathway of ammonia oxidation, and in turn, influence N-use efficiency (Sun et al. 2016).

Rhizosphere microbiota structure of R. pseudoacacia plantations varies with stand age

Vegetation restoration can alter the environmental conditions and microbial diversity of bulk soil (Jiao et al. 2018, Wang et al. 2018, Zhang et al. 2019). In the present study, the rhizosphere microbiota associated with *R. pseudoacacia* plantation stands clustered into three distinct groups (i.e., RS15–RS25, RS35 and RS45), with increasing dissimilarity over time (Figure S1b available as Supplementary data at *Tree Physiology* Online). The result confirms that the plantation stands of different ages could recruit distinct microbial taxa in the rhizosphere. Furthermore, the present study demonstrates temporal shifts in rhizosphere microbiota structure and identifies biomarker microbial taxa (classes) associated with stand age (Figure 2c and d). The relative abundances of biomarker taxa prominently changed over time. Notably, with an increase in stand age, more biomarker taxa were depleted, rather than enriched (Figure 3a), which indicates that exclusion of microbial taxa in the rhizosphere is sensitive to stand age.

In the present study, some biomarker microbial taxa related to N and C cycling, such as *Nitrospira*, *Alphaproteobacteria* and *Acidobacteria*, were enriched in the rhizosphere of older stands (Figure 3). In the course of vegetation restoration, the bacterial groups may be required to meet the nutrient demands of older *R. pseudoacacia* plantations. Specifically, *Nitrospira* species participate in nitrification by oxidizing nitrite into nitrate (Kowalchuk and Stephen 2001). In the present study, variation

in the relative abundance of *Nitrospira* was mainly attributed to glycerol-3-phosphate and terbutaline (Figure S3 available as Supplementary data at *Tree Physiology* Online). Terbutaline belongs to phenols, a natural mixture of phytochemicals in root exudates that modulates the soil microbiome (Badri et al. 2013). However, the underlying mechanisms via which terbutaline and glycerol-3-phoshate influence *Nitrospira* abundance still need to be clarified.

The class *Alphaproteobacteria* harbors many rhizobia (Reinhold-Hurek et al. 2015). In the present study, variation in the relative abundance of *Alphaproteobacteria* was mainly attributed to N-acetylneuraminic acid, 2-Dexoxyuridine, gluconic acid, terbutaline and glycerol-3-phoshate (Figure S3 available as Supplementary data at *Tree Physiology* Online), all of which have low molecular weights. Recently, root exudates and small-molecular-weight organic acids have been reported to enhance the desorption of soil organic contaminants (available fractions) by increasing DOC content and predicted microbiome diversity, composition and function (Du et al. 2020, Gu et al. 2020). Moreover, *Acidobacteria* is a ubiquitous group of bacteria widely found in terrestrial ecosystems and they mediate C cycling by degrading complex plant-derived polysaccharides (Barns et al. 1999). Furthermore, the class *Gammaproteobacteria* was depleted in the rhizosphere of older stands (Figure 3). Both *Acidobacteria* and *Gammaproteobacteria*, which comprise pathogens and are related to host health, have been found in the human gut and soil (Shin et al. 2015).

The above findings indicate that shifts in root exudate profiles altered rhizosphere microbiota structure, which is consistent with our second hypothesis. In summary, the microbiome biomarkers identified in the rhizosphere of *R. pseudoacacia* plantations are closely related to soil nutrient cycling and plant health. Consequently, the rhizosphere acts as a key hub of plant–soil–microbiota interactions, driving soil nutrient availability and plant growth in the restored ecosystem.

Plant–soil–microbiota interactions regulate potential functional genes in restored plantations

Root exudates mediate coevolution between host plants and rhizosphere microbiota by promoting plant growth and conveying nutritional benefits to soil microorganisms (Lu et al. 2018, Lian et al. 2020, Zhao et al. 2021). Plant, soil and rhizosphere microbiota can be manipulated or engineered in natural ecosystems to shift direction in favor of vegetation restoration for sustainable gains (Philippot et al. 2013). Because rhizosphere microbiota act as a biological mediator of plant–soil interactions in restored ecosystems, unraveling relationships among soil nutrients, rhizosphere microbiota, potential functional genes and root exudates could enhance our understanding of the mechanisms driving forest ecosystem restoration (Zhang et al. 2017, Coban et al. 2022).

In the present study, a significant positive correlation was observed between the relative contents of biomarker root metabolites and the relative abundances of C fixation and S cycling-related genes (Figure S5 available as Supplementary data at *Tree Physiology* Online). When *R. pseudoacacia* roots recruit specific microbial taxa in the rhizosphere, the plants may stimulate the functional potential of rhizosphere microbiota by regulating the metabolic profiles of root exudates (Badri et al. 2013, Zhao et al. 2021). Indeed, the addition of root exudates stimulated soil microbial communities to decompose labile organic matter and release N in a loblolly pine (*Pinus taeda*) plantation (Meier et al. 2017). In degraded grassland, the contribution of root exudates to soil nutrient inputs was enhanced (Shen et al. 2020). Accordingly, the biomarker root metabolites of *R. pseudoacacia* plantations might facilitate microbially mediated C fixation and S cycling in the rhizosphere during vegetation restoration.

According to the PLS-PM results, the key root exudates influenced the relative abundances of C fixation, P cycling and S cycling-related functional genes through direct positive effects, or through indirect negative effects via biomarker microbial taxa (Figure 4). Nitrogen application rate has been reported to affect the metabolic profiles of maize root exudates (Hao et al. 2022), whereas microbial community variation in bulk soil increased the abundance of C and N cycling-related functional genes in *Quercus liaotungensis* forest (Yan et al. 2020). Consistent with our third hypothesis, the results indicate that changes in the metabolic profiles of root exudates could alter soil available nutrients, thereby influencing rhizosphere microbiota structure and gene function.

Plant development, which is influenced by soil nutrient availability, leads to dynamic patterns in the chemical composition of root exudates. Conversely, rhizosphere microbiota utilize available nutrients through complex mechanisms. Therefore, root metabolite profile features and microbial metabolite substrate preferences drive microbial community assembly patterns in the rhizosphere (Zhalnina et al. 2018). It has been reported that phenolic root exudates affect soil C cycling in forest ecosystems and root phenolic profiles are species specific (Zwetsloot et al. 2018). In addition, *A. thaliana* root exudates upregulate functional genes in rhizosphere microbiota associated with N cycling (N fixation, denitrification and dissimilatory N reduction), but downregulated P cycling-related genes (Zhao et al. 2021). Furthermore, *Gammaproteobacteria* species participate in the catalysis of S oxidation and C fixation in coastal intertidal sediments (Lenk et al. 2011). *Thermoanaerobaculia* has potential roles in seafloor S cycling (Flieder et al. 2021), whereas *Nitrososphaeria* is a class of bacteria associated with N cycling (Saghaï et al. 2022). Overall, our data support that key root exudates and rhizosphere microbiome biomarkers play a non-negligible role in the maintenance of soil functions in the course of *R. pseudoacacia* plantation vegetation restoration.

The present study deciphers rhizosphere dynamics in the course of *R. pseudoacacia* restoration based on multi-omics approaches. It provides novel perspectives on the aboveground–belowground interactions in relation to long-term vegetation restoration, despite the limitations of untargeted metabolomic profiling in identifying specific metabolites (e.g., lack of absolute qualitative and quantitative data). In future studies, a combination of targeted metabolome, metagenomics and isotopic labeling techniques could facilitate the unraveling of the feedbacks between the belowground and aboveground parts of plants, and associated driving factors.

Conclusions

Using space-for-time substitution, the present study investigated the root exudate metabolome and rhizosphere microbiome of *R. pseudoacacia* plantations in the course of vegetation restoration. Despite no distinct changes in chemodiversity of root exudates, the metabolic profiles were complex. Age-related metabolites were obtained from a key module of root exudates. Biomarker metabolites, such as fatty acyls, organooxygen compounds, and carboxylic acid and derivatives, exhibited increasing trends over time. In addition, age-related exclusion of microbial taxa was observed in the rhizosphere, with more biomarker taxa (e.g., *Anaerolineae* and *Nitrososphaeria*) depleted rather than enriched, over time. The metabolic profiles of root exudates shifted with change in stand age, suggesting that the rhizosphere chemistry was regulated by plant responses to soil environmental changes. Temporal shifts in root exudates altered soil nutrient availability, whereas available nutrients and key metabolites jointly shaped rhizosphere microbiota structure with varied biomarker taxa related to soil nutrient cycling and plant health. Furthermore, soil–plant–microbiota interactions modulate nutrient cycling-related functional genes in the rhizosphere. The findings provide insights into the coupling between rhizosphere microbiota and potential soil functions, such as nutrient cycling driven by root exudates under forest vegetation succession.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online. Supplementary file 1 contains methods and figures. Supplementary file 2 includes four Excel sheets (Tables S1–S4).

Conflict of interest

No conflict of interest.

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Date availability

All data of this are available from the corresponding author upon request.

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