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RESEARCH ARTICLE

The synthesis and secretion of key substances in the flavonoid metabolic pathway responding to different nitrogen sources during early growth stages in *Robinia pseudoacacia*

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Abstract

Background and aims Legumes can obtain nitrogen from mineral nitrogen and symbiotic nitrogen; however, the regulatory mechanisms by which legume plants respond to various nitrogen sources are still unclear.

Methods Here, the flavonoid metabolism profiles of *Robinia pseudoacacia* (black locust) in response to different nitrogen sources during the early growth stages were performed using the hydroponics method.

Results The flavonoids were accumulated specifically responding to different nitrogen sources (symbiotic nitrogen and NO_3^-) in the roots and root exudates of *R. pseudoacacia* and showed growth-stage-dependent changes in the plant growth process. More flavonoids were synthesized and secreted under nitrogen deficiency. The differential flavonoids among different treatments were significantly enriched in the flavone and flavonol biosynthesis pathway and isoflavonoid biosynthesis pathway. Liquiritigenin, naringenin, apigenin and kaempferol were not only the metabolic nodes of these two metabolic pathways but also the key nodes in the differential flavonoid network. Therefore, these four flavonoids may be the key flavonoids of *R. pseudoacacia* responding to different nitrogen sources.

Conclusion Our findings provide an important insight into the adaptability of legumes to different nitrogen environments and are of great significance for understanding the molecular mechanisms underlying nitrogen utilization in legumes and will contribute to the development of sustainable agricultural practices and crop production.

Keywords Nitrogen sources · Flavonoid metabolomics · Root and root exudate · *Robinia pseudoacacia*

Abbreviations

NF	Nitrogen free
SN	Symbiotic nitrogen
MN	Mineral nitrogen
dpt	days post-treatment
UPLC-MS/MS	Ultra performance liquid chromatography tandem mass spectrometry
PCA	Principal component analysis
HCA	Hierarchical clustering analysis
OPLS-DA	Orthogonal partial least squares discriminant analysis
FC	Fold change

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VIP	Variable importance for the projection
KEGG	Kyoto Encyclopedia of Genes and Genomes
Chl	Leaf chlorophyll content
SL	Shoot length
SDW	Shoo t dry weight
RL	Root length
RDW	Root dry weight
RN	Root nitrogen content
ShN	Shoot nitrogen content
SD	Standard deviation
ANOVA	One-way analysis of variance
DFs	Differential flavonoids

Introduction

It is well known that secondary metabolites are vital molecules in plant life, acting as protective agents against environmental factors. In particular, flavonoids, phenylpropanoid-derived compounds, play important roles in plant growth and development, as well as in resistance to biotic and abiotic stress. For example, gibberellin boosted root growth by directly decreasing flavonol biosynthesis in *Arabidopsis* (Tan et al. 2019). The synthesis of anthocyanin rhamnoside improved the cold resistance of *A. thaliana* (Li et al. 2017). The accumulation of flavonoids elevated the resistance to the brown planthopper in rice (Dai et al. 2019). More than 8000 distinct flavonoids have been discovered to date and the majority of these flavonoids can be divided into six major categories, namely flavones, flavonols, flavanones, isoflavones, flavanols and anthocyanins (Wang et al. 2022). Among them, isoflavones such as daidzein and genistein widely exist in legumes.

Legumes are one of the most important crop plants for humans because they provide raw materials for industry, feed for livestock and sources of food (Mathesius 2018). Legumes are important for sustainable agriculture because of their ability in fixing nitrogen from the atmosphere and ultimately help reduce the use of mineral fertilizers in the agro-ecosystems. Under low available nitrogen conditions, the neighboring plants can also stimulate flavonoid production in the roots and root exudates of the legumes. For example, the legumes co-cultivated with cereals like wheat have been shown to increase flavonoid biosynthesis and secretion in the roots (Leoni et al. 2021). The specific flavonoids secreted by the legume roots recruit rhizobia to colonize the rhizosphere. These flavonoids are perceived and bound by NodD, a transcriptional activator of the LysR family from the compatible rhizobia. Subsequently, NodD activates the expression of bacterial *nod* genes, resulting in the synthesis and secretion of the specific lipo-chitooligosaccharide signaling compounds, Nod factors (Peck et al. 2006). The perception of specific Nod factors triggers a signaling cascade in the host to initiate the nodule formation. Flavonoids play a role in different stages of nodule formation. Firstly, flavonoids are able to act as the selectors for compatible symbiotic organisms. Flavonoids can be used as the inducers of the *nod* gene expression of compatible rhizobia and as the phytoalexins inhibiting the infection of incompatible rhizobia (Liu and Murray 2016). Daidzein induced the expression of the *nod* genes in *Bradyrhizobium japonicum* (nodulating soybean), while it inhibited that of those from *Rhizobium leguminosarum* (nodulating clover or peas) (Begum et al. 2001). Secondly, flavonoids are essential for nodule initiation. It was confirmed that the flavonols could inhibit polar auxin transport in *Medicago truncatula* roots, thereby promoting the local accumulation of auxin in the initial site of root nodules and leading to the initiation of root nodules primordium (Wasson et al. 2006; Zhang et al. 2009). Finally, flavonoids may also be involved in the systemic regulation of the nodule number, for example, the exogenous supply of either daidzein or coumestrol increased the nodule number (Abd-Alla 2011). Although the molecular mechanisms that regulate the production of flavonoids during nodulation in plants are not well understood, the interconnection between the availability of nitrogen and the metabolism of flavonoids has been demonstrated. Nitrogen supply has a negative effect on flavonoid biosynthesis in plants. An obvious inverse relationship between nutrient availability and flavonoid accumulation was exhibited in *Arabidopsis* and tomato, with nitrogen limitation causing the maximum increase of flavonoids (Stewart et al. 2001).

Robinia pseudoacacia (black locust), one of the leguminous plants, can establish symbiosis with various rhizobia from different genera, endowing black locust with strong adaptability to the environment. Because of its prominent symbiotic nitrogen fixation ability, fast growth, the capacity of soil and water conservation

and other advantages, *R. pseudoacacia* has become an important ecological afforestation pioneer tree species in China (Fan et al. 2018). Black locust also is used to develop new biological products for multi-directional uses, including lubricants, polymers, as well as bioenergy and fuels (Tyśkiewicz et al. 2019). In addition, it has also been reported that black locust has pharmacological benefits, owing to the activities of its flavonoids in living organisms (Tyśkiewicz et al. 2019). The main flavonoids in *R. pseudoacacia* are quercetin, rutin, kaempferol, robinin and acacetin (Guo et al. 2019; Tyśkiewicz et al. 2019; Veitch et al. 2010), which play vital roles in resistance to environmental stresses (Khalid et al. 2019; Zhao et al. 2016).

Flavonoids attracted *Aeromonas* sp. to colonize the roots of *Arabidopsis* and thus improved the ability of plants to resist dehydration (He et al. 2022). The higher concentration of quercetin in root exudates of the invasive plant *Triadica sebifera* enhanced the colonization of arbuscular mycorrhizal fungal and promoted its growth (Tian et al. 2021). Although the ecological functions of flavonoids have been investigated, the response of synthesis and secretion of flavonoids to different nitrogen nutrition from different sources during early growth stages in the leguminous tree species remains largely unexplored. In recent years, metabonomics methodology has become an effective tool for the comprehensive and efficient study of complex metabolic components, which has been widely used to investigate the detailed metabolite profile in plants (Yang et al. 2021; Zhao et al. 2020). However, due to the complexity of soil and plant interactions, current methods for collecting root exudates are limited. Hydroponics, the main method to study plant metabolism, allows accurate control over the chemical background of the plant growth, facilitates the collection of a sufficient amount of freshly produced exudates, avoids problems related to non-uniform mineral adsorption of exudate components and minimizes the microbial transformation of exuded metabolites (Zhalnina et al. 2018). Consequently, we initiated a study on the metabolism of flavonoids mediated by different nitrogen sources in the roots and root exudates of *R. pseudoacacia* during early growth stages by hydroponics. We hypothesized that *R. pseudoacacia* could regulate the synthesis and secretion of key compounds in the flavonoid metabolic pathway to adapt different nitrogen environments.

Materials and methods

Plant growth and bacterial cultivation

R. pseudoacacia seeds (Northwest A&F University, Yangling, China) were treated with 75% ethanol for 1 min, rinsed twice with sterile water, and sterilized with 50% sodium hypochlorite for 10 min. Then the seeds were washed with sterile water several times. The surface-sterilized seeds were placed in an Erlenmeyer flask containing 70℃ aseptic hot water and incubated overnight at 28℃ with shaking at 150 rpm. Finally, the sterile seeds were transferred to aseptic petri dishes covered with moistened sterile filter papers and incubated in the dark at 28℃ for 72 h.

The germinated seedlings were transferred to the cracks between the sterile filter paper rolls and the holders, which were placed in the sterile trays containing the nitrogen-free one-quarter strength Hoagland's nutrient solution (in brief, the nutrient solution was modified by substituting 5 *mM* KCl and $CaCl_2$ for 5 *mM* KNO_3 and $Ca(NO_3)_2$, respectively) (Hoagland and Arnon 1950). Plants were cultivated in a glasshouse with a cycle of 16 h light at 24℃ and 8 h dark at 18℃. During this period, the nutrient solution was changed every 5 days.

The rhizobia strain used in this study, *Mesorhizobium amorphae* CCNWGS0123, was isolated from the nodules of *R. pseudoacacia* grown in heavy metal tailings in Gansu Province, China (Mohamad et al. 2012), and the strain specifically nodulated *R. pseudoacacia* (Chen et al. 2013; Wang et al. 2019). *Mesorhizobium amorphae* CCNWGS0123 was cultured in tryptone-yeast extract (TY) liquid medium at 28℃ with shaking at 200 rpm for 2–3 days. The bacteria were collected by centrifugation at 3300 *g* for 6 min. Rhizobial cell pellets were resuspended in sterile double-distilled water and collected by centrifugation and then repeated this step three times. In the end, rhizobial cell pellets were resuspended and adjusted to OD600 ≈ 0.3 with sterile double-distilled water.

Fifteen-day-old seedlings were treated with three nutrient solutions. The treatment regimes were: (1) the sterile nitrogen-free nutrient solution (nitrogen free, NF); (2) the nitrogen-free nutrient solution containing *M. amorphae* CCNWGS0123 suspension (symbiotic nitrogen, SN); (3) the sterile one-quarter strength Hoagland's nutrient solution (mineral nitrogen, MN).

Measurements of growth phenotype and nitrogen content

The plants were sampled at 1 (infection initiation: refers to the rhizobial attachment to root hairs, I), 3 (nodule primordium initiation and formation, N), 15 (early stage of nitrogen-fixing nodules, E), and 28 (mature nitrogen-fixing nodules, M) days post-treatment (dpt). The sampling time was determined based on previous studies (Chen et al. 2013; Haynes et al. 2004; Vega-Hernández et al. 2001; Wang et al. 2019; Xiao et al. 2014). Chlorophyll content (Chl), the root length (RL), the shoot length (SL), the dry weights of both root and shoot (RDW and SDW) were determined. Ten plants were collected per treatment at each time point. The chlorophyll meter (SPAD-502 Plus, Konica Minolta, Japan) was used to measure the chlorophyll content of leaves. The length of each root and shoot were measured with a ruler. Plants were washed with water and dried to constant weight at 65℃. The nitrogen content of both shoot and root (ShN and RN) was measured. Three biological replicates were conducted for per treatment at each time point, and each biological replicate contained at least five individual plants. Dry samples of the plants were ground into powder and then digested with sulfuric acid and hydrogen peroxide to determine the nitrogen content (Sun et al. 2021).

Preparation and extraction of the roots and root exudates

On each sampling date, the roots were washed with sterile deionized water and freeze-dried using a vacuum freeze-dryer (Scientz-100 F). Three biological replicates were performed for per treatment, and each biological replicate contained mixed roots from at least five different plants. The freeze-dried samples were crushed by a mixer mill (MM 400, Retsch, Haan, Germany) with a zirconia bead for 1.5 min at 30 Hz. Samples (100 mg of lyophilized powder) were dissolved in 1.2 ml of 70% methanol solution, vortexed for 30 s every 30 min for 6 times in total, and then stored at 4℃ overnight. The following mixtures were centrifuged at 13,201 *g* for 10 min at 4℃. The extracts were filtrated by SCAA-104 with a 0.22 μm pore size (ANPEL, Shanghai, China) and stored in a sample flask for the ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis.

On each sampling date, root exudates were collected according to a modification of the method of Lv et al. and Sun et al. (Lv et al. 2020; Sun et al. 2016). Briefly, the whole plants were washed with sterile deionized water and then put the roots into a conical flask with 30 ml of sterile deionized water for 6 h. The conical flasks were covered with aluminium foil to create a dark environment for the roots. Then the root exudates were filtered by a 0.22 μm pore size (Millipore) to remove root debris and microorganisms. The filtrates were collected, frozen with liquid nitrogen, and freeze-dried using a vacuum freeze-dryer (Scientz-100 F). Three biological replicates were conducted for per treatment, and each biological replicate contained mixed root exudates from at least five different plants. The freeze-dried root exudates were extracted with 500 μl of 70% methanol solution and vortexed for 3 min. The following mixtures were centrifuged at 13,201 *g* for 10 min at 4℃. The extracts were filtrated by SCAA-104 with a 0.22 μm pore size (ANPEL, Shanghai, China) and stored in a sample flask for UPLC-MS/MS analysis.

UPLC-MS/MS conditions

The extracts were analyzed using a UPLC-ESI-MS/MS system (UPLC, SHIMADZU Nexera X2; MS, Applied Biosystems 4500 Q TRAP) equipped with an Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm). The mobile phase was consisted of solvent A, pure water with 0.1% formic acid, and solvent B, acetonitrile with 0.1% formic acid. The gradient program was performed as follows: 95:5 V/V for 0 min, 5:95 V/V for 9.0 min, 5:95 V/V for 10.0 min, 95:5 V/V for 11.1 min, and 95:5 V/V for 14.0 min. The flow rate and injection volume were 0.35 ml min^{-1} and 4 μl, respectively. The column oven was set to 40℃. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS.

A triple quadrupole-linear ion trap mass spectrometer (Q TRAP), AB4500 Q TRAP UPLC/MS/MS system, equipped with linear ion trap (LIT) and triple quadrupole (QQQ) scans were used to detect metabolites. This system was equipped with an ESI turbo ion-spray interface operating in positive and negative ion mode and controlled using Analyst 1.6.3 software (AB Sciex). The ESI source operation parameter was

an ion source (turbo spray, 550℃, 5500 V/-4500 V). Gas I, gas II, and curtain gas were set at 50, 60, and 25.0 psi, respectively. The collision-activated dissociation (CAD) was high. In the QQQ and LIT modes, instrument tuning and mass calibration were carried out with 10 and 100 μmol/l polypropylene glycol solutions, respectively. QQQ scans were acquired as MRM experiments with collision gas (nitrogen) set to medium. Declustering potential (DP) and collision energy (CE) were done with further optimization for individual MRM transitions. According to the metabolites eluted within this period, a specific set of MRM transitions were monitored for each period.

Metabolite analyses

Qualitative analysis of primary and secondary MS data was performed by comparison of the retention time (RT), the accurate precursor ions (Q1), product ions (Q3) values and the fragmentation patterns with those obtained by injecting standards using the same conditions if the standards were available (Sigma-Aldrich, St. Louis, MO, USA). The data were analyzed using a self-compiled database, MWDB (MetWare Biological Science and Technology Co., Ltd., Wuhan, China) and publicly available metabolite databases if the standards were unavailable. Metabolite quantification was performed using the MRM mode. The QQQ-MS eliminated the interference of non-target ions and screened the characteristic ions of each metabolite to obtain the signal strengths. After getting the mass chromatographic analysis data of metabolites in different samples, the mass chromatographic peaks of all substances were integrated by peak area, and the mass chromatographic peaks of the same metabolite in different samples were integrated and corrected. The corresponding relative metabolite contents were expressed as chromatographic peak area integrals. The identification of metabolites was performed using isotopic distribution, the accurate mass of metabolites, secondary fragments (MS2) and retention time (RT) of MS2 fragments. Through the intelligent secondary spectrum matching method independently developed by MetWare, the secondary spectrum and RT of the metabolites in the samples were matched intelligently with the secondary spectrum and RT of the MWDB database one by one. The MS tolerance and MS2 tolerance were set to 20 ppm (parts per million), respectively.

Principal component analysis (PCA), hierarchical clustering analysis (HCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were executed on the metabolites of 72 samples using R software to study metabolite accession-specific accumulation. PCA and HCA were performed using the "vegan" package and "ComplexHeatmap" package, respectively (Gu et al. 2016), significance for PCA analyses was checked with "Adonis" (999 permutations). OPLS-DA was conducted based on the variable importance in projection (VIP) value in the test model by the "MetaboAnalystR" package (Chong and Xia 2018). Differential metabolites define as metabolites with P-value <0.05, fold change (FC) values ≥ 2.0 or ≤ 0.5 and VIP scores >1.0. The differential metabolites among the three treatment groups (NF, SN and MN) were analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database with a P-value <0.01. Network analysis was carried out to investigate the interactions of the differential metabolites using R software and the interactive platform Gephi. The relative content exceeding 0.01% was opted to calculate Pearson's correlation between differential metabolites. An interaction was considered to be robust when Pearson's correlation coefficient was >0.6 and the P-value was <0.01.

Statistical analysis

Before conducting statistical analysis, all data were investigated for normality using the Shapiro-Wilk test and were tested for the homogeneity of variances using Bartlett's test. All statistical analyses were performed with R software (v 3.5.0). Unless stated, visualization of data relied on the ggplot2 package. The significance among the three treatments was tested by one-way analysis of variance (ANOVA) and Tukey's HSD post hoc test in the stats and multcomp packages of R software (v 3.5.0) and significance was accepted at P-value <0.05. The data of growth phenotype and nitrogen content were expressed as mean ± standard deviation (SD) and were plotted using Origin 8.0 software.

Results

The analyses of growth phenotype and nitrogen content under different nitrogen conditions

To explore the effect of three treatments (nitrogen free (NF), symbiotic nitrogen (SN) and mineral nitrogen (MN)) on the growth of the plant, the plants of all

groups at 1 (I), 3 (N), 15 (E) and 28 (M) days post-treatment (dpt) were collected for analysis. Unsurprisingly, the mature nodules only appeared on SN roots at stage M (Fig. 1A), indicating that other treatments were not contaminated with rhizobia, hence the subsequent experimental results were credible. The growth of the plant among different treatments showed significant differences ($P < 0.05$) at the E and M stages (Fig. 1B). The leaf chlorophyll content (Chl), the shoot length (SL) and the shoot dry weight (SDW) in MN were dramatically higher than that in NF and SN at the E and M stages, and they were also markedly higher in SN than those in NF at stage M (Fig. 1B). However, the root length (RL) and the root dry weight (RDW) were significantly higher in NF than those in MN at the M stage (Fig. 1B). The nitrogen content of both shoot and root (ShN and RN) showed significant differences among different treatments at four stages ($P < 0.05$, Fig. 1C). ShN and RN in MN were significantly higher than those in the other two treatments (Fig. 1C). Also, ShN and RN in SN were significantly higher than those in NF at the I and M stages, as well as RN in SN at stage N (Fig. 1C). In addition, both ShN and RN in NF gradually decreased with plant growth, while ShN and RN in SN showed a decline in the first three stages and increased at stage M (Fig. 1C). In MN, ShN decreased gradually in the first three stages and increased at the M stage, and RN initially increased and then gradually decreased from the N stage (Fig. 1C). Overall, the nitrogen content in SN was lower than that in MN and was comparable to that in NF in the first three stages.

Flavonoid metabolism profile responding to different nitrogen sources

Given that the special role of flavonoids in legumes responding to environmental nitrogen alterations, the flavonoid metabolism of the roots and root exudates of the three groups of plants described above were analyzed. A total of 295 flavonoids were detected in the roots, including 20 chalcones, 36 flavanones, 7 flavanonols, 5 anthocyanidins, 99 flavones, 59 flavonols, 21 flavonoid carbonosides, 3 flavanols, 3 tannins, 1 biflavones, and 41 isoflavones. In total, 242 flavonoids were identified in root exudates, containing 16 chalcones, 35 flavanones, 5 flavanonols, 2 anthocyanidins, 80 flavones, 40 flavonols, 20 flavonoid carbonosides, 2 flavanols, 2 tannins, 1 biflavones, and 39 isoflavones (Fig. 2A; Tables S1 and S2). It's worth noting that some of these identifications, particularly those marked as Level 3 in the supplemental table, are less accurate and can be considered tentative identifications. PCA results showed that the metabolic changes in *R. pseudoacacia* roots and root exudates over time could be indicated by the time-dependent shifts at the level of the first principal component (PC1), which accounted for 28.69% and 46.59% of the total variation, respectively (Fig. 2B). Furthermore, the metabolite profiles of three treatments were separated by the second principal component (PC2), accounting for 15.22% and 18.36% of the total variance, respectively. Adonis analyses revealed that the flavonoid metabolism profiles in the roots ($R^2 = 47.84\%$, $P = 0.001$) and root exudates ($R^2 = 49.02\%$, $P = 0.001$) changed significantly with time. The flavonoid metabolism profiles in the roots ($R^2 = 19.73\%$, $P = 0.001$) and root exudates ($R^2 = 24.10\%$, $P = 0.001$) also showed significant differences among treatments. The four main clusters were obtained by hierarchical clustering analysis based on the relative differences in flavonoid accumulation patterns in the roots and root exudates (Fig. 2C). Flavonoids in cluster 1 were accumulated more in the inoculated roots at 28 dpt (SNM) and in the root exudates of all three treatments at 28 dpt (SNM, NFM and MNM). The flavonoids in cluster 2 were significantly enriched in the roots of nitrogen-free treatment and inoculation treatment at 15 and 28 dpt (SNE, NFE, SNM and NFM) and in the root exudates from nitrogen-free treatment and inoculation treatment at 15 dpt (SNE and NFE). In the roots and root exudates of all three treatments, the accumulation of flavonoids in cluster 3 was higher at 1 dpt (SNI, NFI and MNI). Flavonoids in cluster 4 were obviously enriched in the roots of nitrogen-free treatment at 3 and 15 dpt (NFC and NFE) and in the inoculated roots at 3 dpt (SNC), and in the root exudates from the three treatments at 1 dpt (SNI, NFI and MNI). Then, the differences in metabolites among the three treatments in the roots and root exudates were preliminarily screened with VIP > 1 (Fig. 3). In the three pairwise comparison groups (NF vs. MN, NF vs. SN, and MN vs. SN), up- and down-regulated flavonoids are for the latter. In NF vs. MN, 226 and 209 differential flavonoids (DFs) were derived from the roots and root exudates, respectively (Fig. 3A). Biflavones, flavanols, flavanonols and tannin were down-regulated in MN roots and root exudates, as well as anthocyanidins in

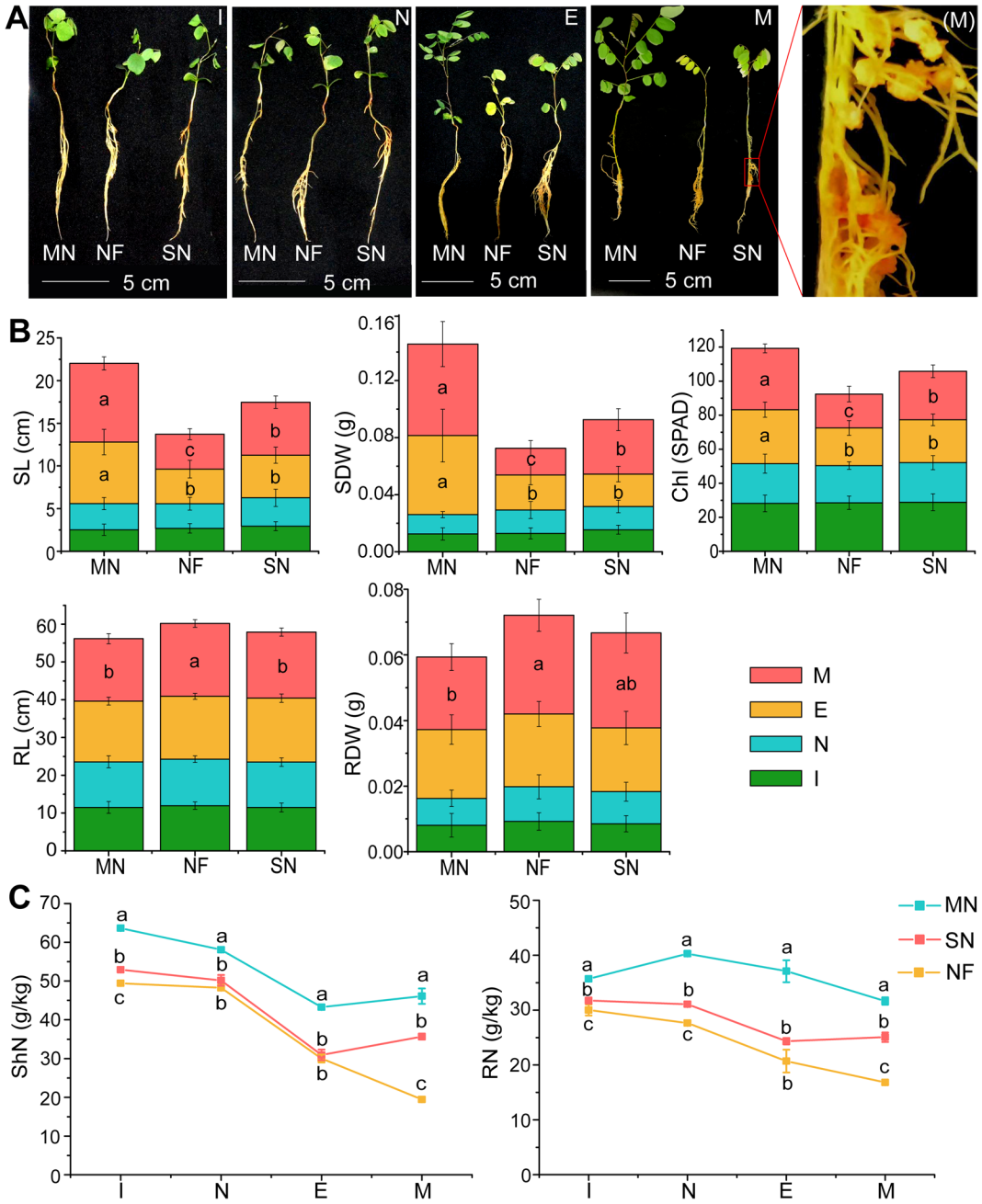


Fig. 1 Plant phenotype and morphological indexes at four growth stages under different nitrogen conditions. **A** Phenotypic photos of the plants. **B** Statistical results of growth phenotype. **C** Statistical results of nitrogen content in the shoots and roots ($n=3$). Data are presented as the mean ± standard deviation (SD). A statistical Tukey test was used, and means with different letters were statistically different at $P<0.05$ in three treatments at the same stage. NF, nitrogen free. SN, symbiotic nitrogen. MN, mineral nitrogen. I, 1 day post-treatment (dpt). N, 3 dpt. E, 15 dpt. M, 28 dpt. (M), magnification of the roots of SN in M. Chl, the chlorophyll content. SL, the shoot length. SDW, the shoot dry weight. RL, the root length. RDW, the root dry weight. ShN, the shoot nitrogen content. RN, the root nitrogen content

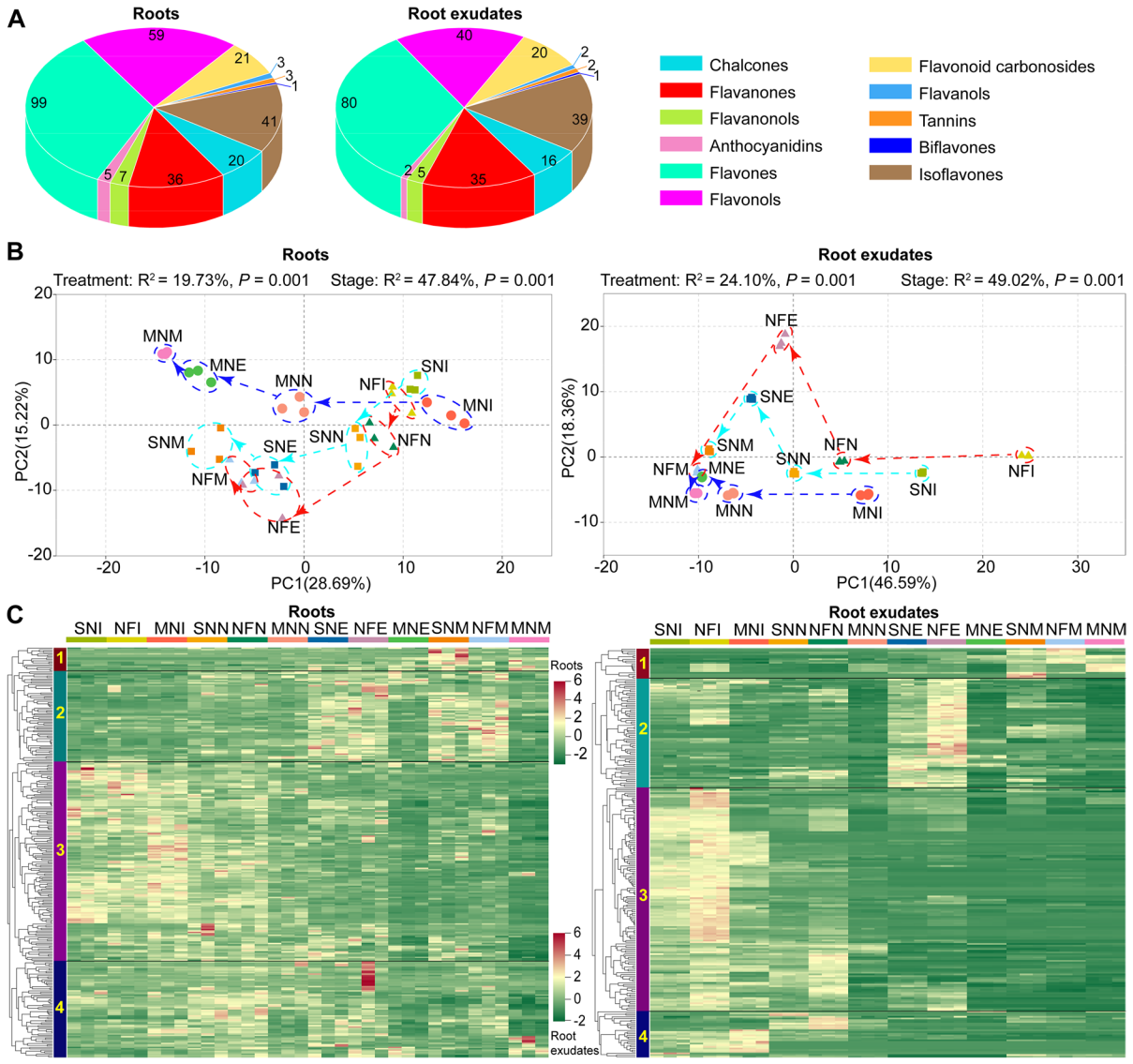


Fig. 2 Flavonoid metabolism profiles of the roots and root exudates of the plants subjected to three treatments during different growth stages ($n = 3$). **A** Classification of flavonoids. **B** Principal component analysis (PCA). **C** Heat map visualization. The profiles of all types of flavonoids were normalized to complete the linkage hierarchical clustering. NFI, NFN, NFE, and NFM, nitrogen-free treatment at 1, 3, 15 and 28 days post-treatment (dpt), respectively. SNI, SNN, SNE, and SNM, inoculation treatment at 1, 3, 15 and 28 dpt, respectively. MNI, MNN, MNE, and MNM, nitrogen addition treatment at 1, 3, 15 and 28 dpt, respectively

MN roots and chalcones, flavonoid carbonoside, and isoflavones in MN root exudates (Fig. 3B). In NF vs. SN, 137 and 162 DFs were obtained from the root and root exudates, respectively (Fig. 3A). Biflavones were up-regulated, while flavanols and tannin were down-regulated in SN roots. Biflavones and flavanonols were down-regulated in SN root exudates (Fig. 3B). Besides, anthocyanidins, flavanols and tannin were not DFs in NF vs. SN (Fig. 3B). In MN and SN, 212 and 214 DFs were identified in the roots and root exudates, respectively (Fig. 3A). Biflavones, flavanols and flavanonols were up-regulated in SN roots (Fig. 3B). Flavanols and flavanonols and tannin were up-regulated, while biflavones were down-regulated in SN

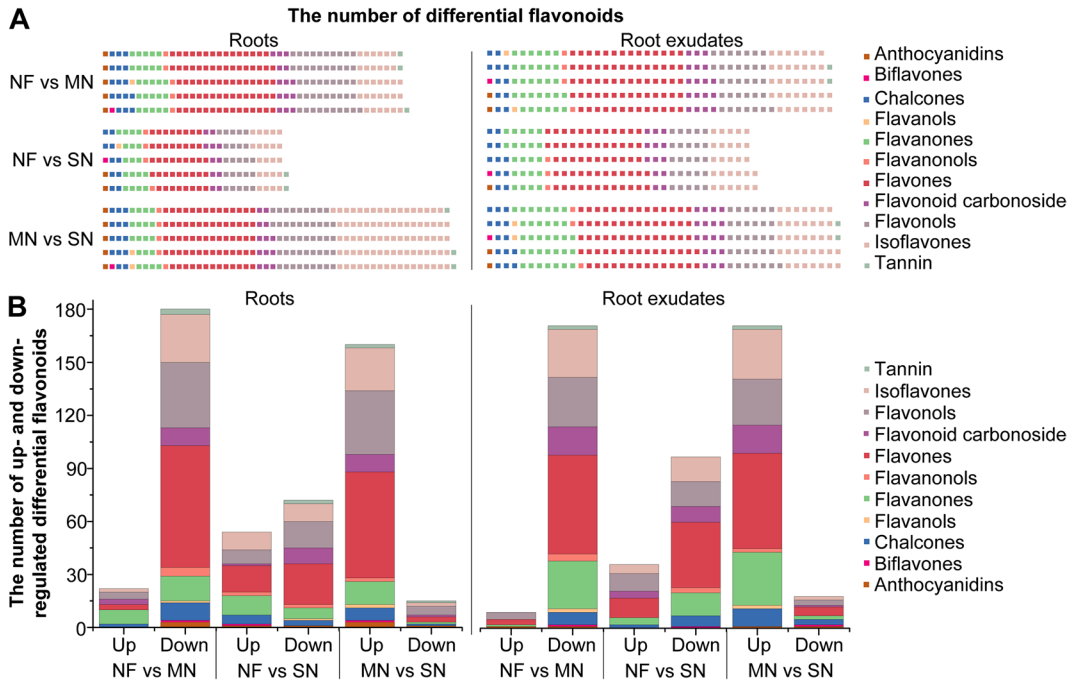


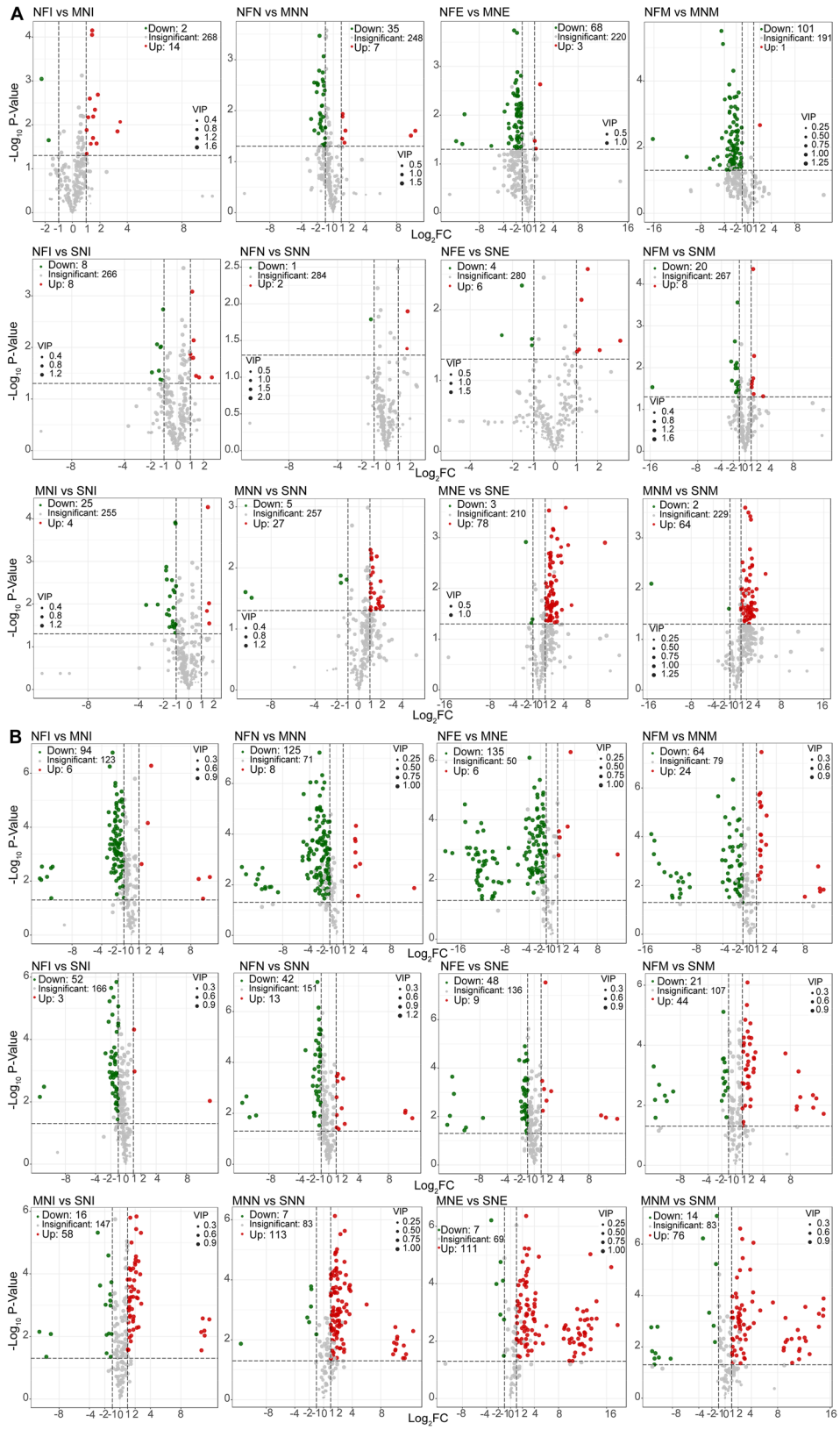
Fig. 3 Differential flavonoid metabolism profiles of the roots and root exudates of the plants subjected to three pairwise comparison groups in all stages. **A** The number of differential flavonoids. **B** The number of up- and down-regulated differential flavonoids. NF, nitrogen free. SN, symbiotic nitrogen. MN, mineral nitrogen. In all pairwise comparison groups, up- and down-regulated compounds are for the latter

root exudates (Fig. 3B). Additionally, flavanones, flavones and flavonols exhibited both up-regulation and down-regulation within the same comparison group (Fig. 3B). In NF vs. MN and NF vs. SN, the number of down-regulated DFs in the roots and root exudates of MN and SN is greater than the number of up-regulated DFs, while in MN vs. SN, the number of up-regulated DFs in SN roots and root exudates is greater than the number of down-regulated DFs (Fig. 3B). These results showed that in the roots and root exudates, the accumulation patterns of flavonoids were different in response to distinct nitrogen sources and showed growth-stage-dependent changes.

Metabolic changes of flavonoids at different growth stages

According to the above results, we speculated that in the growth process, plants would produce and secrete more flavonoids in the case of nitrogen deficiency, while produce and secrete less flavonoids in the case of sufficient nitrogen nutrition. To verify this conjecture, we next investigated the significant differences in metabolites among the three treatments in the plant growth process, and volcano plots were used to recognize the down-regulated (FC $\leq$ 0.5, VIP > 1, and P-value < 0.05) or up-regulated (FC $\geq$ 2, VIP > 1, and P-value < 0.05) flavonoids (Fig. 4).

Firstly, the DFs in the roots and root exudates during the plant growth process were analyzed. In NF vs. MN, there were 16, 42, 71 and 102 DFs in the roots (Fig. 4A) and 100, 133, 141 and 88 DFs in the root exudates (Fig. 4B) at the four sampling time points, respectively, indicating that the number of DFs increased gradually with plant growth in the roots and increased gradually in the first three stages but decreased at stage M in the root exudates. In NF vs. SN, there were 16, 3, 10 and 28 DFs in the roots (Fig. 4A) and 55, 55, 57 and 65 DFs in the roots exudates (Fig. 4B) at the four stages, respectively, suggesting that in the roots and root exudates, the number of DFs increased gradually from stage N. In MN vs. SN, there were 29, 32, 81 and 66 DFs in the roots (Fig. 4A) and 74, 120, 118 and 90 DFs


A
NFI vs MNI
Down: 2
Insignificant: 268
Up: 14
NFN vs MNN
Down: 35
Insignificant: 248
Up: 7
NFE vs MNE
Down: 68
Insignificant: 220
Up: 3
NFM vs MNM
Down: 101
Insignificant: 191
Up: 1
NFI vs SNI
Down: 8
Insignificant: 266
Up: 8
NFN vs SNN
Down: 1
Insignificant: 284
Up: 2
NFE vs SNE
Down: 4
Insignificant: 280
Up: 6
NFM vs SNM
Down: 20
Insignificant: 267
Up: 8
MNI vs SNI
Down: 25
Insignificant: 255
Up: 4
MNN vs SNN
Down: 5
Insignificant: 257
Up: 27
MNE vs SNE
Down: 3
Insignificant: 210
Up: 78
MNM vs SNM
Down: 2
Insignificant: 229
Up: 64
B
NFI vs MNI
Down: 94
Insignificant: 123
Up: 6
NFN vs MNN
Down: 125
Insignificant: 71
Up: 8
NFE vs MNE
Down: 135
Insignificant: 50
Up: 6
NFM vs MNM
Down: 64
Insignificant: 79
Up: 24
NFI vs SNI
Down: 52
Insignificant: 166
Up: 3
NFN vs SNN
Down: 42
Insignificant: 151
Up: 13
NFE vs SNE
Down: 48
Insignificant: 136
Up: 9
NFM vs SNM
Down: 21
Insignificant: 107
Up: 44
MNI vs SNI
Down: 16
Insignificant: 147
Up: 58
MNN vs SNN
Down: 7
Insignificant: 83
Up: 113
MNE vs SNE
Down: 7
Insignificant: 69
Up: 111
MNM vs SNM
Down: 14
Insignificant: 83
Up: 76
VIP
-Log10 P-Value
Log2FC

◂**Fig. 4** Volcano plots of the roots (**A**) and root exudates (**B**) of the plants subjected to different comparison groups during different growth stages. NFI, NFN, NFE, and NFM, nitrogen-free treatment at 1, 3, 15 and 28 days post-treatment (dpt), respectively. SNI, SNN, SNE, and SNM, inoculation treatment at 1, 3, 15 and 28 dpt, respectively. MNI, MNN, MNE, and MNM, nitrogen addition treatment at 1, 3, 15 and 28 dpt, respectively. In all pairwise comparison groups, up- and down-regulated compounds are for the latter and are shown with red and green dots, respectively, and grey dots represent insignificant compounds

in the root exudates (Fig. 4B) at the four sampling time points, respectively, indicating that the number of DFs increased gradually in the first three stages but decreased at stage M in the roots and decreased gradually from the N stage in the root exudates.

Then, the cases of up-regulated and down-regulated DFs with plant growth were analyzed. In NF vs. MN, the number of up-regulated DFs in NF roots (2, 35, 68, and 101) gradually increased with the plant growth process, while the number of up-regulated DFs in MN roots (14, 7, 3, and 1) gradually decreased over time (Fig. 4A). Similarly, in NF vs. MN, the number of up-regulated DFs in NF root exudates (94, 125, 135, and 64) showed a progressive increase in the first three stages and decreased at stage M, whereas the number of up-regulated DFs in MN root exudates (6, 8, 6, and 24) fluctuated with plant growth (Fig. 4B). In NF vs. SN, the number of up-regulated DFs in both NF roots (8, 1, 4, and 20) and SN roots (8, 2, 6, and 8) showed the trend of first decline and then rise (Fig. 4A), and the number of up-regulated DFs in both NF root exudates (52, 42, 48, and 21) and SN root exudates (3, 13, 9, and 44) fluctuated with plant growth (Fig. 4B). In addition, in MN vs. SN, the number of up-regulated DFs in MN roots (25, 5, 3, and 2) gradually declined with plant growth, whereas the number of up-regulated DFs in SN roots (4, 27, 78, and 64) gradually increased in the first three stages and decreased at stage M (Fig. 4A). And the number of up-regulated DFs in SN root exudates (58, 113, 111, 76) was higher than that in MN root exudates (16, 7, 7, 14) at four stages (Fig. 4B).

As anticipated, the plants produced more flavonoids under nitrogen-free conditions than the plants inoculated with rhizobia or supplied with NO_3^- in the plant growth process. Generally, a nodule capable of fixing nitrogen is formed about 15 to 20 days after rhizobia infection; until then, the plants are deficient in nitrogen. Thus, compared with that in the plants supplied with NO_3^-, the inoculated plants produced and secreted more flavonoids during the first three stages, similar to the NF treatment, after which (15 days after inoculation) the synthesis and secretion of flavonoids were decreased. Hierarchical clustering analysis showed the same results (Fig. S1). The data provide strong support for the conjecture described above.

Enrichment analysis of DFs

To understand the case of enrichment of all the above DFs in the metabolic pathway, the KEGG pathway enrichment analysis was performed. The results showed that in the plant growth process, the metabolic pathways involved in the DFs significantly enriched responding to different nitrogen sources were mainly the biosynthesis of secondary metabolites, the flavonoid biosynthesis and the isoflavonoid biosynthesis in the roots, while were mainly the flavone and flavonol biosynthesis, and the isoflavonoid biosynthesis in the root exudates (Fig. S2).

Then, the metabolic profiles involved in the DFs significantly enriched (46 in the roots, 32 in the root exudates) with plant growth were summarized (Fig. 5), and whether each DFs was up-regulated or down-regulated in each pairwise comparisons (NF vs. MN, NF vs. SN, and MN vs. SN) was also shown in Fig. 5. In the roots and root exudates, some of the DFs were present in all three pairwise comparisons (shown in the large boxes with three colors in Fig. 5), suggesting that the synthesis and secretion of these flavonoids (7 in the roots, 14 in the root exudates) could be related to the changes in the environmental nitrogen status and the plant nitrogen nutrient status, including formononetin, calycosin, pseudobaptigenin, eriodictyol, dihydroquercetin, luteolin and dihydrokaempferol in the roots (Fig. 5A); and daidzein, calycosin, pseudobaptigenin, prunetin, biochanin A, formononetin-7-*O*-glucoside, biochanin A-7-*O*-glucoside, apigenin, formononetin-7-*O*-glucoside-6″-*O*-malonate, cosmosiin, rhoifolin, vitexin, nicotiflorin and apigenin-7-*O*-[*β*-D-apiosyl-(1->2)-(6-malonyl-*β*-D-Glc)] in the root exudates (Fig. 5B).

The responses of DFs to different nitrogen sources were further analyzed. Some flavonoids showed significant changes in only one comparison group of roots or root exudates, and most of them changed significantly

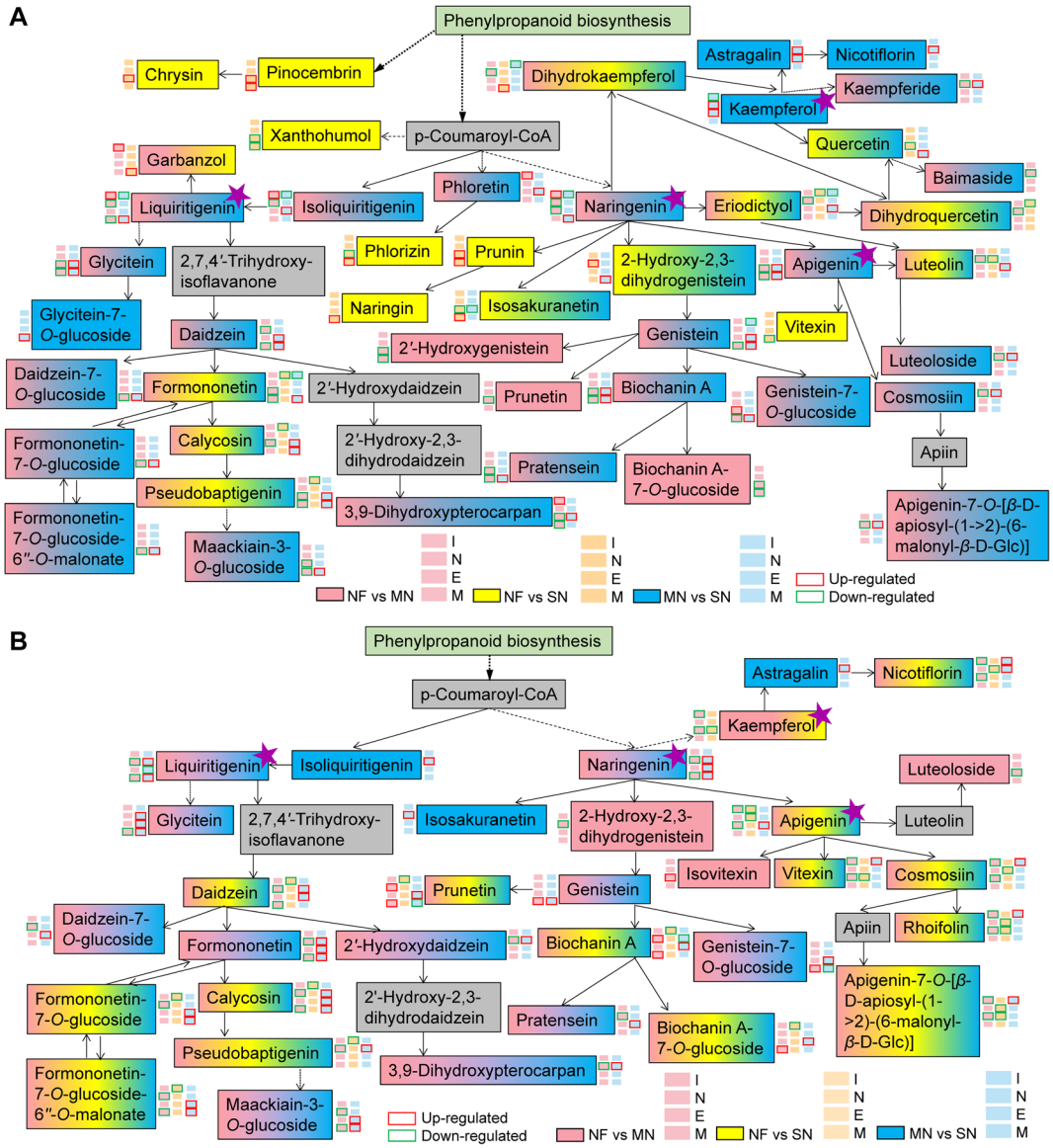


Fig. 5 Metabolic pathways of the differential flavonoids in the roots (**A**) and in the root exudates (**B**). The large gray boxes indicate that the substance was not detected. The large boxes with different colors represent that the substance in the box was detected in two or more pairwise comparisons. Different comparison groups are shown with different colors in the boxes (pink represents NF vs. MN, yellow represents NF vs. SN, and blue represents MN vs. SN). The four small boxes of the same color arranged vertically represent different stages of plant growth, with stage I at the top and stage M at the bottom. The red and green borders of the small boxes indicate the up-regulation and down-regulation of compounds, respectively. In all pairwise comparison groups, up- and down-regulated compounds are for the latter. Note: flavanones liquiritigenin and naringenin are metabolic nodes at the intersection of the flavanone biosynthesis and the isoflavonoid biosynthesis; apigenin (flavone) is a metabolic node at the intersection of the flavanone biosynthesis and the flavone biosynthesis; kaempferol (flavonol) is a metabolic node at the intersection of the flavanonol biosynthesis and the flavonol biosynthesis. Purple star, metabolic nodes. NF, nitrogen free. SN, symbiotic nitrogen. MN, mineral nitrogen. I, 1 day post-treatment (dpt). N, 3 dpt. E, 15 dpt. M, 28 dpt. Solid arrows, single biosynthetic steps. Dotted arrows, multiple steps

during the E and M stages, which was consistent with the variation in the plant growth phenotypes (Fig. 1). For example, in NF vs. MN, 2′-hydroxygenistein and biochanin A-7-*O*-glucoside at the E and M stages, and prunetin at stage E were significantly down-regulated in MN roots (Fig. 5A), demonstrating that the three flavonoids could be produced responding to nitrogen deficiency at the E and M stages; isovitexin was markedly up-regulated in MN root exudates at stage E (Fig. 5B), suggesting isovitexin could be involved in nitrogen uptake at stage E. In NF vs. SN, chrysin at stage E, pinocembrin and prunin at the E and M stages, and naringin at stage M were dramatically up-regulated in SN roots (Fig. 5A), indicating that they could be specifically interrelated to symbiotic nitrogen fixation; xanthohumol at the E and M stages and vitexin at stage M in SN roots were down-regulated (Fig. 5A), suggesting that these compounds could be produced responding to nitrogen deficiency at the E and M stages. Importantly, in MN vs. SN, six flavonoids, containing glycitein-7-*O*-glucoside at stage M, astragalin at the N and E stages, and nicotiflorin at stage N in the roots (Fig. 5A), and isoliquiritigenin, isosakuranetin and astragalin in the root exudates at stage N (Fig. 5B), were significantly up-regulated in SN, demonstrating that they could be mainly associated with the symbiotic infections. Besides, some flavonoids were present in multiple comparison groups and fluctuated with plant growth, suggesting that their response to different nitrogen nutrition of different sources were related to plant growth and development, such as genistein, isoliquiritigenin and eriodictyol in the roots (Fig. 5A), and prunetin and biochanin A in the root exudates (Fig. 5B).

Finally, two main metabolic pathways were identified (Fig. 5), including isoflavonoid biosynthesis, and flavone and flavonol biosynthesis. Concurrently, four compounds are metabolic nodes at the intersection of different flavonoid metabolic pathways, including liquiritigenin, naringenin, kaempferol and apigenin. In the plant growth process, node flavonoids were significantly enriched in response to different nitrogen sources. Compared with those in MN, liquiritigenin and naringenin were markedly down-regulated in both the 1-dpt NF and 1-dpt SN roots and were up-regulated in the 3-dpt NF roots, 28-dpt SN roots and in the root exudates of both NF and SN at 3 and 28 dpt (Fig. 5). Moreover, compared with those in MN, liquiritigenin was dramatically up-regulated in the 28-dpt NF roots and was down-regulated in the 15-dpt SN root exudates (Fig. 5), while naringenin was up-regulated in the 15-dpt NF roots and in the 15-dpt SN root exudates (Fig. 5). Apigenin was dramatically up-regulated in both NF roots and SN roots at 15 and 28 dpt, in the 1-dpt and 15-dpt NF root exudates and in the 15-dpt SN root exudates as compared to that in MN (Fig. 5). Compared with that in MN, kaempferol was significantly down-regulated at 1 dpt and subsequently up-regulated at 3 and 15 dpt in the SN roots, and also was up-regulated in the 1-dpt and 15-dpt NF root exudates (Fig. 5). Compared with that in NF, apigenin was markedly down-regulated in the 1-dpt, 3-dpt and 15-dpt SN root exudates, while kaempferol was dramatically down-regulated in the 15-dpt SN root exudates (Fig. 5). These results indicated that liquiritigenin, naringenin, apigenin and kaempferol were synthesized and secreted mainly responding to nitrogen deficiency and were associated with symbiotic nodulation, among which apigenin and kaempferol seemed to have a stronger response to nitrogen deficiency.

Network analysis of DFs in significantly enriched metabolic pathways

To further obtain the key DFs in response to different nitrogen sources, network analysis was performed on DFs in Fig. 6. In the network, nodes represent individual compounds. Edges represent pairwise correlations among nodes in the network, indicating biologically or biochemically meaningful interactions. There were 38 nodes and 97 edges in the roots network (Fig. 6A), and 32 nodes and 179 edges in the root exudates network (Fig. 6B), suggesting that the interactions among flavonoids in root exudates were stronger than that in the roots. There were 6 key nodes (degree > 10 and closeness centrality > 0.36) in the roots network, and the corresponding flavonoids were liquiritigenin, naringenin, daidzein-7-*O*-glucoside, formononetin-7-*O*-glucoside-6″-*O*-malonate, genistein and formononetin-7-*O*-glucoside (Fig. 6A). There were 10 key nodes (degree > 15 and closeness centrality > 0.58) in the root exudates network, and the corresponding flavonoids were 3,9-dihydroxypterocarpan, apigenin, cosmosiin, calycosin, daidzein-7-*O*-glucoside, genistein, kaempferol, luteoloside, rhoifolin, liquiritigenin (Fig. 6B). Two key nodes (liquiritigenin and naringenin) from the roots network and 3 key nodes (apigenin, kaempferol and liquiritigenin) from the root exudates network were consistent with metabolic nodes in Fig. 5, suggesting that plants may respond to

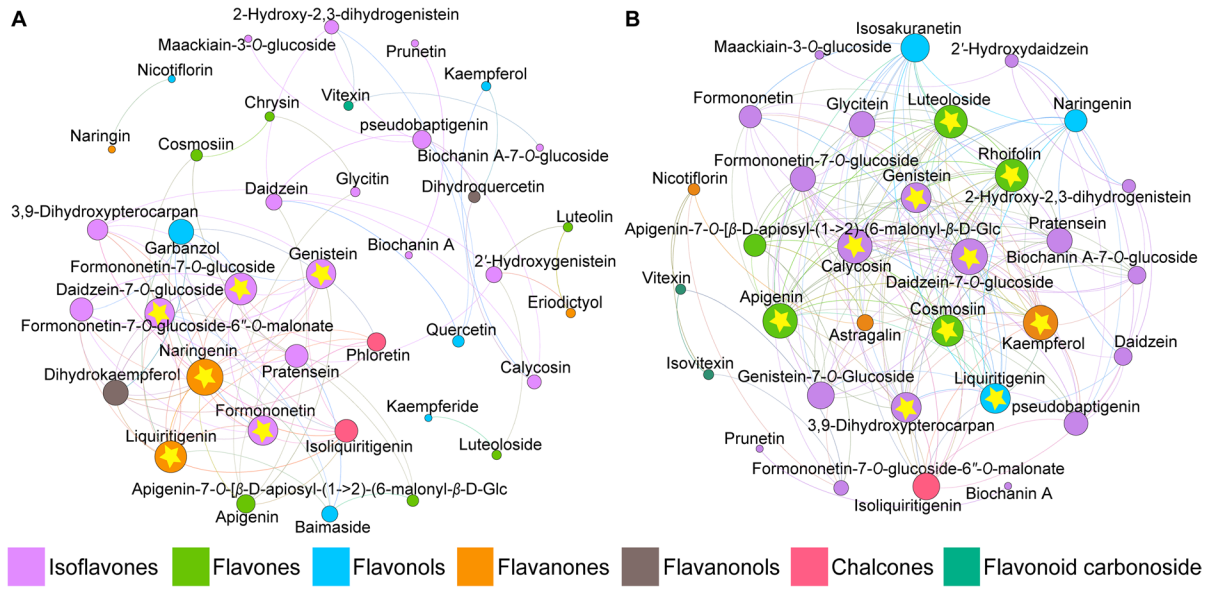


Fig. 6 Interaction networks of the differential flavonoids in the roots (**A**) and in the root exudates (**B**). Size of node represents relative number of direct connections made by individual. Node size represents degree. The yellow star represents the key nodes in the network

different nitrogen sources by regulating the synthesis and secretion of these four key flavonoids.

Relative contents of key flavonoids

To obtain detailed information on the response of four key flavonoids described above (liquiritigenin, naringenin, apigenin, kaempferol) to different nitrogen sources, their relative contents were analyzed (Fig. 7). The content changes of liquiritigenin and naringenin in the roots and liquiritigenin in the root exudates under the different treatments varied with plant growth but showed similar trends under the same treatment (Fig. 7). For instance, the accumulation of liquiritigenin and naringenin in SN roots and liquiritigenin in SN root exudates fluctuated with plant growth and the trends of their content variation in SN were consistent with those in NF (Fig. 7). In MN, the accumulations of liquiritigenin and naringenin in the roots and liquiritigenin in the root exudates were at

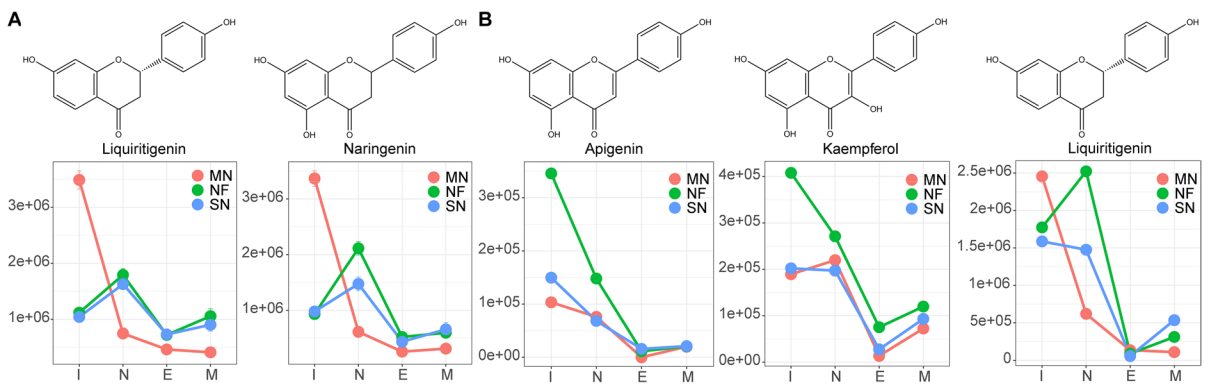


Fig. 7 Relative contents of the key flavonoids in the roots (**A**) and in the root exudates (**B**). NF, nitrogen free. SN, symbiotic nitrogen. MN, mineral nitrogen. I, 1 day post-treatment (dpt). N, 3 dpt. E, 15 dpt. M, 28 dpt

their highest level at stage I and much higher than those of other treatments, and then decreased rapidly with the plant growth (Fig. 7A). By contrast, the contents of liquiritigenin and naringenin in the roots and liquiritigenin in the root exudates of NF and SN were highest at stage N and were higher than those in MN at the last three stages (Fig. 7). These results indicated that the synthesis and secretion of liquiritigenin and naringenin could be mainly in response to nitrogen deficiency and symbiotic nitrogen fixation. In the root exudates, the content changes of apigenin and kaempferol under the different nitrogen conditions varied with plant growth but showed similar trends (Fig. 7B). In NF root exudates, the contents of apigenin and kaempferol were higher than or comparable to that of other treatments at the different stages of plant growth and their highest levels at the I stage (Fig. 7B), suggesting that apigenin and kaempferol were secreted to respond to nitrogen deficiency. Taken together, these results were similar to those of the metabolic nodes in Fig. 5.

Discussion

As a major component of protein, nucleic acids, and enzyme cofactors, nitrogen is an important macronutrient and plays a crucial role in plant growth and development (Chen et al. 2020). In general, nitrogen deficiency generally reduces plant leaf Chl concentration, yield, and biomass (Zhao et al. 2005). In our study, nitrogen deficiency inhibited the growth of aboveground parts of the plants, while the root nodule symbiosis and nitrogen addition can alleviate the inhibition of plant growth caused by nitrogen deficiency (Fig. 1). Furthermore, RL and RDW were significantly higher in NF than those in MN at stage M (Fig. 1B), which is an important adaptive strategy for plants in response to nitrogen deficiency (Sun et al. 2020). Nitrogen fixation cannot be performed in the early stages of the symbiotic relationship between legumes and rhizobia, so the ShN and RN of SN and NF should be comparable in the first three stages. However, the values of ShN at stage I and RN at the I and N stages of SN were markedly higher than those in NF, though the differences were not as significant as in the last stage (Fig. 1C), which may potentially be due to the release of nitrogen-containing compounds for plant growth after the breakdown of inoculated bacteria. Mature nodules provide nitrogen to plants, but cannot reach the comparable level as nitrate supply in a short period, so the ShN and RN of MN were significantly higher than those in SN at four stages (Fig. 1C). In NF, where external nitrogen sources were lacking, plants relied on internal nitrogen reservoirs to meet their growth demands, resulting in a gradual decline of both ShN and RN with plant growth (Fig. 1C). Unlike NF, the plants can acquire nitrogen through symbiotic nitrogen fixation by rhizobia in SN. However, in the initial stage of symbiotic organogenesis, young nodules do not have the ability to fix nitrogen until the nodules mature. Therefore, the substantial increase in ShN and RN at stage M may be associated with the nitrogen-fixation ability of the mature nodules (Fig. 1C). In MN, mineral nitrogen was acquired by the plants through root uptake and the long-distance transport between roots and shoots. Thus, the RN showed the trend of first rise and then decline (Fig. 1C). In summary, the changes of both ShN and RN in NF, SN and MN could be attributed to the different responses of plants to nitrogen availability and utilization during various growth stages.

The flavonoids secreted by plants into the rhizosphere have various ecological effects. For instance, medicarpin, pisatin and quercetin inhibited the growth of plant pathogens (Guenoune et al. 2001; Parvez et al. 2004; Wu and VanEtten 2004). Certain flavonoids acted as repellents for specific nematode species (Wuyts et al. 2006). Several flavonoids in root exudates of forage legume *Desmodium uncinatum* significantly inhibited the post-germination and attachment of the parasitic weed *Striga* (Hooper et al. 2010; Khan et al. 2010). Flavonoids are also classified as antimicrobials, insecticides and herbicides to promote plant growth based on their ecological effects in the rhizosphere. It has been identified that there were the flavonoids including acacetin, robinin, quercetin, rutin, kaempferol, apigenin and luteolin in *R. pseudoacacia* (Guo et al. 2019; Tyśkiewicz et al. 2019; Veitch et al. 2010), and apigenin, naringenin and isoliquiritigenin were found in its root exudates (Scheidemann and Wetzel 1997). In the current study, the flavonoid metabolism of the roots and root exudates in black locust were detected based on UPLC-MS/MS, and 295 and 242 flavonoids were characterized, respectively (Fig. 2A). These flavonoids were categorized into 11 major classes, including chalcones, flavanones,

flavanonols, anthocyanidins, flavones, flavonols, flavonoid carbonoside, flavanols, tannin, biflavones, and isoflavones. This greatly expands our understanding of the specialized flavonoids in the roots and root secretions of *R. pseudoacacia* in response to different nitrogen environments.

The accumulation of specialized metabolites is one of the characteristics of various nutrient deficiencies in plants and the up-regulation of specialized metabolic pathways is considered to be the stress response mechanisms in plants, such as nutrient stress, drought stress and salt stress (Cao et al. 2019). Flavonoids, as one of these specialized metabolites, play a crucial role in resisting environmental stresses. It is worth mentioning that although different plant species often develop specific branches to adapt to diverse environmental conditions, they share a common core flavonoid pathway in higher plants (García-Calderón et al. 2020). The dynamic change analysis of flavonoids also showed that the synthesis and secretion of flavonoids varied with the different growth stages of plants in this study, which is consistent with the result that the secretion of isoflavones in soybeans showed growth-stage-dependent changes under normal conditions (Fig. 2B) (Sugiyama et al. 2016).

The flavonoid synthesis or flavonoid content was negatively correlated with increasing nitrogen supply. For instance, the levels of rutin and quercetin (flavonols), luteolin (flavones) and catechin (flavanols) were significantly increased due to nitrogen deficiency in inflorescences of *Coreopsis tinctoria Nutt.* (Li et al. 2021). Genistein and daidzein (isoflavones) secretions from soybean roots were significantly increased under nitrogen-deficient growth conditions, and glycitein (isoflavones) and isorhamnetin (flavonols) were accumulated to higher levels in soybean roots and shoots under low nitrogen conditions (Nezamivand-Chegini et al. 2022; Sugiyama et al. 2016). We found that more flavonoids were synthesized and secreted under nitrogen deficiency (Fig. 4). Due to the limitation of protein synthesis under nitrogen-deficient conditions, the availability of phenylalanine as a synthetic precursor of flavonoids increases that leading to the increased synthesis of flavonoids. Our results also provide evidence for previous research findings that nitrogen supply has a negative effect on flavonoid biosynthesis in plants.

As one of the responses to low nitrogen provision, plants produce and secrete flavonoids to increase nitrogen acquisition. For example, the flavonoids secreted by maize roots under nitrogen deprivation predominantly recruited Oxalobacteriaceae, which induced lateral root formation and thus facilitated nitrogen uptake from soil (Yu et al. 2021). Here, some flavonoids were identified to be markedly accumulated under nitrogen-free conditions compared to the two nitrogen treatments (Fig. 5), such as isoflavones (daidzein at N and M stages and formononetin at stage M), flavones (apigenin at stage E and cosmosiin at stage N) and flavanones (liquiritigenin at N and M stages and naringenin at stage N), suggesting these flavonoids were produced and secreted to respond to nitrogen deficiency and could be involved in nitrogen or other nutrition acquisition. In addition to getting nutrients, flavonoids are secreted by plants to defend against biotic and abiotic stresses by recruiting beneficial microorganisms and inhibiting harmful microorganisms. Acting as a repellent, daidzein (isoflavone) was secreted by soybean roots into the soils to induce the formation of a healthy microbial population (Okutani et al. 2020). Kaempferide 3-*O*-[2^{G}-*β*-D-glucopyranosyl]-*β*-rutinoside (flavonols / flavanonols) from *Dianthus caryophyllus* had an obvious defense effect against *Fusarium oxysporum* (Curir et al. 2005). Therefore, in this study, the flavonoids synthesized and secreted by black locust under nitrogen-free conditions may also recruit beneficial microorganisms contributing to nitrogen acquisition, which needs to be further explored.

In addition to inhibiting harmful microorganisms, flavonoids can also promote the absorption of nutrient elements. The flavonoids, such as hesperetin (flavanones), luteolin and chrysin (flavones), enhanced mycorrhizal colonization in the roots, which led to the solubilization of mineral phosphate and may contribute to the mineralization of nutrients such as nitrogen (Bag et al. 2022). Flavonols (such as kaempferol, nicotiflorin, and rutin) also acted as metal-chelating agents, making it easier for plants to obtain certain trace elements (Gupta and Chakrabarty 2013). In the rhizosphere, flavonoids including genistein (isoflavones), quercetin and kaempferol (flavonols), improved Fe utilization by chelating and converting oxides of iron from Fe^{3+} to Fe^{2+} (Cesco et al. 2010). We also found some flavonoids such as isovitexin (flavonoid carbonoside) at stage E, genistein and genistein-7-*O*-glucoside (isoflavones) at stage M, and biochanin A (isoflavones) at N and M stages, were

dramatically accumulated in MN root exudates compared to those in NF and SN, and the aboveground biomass of the plants was significantly increased (Fig. 5), suggesting these flavonoids may boost the plant's uptake of mineral nitrogen and other nutrients, such as iron and phosphorus.

Low nitrogen level promotes the secretion of *nod* gene-inducing flavonoids from the legume roots. In chickpea roots inoculated with *Rhizobium*, a very high accumulation of the isoflavones, formononetin and biochanin A and their glycoside conjugates were found (Arfaoui et al. 2007). After inoculation with *Bradyrhizobium japonicum*, elevated concentrations of daidzein and genistein (isoflavones) were detected in soybean root exudate (Cooper 2004). The secretion of flavonoids varied at diverse stages of the development of the symbiotic relationship. The *nod* gene-inducing flavonoids, specifically luteolin (flavones), had dual actions as chemoattractants, and different flavonoids attracted different *Rhizobium* species (Hassan and Mathesius 2012). Kaempferol (flavonols) inhibited the polar transport of auxin in *M. truncatula* roots, leading to initiate the formation of nodule primordia. Meanwhile, the *nod* genes inducer 7,4′-dihydroxyflavone (flavones) inside the roots seemed to sustain Nod-factor synthesis in the infection thread (Zhang et al. 2009). Moreover, the levels of formononetin and ononin were systematically inhibited responding to *Sinorhizobium meliloti* and Nod factors, indicating that these isoflavones probably participated in the systemic modification of nodulation (Catford et al. 2006). Here, we found that the *nod* gene-inducing flavonoids were significantly accumulated in SN root exudates, including isoliquiritigenin (chalcones) at stage N, daidzein (isoflavones) at N and E stages, apigenin (flavones), daidzein-7-*O*-glucoside and genistein-7-*O*-glucoside (isoflavones) at stage E, genistein (isoflavones) at stage M, liquiritigenin (flavanones) at N and M stages, naringenin (flavanones) at N to M stages, formononetin-7-*O*-glucoside (isoflavones) at E and M stages. Additionally, flavonoids enriched significantly in the roots and root exudates in plants inoculated with rhizobia could be involved in different stages of symbiosis (Fig. 5). For instance, flavones (rhoifolin, cosmosiin and apigenin-7-*O*-[*β*-D-apiosyl-(1->2)-(6-malonyl-*β*-D-Glc)]) and flavonoid carbonoside (vitexin) were abundantly secreted at stage I in SN root exudates, suggesting they could be related to the recruitment or recognition of rhizobia; flavones (apigenin-7-*O*-[*β*-D-apiosyl-(1->2)-(6-malonyl-*β*-D-Glc)], luteoloside and cosmosiin) and flavonols (nicotiflorin, kaempferide, kaempferol and baimaside) were massively accumulated at stage N in SN roots, indicating they could be interrelated to both symbiotic infection and nodule initiation; chalcones (isoliquiritigenin and phloretin), isoflavones (3,9-dihydroxypterocarpan), flavonols (kaempferol and apigenin), flavanones (eriodictyol) and flavones (luteolin and chrysin) were largely synthesized at stage E in SN roots, demonstrating they could be associated with nodule development; chalcones (phlorizin), flavanones (liquiritigenin, naringenin, naringin and isosakuranetin), flavonols (quercetin, garbanzol and apigenin) and isoflavones (maackiain-3-*O*-glucoside, daidzein-7-*O*-glucoside, formononetin-7-*O*-glucoside-6″-*O*-malonate, formononetin, formononetin-7-*O*-glucoside, genistein-7-*O*-glucoside, glycitein-7-*O*-glucoside and pratensein) were abundantly accumulated at stage M in SN roots, suggesting they could be related to the maturation of nodules, the maintenance of nitrogen fixation function or systemic regulation of nodulation. Higher flavonoid levels in legume roots strongly facilitated infection via including up-regulation of *nod* genes and other responses in rhizobia (Liu and Murray 2016). Moreover, the expression of the *nod* genes in rhizobia (such as *S. meliloti* and *B. japonicum*) was suppressed by the presence of ammonium (Liu and Murray 2016). Studies on *M. truncatula* showed that the genes encoding chalcone-*O*-methyltransferase were expressed in the root hairs infected with rhizobia and in the infection zone but not in the nitrogen fixation zone of the root nodules (Chen et al. 2015). The nitrogen fixation and ammonia formation in nodules occur only in the nitrogen fixation region, so the expression of the genes involved in flavonoid synthesis may be inhibited by a high level of ammonia. Therefore, the level of available nitrogen may regulate the symbiotic nodulation between legumes and rhizobia by controlling the production of flavonoids in plants.

Flavonoid biosynthesis originates from phenylalanine. There are four pathways involved in flavonoid metabolism on the KEGG website, including flavonoid biosynthesis, flavone and flavonol biosynthesis, isoflavonoid biosynthesis and anthocyanin biosynthesis. In our study, we found that the main metabolic pathways involved in the DFs significantly enriched were the isoflavonoid biosynthesis

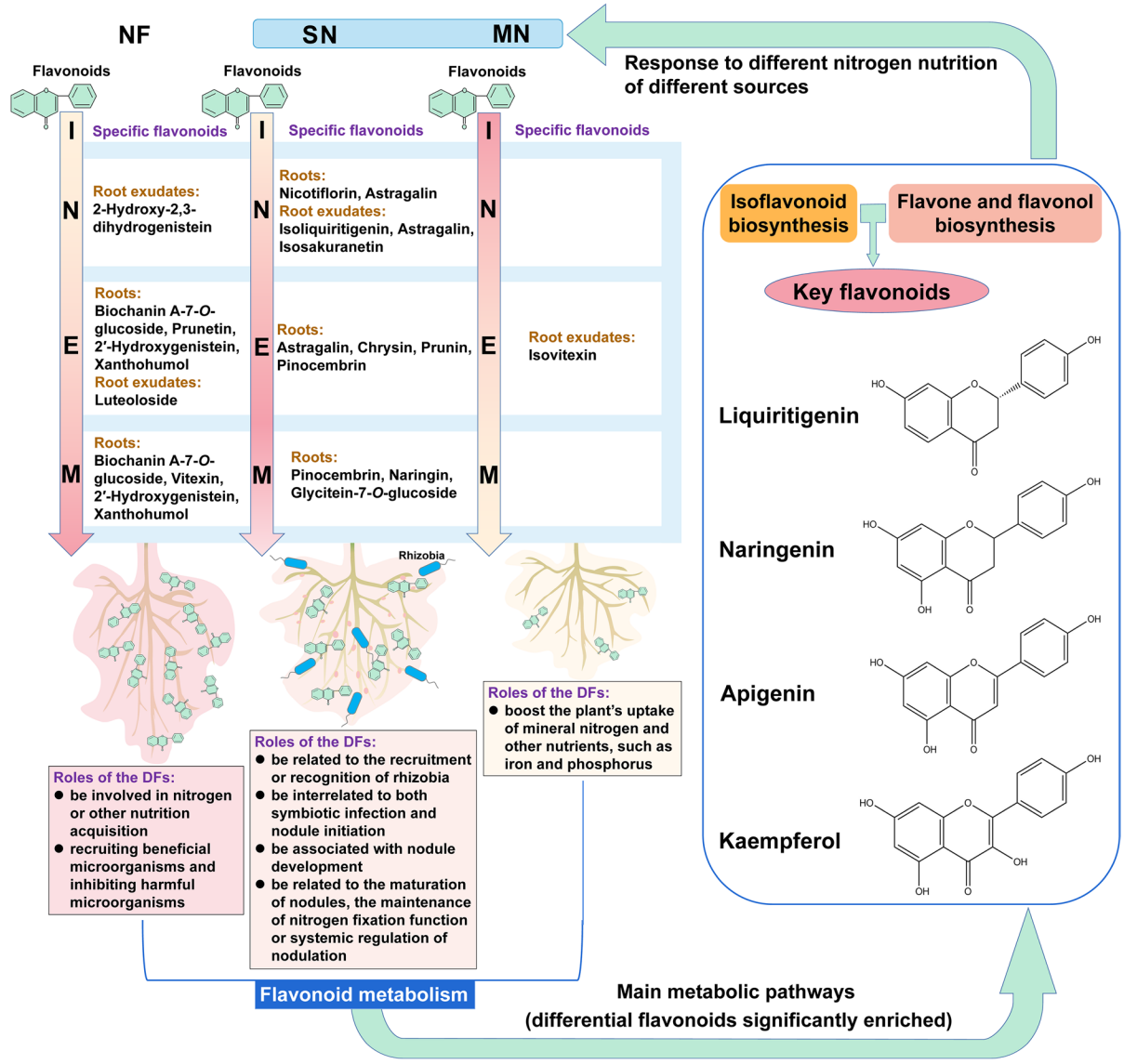


Fig. 8 Proposed mechanisms underlying how plants regulate flavonoid metabolism in response to different nitrogen sources. The big arrows with the pink gradient represent the plant roots and the light to dark color indicates the increased flavonoid synthesis and secretion at different stages of plant growth. DF, differential flavonoids. I, 1 day post-treatment (dpt). N, 3 dpt. E, 1 dpt. M, 28 dpt

and the flavone and flavonol biosynthesis (Fig. S2). Four key flavonoids (liquiritigenin, naringenin, kaempferol and apigenin) have been identified, and their responses to different nitrogen sources were distinct (Figs. 5, 6 and 7). Liquiritigenin, naringenin, apigenin and kaempferol were synthesized and secreted mainly responding to nitrogen deficiency and were associated with symbiotic nodulation, among which apigenin and kaempferol seemed to have a stronger response to nitrogen deficiency (Figs. 5, 6 and 7). Moreover, liquiritigenin, naringenin and kaempferol were associated with the absorption of nitrogen, which is consistent with previous findings that naringin and apigenin contributed to the mineralization of nutrients and kaempferol promoted the uptake of certain trace elements (Cesco et al. 2010; Gupta and Chakrabarty 2013; Masaoka et al. 1993).

Conclusion

In summary, our data point to a model (Fig. 8). The responses of flavonoids in the roots and root exudates of *R. pseudoacacia* to different nitrogen sources in the plant growth process were analyzed by metabolomic analysis. The results showed that there were significant differences in the synthesis and secretion of flavonoids in response to different nitrogen sources at various stages of plant growth. The synthesis and secretion of flavonoids were significantly increased in the plant under nitrogen deficiency conditions. Plants could respond to different sources of nitrogen by regulating the synthesis and secretion of key flavonoids. These findings provide important insights into the adaptability of legumes to different nitrogen environments and could aid in developing sustainable agricultural practices that optimize nitrogen resource utilization. Future work needs to elucidate the molecular mechanisms by which these flavonoids regulate the responses of *R. pseudoacacia* to different nitrogen sources and investigate the potential effects of these flavonoids on symbiotic nitrogen fixation and the growth of legume crops under varying nitrogen conditions.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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