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RESEARCH ARTICLE

Rhizosphere flavonoids alleviate inhibition of soybean nodulation caused by shading under maize–soybean strip intercropping

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Highlights

- Canopy light environment altering in maize–soybean strip intercropping exert stronger regulatory influence on nodule formation than belowground factors.
- Intercropping promotes flavonoids and flavonols exudation and regulates the nodule development.
- Root–root interaction between maize and soybean promotes nodulation and nitrogen fixation by increasing flavonoid secretion.

Abstract

Flavonoids produced by legume roots act as signaling molecules that induce the expression of nod genes in symbiotic rhizobia. However, the role of flavonoids in root exudates under intercropping systems in promoting soybean nodulation remains unclear. Two consecutive years of field experiments were conducted using maize–soybean strip intercropping with interspecific row spacings of 30 cm (MS30), 45 cm (MS45), and 60 cm (MS60), along with sole cropping of soybean (SS) and maize (MM). Root interactions were manipulated using either no root barrier (NB) or a polyethylene plastic barrier (PB) to assess the relationship between flavonoids in root exudates and soybean nodulation. We found that root–root interaction between soybean and maize increased nodule number and fresh weight in intercropped soybean, with enhancement gradually increasing as interspecific distance widened. The proportion of nodules with diameters exceeding 0.4 cm was higher in intercropped soybean under NB compared to PB. Additionally, the expression of nodule-related genes — *GmENOD40*, *GmNIN2b*, and *GmEXPB2* — was up-regulated. Furthermore, compared to monocropping, isoflavone secretion by soybean roots decreased, whereas flavonoid and flavonol secretion by both maize and soybean roots increased under intercropping. The abundance of differentially secreted flavonoid metabolites in the rhizosphere of both species declined when root contact was prevented by the barrier. In soybean roots, the expression of *GmCHS8* and *GmIFS1* was up-regulated, while *GmICHG* was down-regulated under root interaction. Most flavonoid and flavonol compounds showed positive correlations with nodule diameter. Nodule number, fresh weight, and the proportion of nodules larger than 0.2 cm increased in diverse soybean genotypes treated with maize root exudates, which contributed to enhanced nitrogen fixation capacity. Therefore, maize–soybean strip intercropping, combined with optimal row spacing, enhances the positive effects of underground root interactions and improves nodulation and nitrogen fixation in intercropped soybean.

Keywords: maize–soybean strip intercropping, root interaction, flavonoids, nodule

1. Introduction

Soybean (*Glycine max* (L.) Merrill) is one of the most important fodder and oil crops worldwide. In the symbiotic nitrogen fixation (SNF) system between soybean and rhizobium, nodules can fix N_2 from the atmosphere to support host plant growth (Mulder *et al.* 2005; Lyu *et al.*

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2022). Soybean produces up to 77% of the N_2 fixed by all legumes (Herridge *et al.* 2008), and 50–60% of the nitrogen required over its entire growth cycle is derived from N_2 fixed by nodules (Ohwakiy 1997). In agriculture, SNF not only increases soybean yield (Raza *et al.* 2019b) but also reduces dependence on nitrogen fertilizers, thereby lowering input costs and mitigating the ecological impacts associated with large-scale fertilizer application. Intercropping, a traditional crop diversification practice, offers advantages such as biological reciprocity, increased yield, and reduced fertilizer demand. Studies confirm that this diversification enables belowground complementarity, alters root exudation patterns, and reshapes microbial communities, potentially promoting beneficial microbial growth (Brooker *et al.* 2015; Li *et al.* 2024). Soybean is a major legume cultivated under intercropping systems, and its nodulation is a complex process. Therefore, it is essential to investigate the mechanisms by which crop diversification enhances efficient nodulation and nitrogen fixation in intercropped soybeans, particularly for boosting crop productivity under prevailing environmental stresses.

A complex signaling network governs legume–rhizobium recognition and subsequent root nodule development. Research indicates that symbiosis begins with the secretion of signal molecules in the roots, which attract rhizobia and stimulate the production of nodulation factors, thereby activating a cascade of downstream nodulation genes (Huang *et al.* 2014; Poole *et al.* 2018; Roy *et al.* 2020). Key genes such as *NFR1* and nodule inception (*NIN*) function in early nodulation events, including the perception of nodulation factors and the formation of infection threads (IT) (Indrasumunar *et al.* 2011; Fu *et al.* 2022). Mutants meanwhile lacking functional *Gmnin1a nin2a nin2b* or *Gmnin1a nin1b nin2a nin2b* exhibit similar defects in rhizobial infection and nodule formation, although rhizobia are captured within curled root hairs, but rhizobia cell division is limited and infection thread initiation fails (Fu *et al.* 2022). Additionally, early nodulin (*ENOD*) genes play critical roles during nodule development, with *ENOD40* serving as a marker gene for nodule primordium initiation and being up-regulated at nodulation initiation (Nguyen *et al.* 2020; Wang *et al.* 2022). To date, a limited number of expansin (*EXPB*) genes have been identified and localized in the nodule cortex, apoplasts, and IT walls (Giordano and Hirsch 2004; Sujkowska *et al.* 2007). Nodule primordium development is earlier in overexpression of *GmEXPB2* compared to wild-type plants and increases nodule number, nodule mass, and nitrogenase activity (Li *et al.* 2015).

Flavonoids, as key signal molecules, have been identified as regulators of nodule formation. Interestingly, certain legumes release specific flavonoids that induce the expression of NOD genes for rhizobia, thereby promoting its colonization and infection (Rao and Cooper 1995; Yang *et al.* 2022). The expression of genes encoding key biosynthetic enzymes, such as chalcone synthase (CHS), is integral to flavonoid synthesis. Interference with or silencing of MtCHS reduces root flavonoid concentrations and significantly impairs nodulation in *Medicago* truncatula (Wasson *et al.* 2006). In legumes, isoflavone synthase (IFS) redirects flavanone intermediates, naringenin and liquiritigenin, into the isoflavonoid branch of the phenylpropanoid pathway, leading to the synthesis of isoflavones such as genistein and daidzein (Jung *et al.* 2000; Sreevidya *et al.* 2006). Soybean nodulation specifically requires isoflavone synthase (GmIFS), underscoring species-specific differences in isoflavone dependency among legumes (Subramanian *et al.* 2006). Rhizosphere isoflavones also mediate communication with soil microorganisms. In soybean, isoflavones are secreted *via* two pathways: (1) ATP-dependent active transport of isoflavone aglycones (Sugiyama *et al.* 2007), and (2) secretion of isoflavone glycosides into the apoplast, followed by hydrolysis *via* isoflavone conjugate-hydrolyzing β-glucosidase (ICHG) (Suzuki *et al.* 2006).

The intercropping system optimizes aboveground light utilization through niche separation and complementarity, particularly when combining tall and short crops (Liu X *et al.* 2017; Raza *et al.* 2019a; Liu *et al.* 2022). Moreover, the high morphological plasticity of crop roots promotes belowground niche complementarity of underground space in the intercropping systems, enhancing resource utilization efficiency (Zhang *et al.* 2022; Raza *et al.* 2023), for example, legumes intercropped with gramineaes. Previous studies demonstrate that maize–soybean intercropping results in asymmetric root distribution (Gao *et al.* 2010), with both maize and soybean exhibiting enhanced root growth and lateral expansion toward inter-row spaces during co-growth stage (Te *et al.* 2023). Optimization of strip width in maize–soybean strip intercropping further improves nutrient uptake and biomass accumulation (Raza *et al.* 2020; Zheng *et al.* 2022). Thus, root interactions may play a crucial role in underground interspecific communication. In maize–faba bean intercropping, root interaction enhance the nodulation ability of faba bean, and maize root exudates have been shown to induce nodulation and symbiotic gene expression in faba bean (Li *et al.* 2016). However, it remains unclear whether maize root exudates similarly promote soybean nodulation, and how variations in root interaction intensity affect soybean's nodulation capacity.

In this study, we proposed the following hypotheses: (i) reasonable maize–soybean strip intercropping configuration alleviates shading-induced inhibition of nodulation through root interaction, thereby promoting nitrogen accumulation; (ii) the intensity of interspecific root interaction influences the secretion of flavonoid compounds in roots; and (iii) flavonoid compounds that promote nodule formation or development are present in maize root exudates. To evaluate these hypotheses, we established maize–soybean strip intercropping systems with different interspecific distances and root separation treatments, using monocropped maize and soybean as controls. These field experiments were complemented by indoor assays involving irrigation with maize root exudates. Together, these approaches assessed how root interaction intensity affects the secretion of flavonoid compounds in crop roots, as well as nodulation and nitrogen fixation in intercropped soybean. The aim of this study was to evaluate how differences in the rhizosphere secretory environment — driven by root interactions — influence the formation and development of soybean nodules.

2. Materials and methods

2.1. Experimental design and site

The experiment was conducted in Chongzhou (30°56′N, 103°65′E), Sichuan Province, China, from May 2021 to October 2022. Climate data for the research site during the intercropping growing seasons from 2021 to 2022 are presented in Appendix A. Total rainfall from May to October was 1,033.2 mm in 2021 and 608.5 mm in 2022. The soil chemical characteristics were as follows: average pH of 5.90, 1.70 g kg^{-1} of total N, 15.77 mg kg^{-1} of available P, 139.70 mg kg^{-1} of available K, and 27.27 g kg^{-1} of organic matter.

Field study (root barrier experiment) A two-factor split-plot design was implemented with three replications using a soybean (Nandou 12, strong nodulation, ND) and maize (Denghai 605) intercropping system. The main factor was maize–soybean spacing, with levels of 30 cm (MS30), 45 cm (MS45), and 60 cm (MS60); the subplot factor was root interaction, including root no barrier (NB) and root separation using a polyethylene plastic barrier (PB) between maize and soybean rows. Monocropped maize and soybean served as controls. The partition was 6 m long and 40 cm wide, constructed with 0.15 mm thick plastic (Appendix B).

Maize and soybean were planted with an equal row spacing of 100 cm. In the maize–soybean strip intercropping system, maize was grown in narrow rows (40 cm apart) and wide rows (160 cm apart), with two rows of soybean planted within each wide maize row at interrow distances of 100, 70, and 40 cm, respectively (Appendix B). Maize was planted at a density of 5.8×10^5 plants ha^{-1}, corresponding to an inter-plant spacing of 17 cm. Soybean was sown at a density of 11.7×10^5 plants ha^{-1}, with an inter-plant spacing of 8.5 cm. Each plot area was 6 m×6 m and contained three maize and three soybean strips per replication. Maize was sown on June 12, 2021 and May 22, 2022, and harvested on September 13, 2021 and September 1, 2022, respectively. Soybean was sown on June 12, 2021 and June 15, 2022, and harvested on October 22, 2021 and October 30, 2022, respectively.

Greenhouse study This experiment followed a completely randomized design with a 3×2 factorial arrangement: three soybean genotypes — ND (Nandou 12, strong nodulation), GX (Guixia 3, weak nodulation) and NTS (NTS1007, super nodulation) — and two root treatments (maize root exudates *vs*. distilled water control). Maize plants were grown in a mixture of perlite and vermiculite (v/v, 1:1) as culture medium. Soybean was cultivated in pots filled with field soil to maintain natural soil biota; this soil had an average pH of 8.5, 1.10 g kg^{-1} of total N, 4.5 mg kg^{-1} of available P, 139.00 mg kg^{-1} of available K, and 13.89 g kg^{-1} of organic matter. Soybean seeds were surface sterilized with 75% alcohol for 5 min, then sown in plastic pots (40 cm diameter, 55 cm height), each containing 2.5 kg of soil. Each pot had two holes with two plants, and resulting in total 120 experimental units. Hoagland's nutrient solution was applied to support plant growth.

At the maize jointing stage, roots were rinsed with distilled water and deionized water 3–6 times, then immersed in $CaCl_2$ solution for 6 h to collect root exudates. The collected secretions were filtered and stored for later use. From soybean emergence to the five trifoliolate leaf stage, maize root exudates were applied to irrigate soybean roots, once every 2 days at 200 mL per application.

2.2. Crop root exudate extraction and LC-MS analysis

Root exudates were collected from maize at the jointing stage and soybean at full bloom using the field home position method (Liu Y C *et al.* 2017). Crop roots were carefully removed from 25 cm×20 cm×20 cm soil blocks, rinsed with distilled water, and then washed three times with deionized water. The roots were subsequently soaked in a 5% thymol solution for 3 min and transferred into collection bags containing 500 mL of $CaCl_2$ solution for exudation over 6 h. Finally, the root exudates were filtered through a 0.45 μm polytetrafluoroethylene filter and freeze-dried to yield a powder using a freeze dryer (EYELA FDU-2110, Japan).

Frozen samples were extracted with 80% methanol (500 mL), shaken and allowed to stand at 4°C for 5 min, followed by centrifugation at 4°C and 15,000×g for 5 min. The supernatant was filtered through a Minisart 0.45 μm filter. LC-MS analysis was performed using an Exion LC (SCIEX, Germany) Chromatograph System coupled to a QTRAP 6500+ (SCIEX, Germany) mass spectrometer. Separation was achieved on a Xselect HSS T3 column (100 Å, 5 μm, 4.6 mm× 250 mm) maintained at 50°C, with a flow rate of 0.4 mL min^{-1}. Solvent A consisted of 99% water and 0.1% formic acid, while solvent B contained 99% acetonitrile and 0.1% formic acid. The gradient elution program was as follows: 2% B for 2 min, linear increase to 100% B over 13 min, hold at 100% B for 2 min, decrease to 2% B over 1 min, and root exudates (RE)-equilibration at 2% B for 2 min.

2.3. Real-time quantitative PCR

Total RNA was isolated from soybean roots using the UNIQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech, Shanghai) according to the manufacturer's instructions. The RNA was reverse transcribed into cDNA using Hifair®III 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen Biotech, Shanghai). RT-qPCR was performed on a CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad, USA) with Hieff qPCR SYBR Green Mix (Yeasen Biotech, Shanghai, China). Briefly, each 20 μL reaction mixture contained 10 μL of SYBR Green Mix, 2 μL of diluted cDNA, 1 μL of gene-specific primers (10 mmol L^{-1}), and 6 μL of H_2O. Thermal cycling conditions included an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, with a subsequent melting curve analysis to verify amplification specificity. All primer sequences used in this study are listed in Appendix C.

2.4. Legume nodulation

Three representative plants per replication in the field experiment and six soybean plants in the pot experiment were used to determine nodule number, nodule fresh weight, and average nodule diameter. All nodules were collected and rinsed, after which surface moisture was blotted dry for photographed prior to imaging for counting and weighing.

Nodule size was classified using Image-Pro Plus 6.0 software into three categories: class 1 (large nodules) with diameter >4 mm, class 2 (medium nodules) with diameter ranging from >2 to 4 mm, and class 3 (small nodules) with diameter ≤2 mm.

2.5. Nodule nitrogen fixation capacity and nitrogen accumulation

Nitrogenase activity in nodules was determined using the acetylene reduction assay, following the protocol described by Chen *et al.* (2018).

Nitrogenase activity (C_2H_4 nmol g^{-1} h^{-1})=nmol of C_2H_4 produced g^{-1} fresh weight of nodule h^{-1}

Nitrogen fixation potential=Nitrogenase activity×Nodule fresh weight (g per plant)

Plant samples were processed according to Zheng *et al.* (2022). Total nitrogen content was quantified by the Rapid N exceedExceed analyzer (Elementar, Germany).

2.6. Statistical analysis

Experimental data were collated using Microsoft Excel 2013. Statistical analysis and graph generation were performed using GraphPad Prism 9 software. Fisher's least significant difference test (LSD, α=0.05) was used to assess significant differences between individual treatment means. A Pearson correlation test was conducted to examine the relationship between nodulation and root exudates (P<0.05). Metabolome data were processed for pattern identification and Pareto-scaling using SIMCA-P 14.1 (Umetrics, Sweden), after which the data were uploaded to the MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) online platform for multivariate statistical analysis, including unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA), as well as permutation testing of model data to evaluate variable importance in projection (VIP). Differentially expressed metabolites were selected according to the VIP values≥1 obtained from the PLS-DA model. Univariate statistical analysis was employed to compare metabolite abundance differences between the two groups, using Student's *t*-test and multiple of variation analysis.

3. Result

3.1. Soybean nodule formation and development

Based on two years of data, soybean intercropped with maize had fewer nodules per plant compared to sole soybean (SS). However, root interactions under no physical barrier (NB) promoted soybean nodule formation and development (Fig. 1). Compared with the root PB treatment, the number of nodules per plant — averaged across both years — was significantly higher under NB treatment by 24.4 and 21.0% in maize–soybean spacing of 30 cm (MS30) at the five trifoliolate (V5) and full pod (R4) stages, and by 12.7 and 13.2% in maize–soybean spacing of 60 cm (MS60) at full

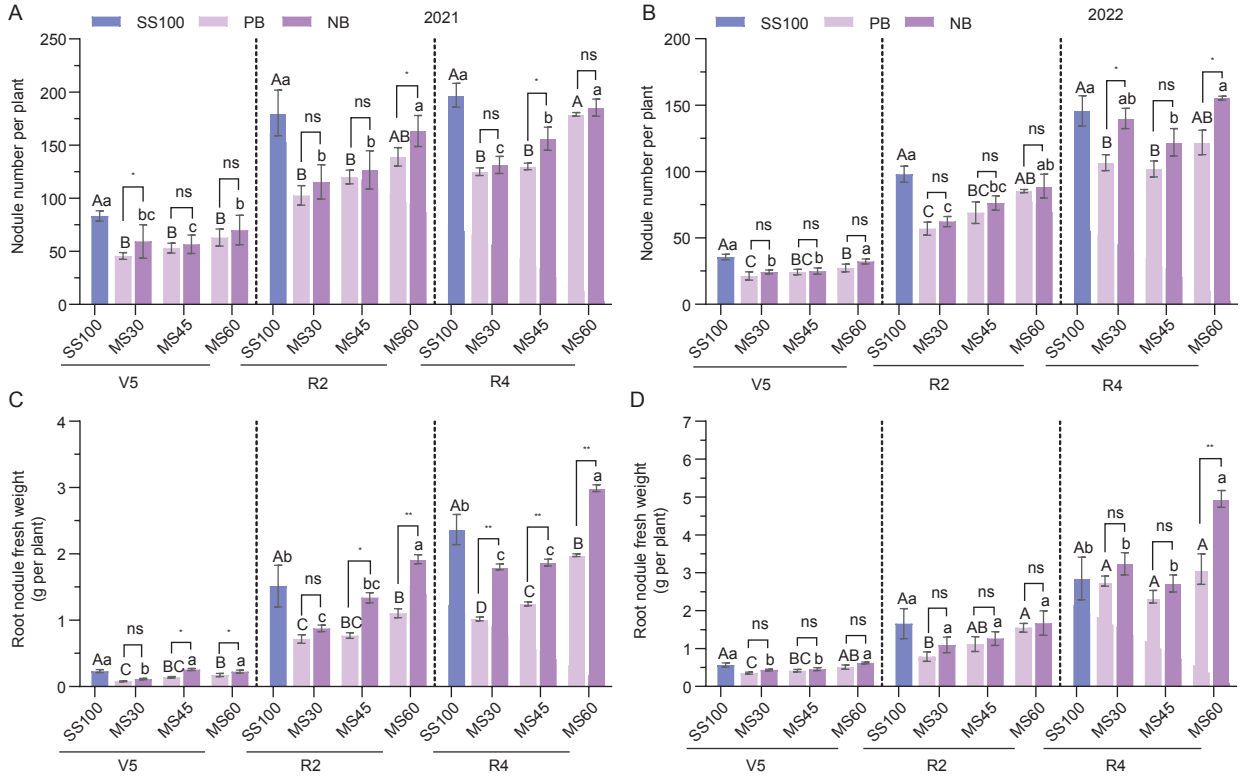


Fig. 1 Nodule number per plant (A and B) and nodule fresh weight (C and D) of soybean treated with different row spacing and root separation in 2021 and 2022. V5, five trifoliolate stage; R2, full bloom stage; R4, full pod stage. SS100, soybean monoculture; MS30, MS45, and MS60, space between maize and soybean is 30, 45, and 60 cm, respectively. PB, root separation using a polyethylene plastic barrier; NB, root no barrier. Bars mean SE (n=9). Letters represent significant differences at P<0.05. ns, not significance; * and ** indicate significant differences at P<0.05 and P<0.01, respectively.

bloom (R2) and R4 stages, respectively (Fig. 1-A and B). At the V5 stage, mean nodule fresh weight (both years average) in MS45 and MS60 under NB treatment increased by 31.4 and 32.7% relative to PB. Nodule fresh weight (both years average) in MS30, MS45, and MS60 under NB treatment was significantly greater than PB by 28.6, 38.0, and 23.6% at R2, and by 24.2, 35.2, and 56.4% at R4, respectively (Fig. 1-C and D).

Additionally, nodule diameter distribution was significantly influenced by row spacing and root interaction in intercropping. From V5 to R4 stages, increasing interspecific row spacing enhanced the proportion of larger nodules (class 1, nodule diameter>4 mm), with root interaction showing greater benefits (Table 1). In 2021 at the V5 stage, nodule diameter distribution under PB and SS100 treatments was concentrated in small nodules (class 3, nodule diameter<0.2 cm), whereas under NB treatment, the distribution included comparable proportions of small and large nodules (classes 2 and 1, 0.2 cm≤nodule diameter≤0.4 cm). In 2022 from V5 to R4 stages and in 2021 from R2 to R4 stages, nodule diameter≤0.4 cm). These results indicate that both interspecific row spacing and root interaction play a critical roles in regulating nodulation.

3.2. Nitrogen fixation and nitrogen accumulation in soybean

To further evaluate interspecific row spacing and root interaction effects on nodule N_2 fixation, our results showed that nitrogenase activity was not significantly altered by increasing interspecific row spacing. However, the NB treatment significantly enhanced nitrogenase activity compared with PB at the R2 and R4 stages (Fig. 2-A and B). These results suggest that root interaction affect nodule N_2 fixation.

In addition, from the V5 to R4 stage, the NB treatment led to a remarkable significant increase in nitrogen fixation potential per plant compared with PB treatment, which exhibited an upward trend with increasing interspecific row spacing (Fig. 2-C and D). Meanwhile, during the V5 to R4 stage, intercropping had adverse effects on soybean nitrogen (N) accumulation, primarily due to reduced leaf N accumulation (Fig. 2-E–G). Expanding interspecific row spacing increased N accumulation, and the NB treatment resulted in higher N accumulation than PB at all developmental stages.

3.3. Analysis of gene expression in soybean roots

In 2022, we analyzed the expression patterns of key genes regulating nodulation and flavonoid biosynthesis in soybean roots and found that root interaction between maize and soybean were more conductive to induce genes expression (Fig. 3). Compared with PB treatment, the key nodulation gene early nodulin 40B (*GmENOD40B*) exhibited significantly increased expression with NB treatment by 0.95-fold in MS45 and 0.35-fold in MS60 at the V5 stage, and a 2.99-fold increase in MS45 at the R2 stage (Fig. 3-A). The expression of the nodule inception gene (*GmNIN2b*) was significantly higher under NB treatment, showing a 3.35-fold increase in MS45 at V5 stage, and increases of 0.34-, 1.69-, and 0.43-fold in MS30, MS45, and MS60, respectively, at R2 stage, relative to PB treatment (Fig. 3-B). Transcripts of *GmEXPB2*, encoding a cell wall β-expansin, were most abundant in MS30 and MS60 under NB treatment at V5 stage (Fig. 3-C), indicating that nodule development had initiated at an early stage, which is consistent with the distribution of nodule diameter (Table 1). The expression of chalcone synthase *GmCHS8* was greater in MS60 than in SS100 (Fig. 3-D). Isoflavone synthase 1 (*IFS1*) gene expression

Table 1 Effects of different row spacing and root separation on the distribution of nodule diameter in soybean[1)]

Year	Treatment[2)]	V5			R2			R4		
		<0.2 cm (%)	0.2–0.4 cm (%)	>0.4 cm (%)	<0.2 cm (%)	0.2–0.4 cm (%)	>0.4 cm (%)	<0.2 cm (%)	0.2–0.4 cm (%)	>0.4 cm (%)
2021	MS30PB	60.35 ab	38.14 ab	1.51 c	70.81 a	24.90 b	4.29 a	19.59 ab	61.70 a	18.72 a
	MS30NB	51.80 ab	45.49 ab	2.71 c	36.05 b	52.29 a	11.66 a	17.39 abc	60.56 a	22.05 a
	MS45PB	57.90 ab	40.15 ab	1.95 c	40.37 b	52.59 a	7.04 a	23.59 a	54.22 a	22.18 a
	MB45NB	45.67 b	42.15 ab	12.18 a	33.62 b	54.39 a	11.98 a	13.43 bcd	52.75 a	33.81 a
	MS60PB	52.77 ab	45.76 ab	1.47 c	38.47 b	53.07 a	8.46 a	16.52 abc	57.39 a	26.10 a
	MS60NB	45.00 b	50.19 a	4.81 b	40.00 b	46.99 a	13.01 a	7.71 cd	61.60 a	30.68 a
	SS100	64.93 a	32.88 b	2.28 bc	29.15 b	59.12 a	11.73 a	6.65 d	65.59 a	27.76 a
2022	MS30PB	25.79 a	66.38 ab	7.83 b	19.18 a	65.34 ab	16.36 b	3.80 bc	52.01 a	44.19 ab
	MS30NB	37.06 a	54.53 bc	8.41 b	20.84 a	58.22 bc	20.95 ab	8.51 abc	57.44 a	34.05 b
	MS45PB	26.20 a	65.40 ab	8.40 b	16.23 a	60.72 abc	23.05 ab	18.44 a	50.29 ab	31.27 b
	MB45NB	37.92 a	48.27 c	13.81 ab	16.11 a	60.58 bc	23.32 ab	3.28 c	56.51 a	40.62 ab
	MS60PB	32.71 a	52.57 c	14.72 ab	20.82 a	56.64 bc	22.54 ab	4.07 bc	48.65 ab	47.28 ab
	MS60NB	28.74 a	49.60 c	21.66 a	14.74 a	54.39 c	30.87 a	2.61 c	38.89 b	58.50 a
	SS100	21.92 a	68.38 a	9.70 b	13.77 a	69.89 a	16.34 ab	14.36 ab	50.36 ab	34.35 b

[1)] V5, five trifoliolate stage; R2, full bloom stage; R4, full pod stage.
[2)] MS30, MS45, and MS60, space between maize and soybean is 30, 45, and 60 cm, respectively. PB, root separation using a polyethylene plastic barrier; NB, root no barrier.
Letters represent significant difference at P<0.05.

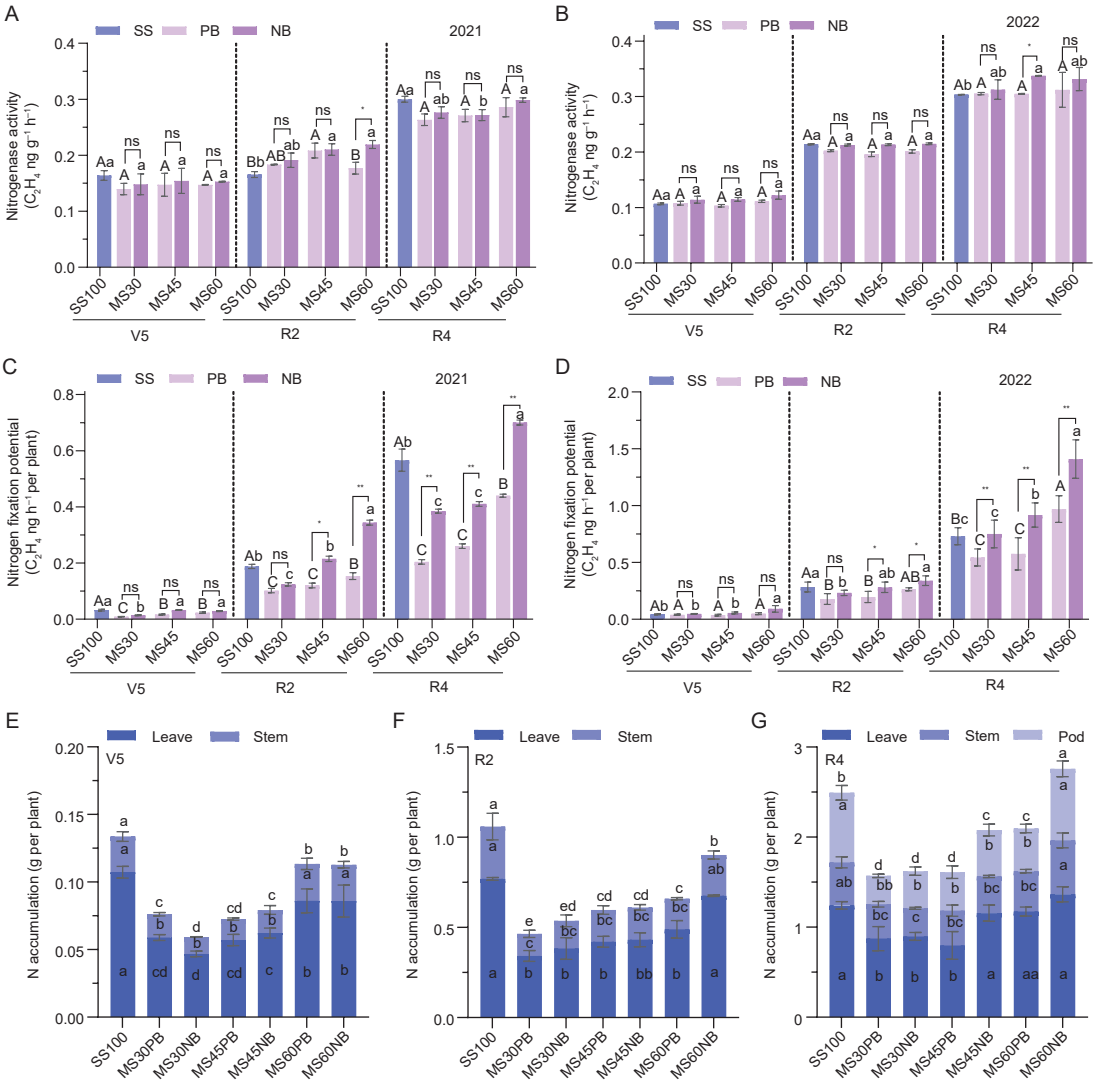


Fig. 2 Nodule nitrogen fixation capacity and N accumulation of soybean treated with different row spacing and root separation. A and B, nitrogenase activity. C and D, nitrogen fixation potential per plant. E–G, N accumulation. V5, five trifoliolate stage; R2, full bloom stage; R4, full pod stage. SS100, soybean monoculture; MS30, MS45, and MS60, space between maize and soybean is 30, 45, and 60 cm, respectively. PB, root separation using a polyethylene plastic barrier; NB, root no barrier. Bars mean SE (*n*=6). Letters represent significant differences $P<0.05$. ns, not significance; * and ** indicate significant differences at $P<0.05$ and $P<0.01$, respectively.

was higher in intercropping with NB treatment than in SS100 at R2 stage, and compared to PB treatment, remarkably increased by 0.24-, 0.81-, and 1.62-fold in MS30, MS45, and MS60, respectively, under NB treatment (Fig. 3-E). During soybean development, the expression of isoflavone conjugates hydrolyzing β-glucosidase (ICHG) in intercropping with NB treatment was higher at V5 stage than in SS100, but declined at R2 stage, except in MS45 under NB treatment (Fig. 3-F). Furthermore, compared with PB treatment, *GmICHG* expression in MS45 with NB treatment was significantly up-regulated by 13.20-fold at V5 stage. Similar up-regulation was also observed in MS30, MS45, and MS60 under NB treatment at R2 stage (Fig. 3-F). These results suggest that the glycoside-hydrolyzing activity of ICHG decreases at R2 stage, which may lead to reduced isoflavone secretion.

3.4. Differences in flavonoid metabolism secreted by crop roots

The flavonoid metabolic differences between planting models and root interaction were analyzed in soybean and maize root exudates using UPLC-MS. Using VIP>1 and $P<0.05$ as thresholds, a total of 20 and 32 differential metabolites were identified in the SS *vs*. MS30PB and SS *vs*. MS30NB groups, respectively (Appendix D). Compared with SS, 20 differential metabolites in soybean root exudates were down-regulated in the MS30PB group, including anthocyanins (rhein), flavanones (isobavachin, fustin, plantagoside, ophiopogonanone C), isoflavonoids (tectorigenin, 3′-hydroxypuerarin, 6′′-*O*-xylosyl-glycitin, pratensein-7-*O*-glucoside, calycosin-7-*O*-beta-D-glucoside, sophoricoside, Calycosin), flavonoids ((–)-epicatechin, gossypetin), flavones and flavonols (di-*O*-methylquercetin, tricin 4′-*O*-beta-guaiacylglycerol, quercetin-3,4′-*O*-di-beta-glucopyranoside, apigenin 5-*O*-glucoside,

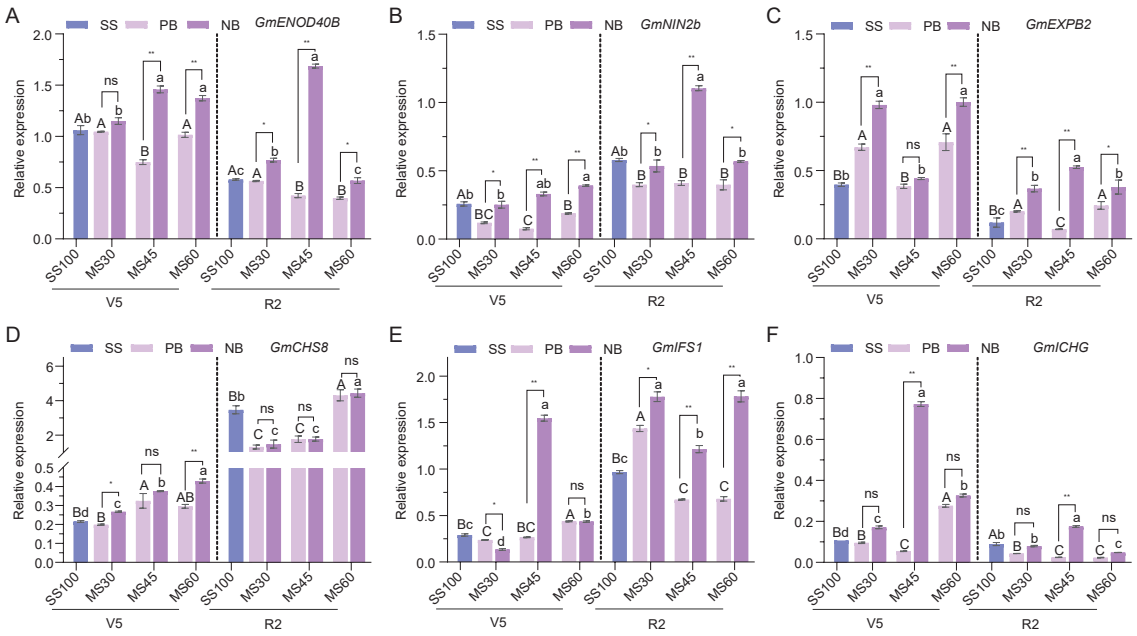


Fig. 3 Relative gene expression of *GmENOD40B* (A), *GmNIN2b* (B), *GmEXPB2* (C), *GmCHS8* (D), *GmIFS1* (E), and *GmICHG* (F) of soybean under different row spacing and root separation. V5, five trifoliolate stage; R2, full bloom stage. SS100, soybean monoculture; MS30, MS45, and MS60, space between maize and soybean is 30, 45, and 60 cm, respectively. PB, root separation using a polyethylene plastic barrier; NB, root no barrier. Bars mean SE (n=3). Letters represent significant differences P<0.05. ns, not significance; * and ** indicate significant differences at P<0.05 and P<0.01, respectively.

liquiritin apioside), chalconesand dihydrochalcones (Phloretin) (Fig. 4-A). In contrast, two metabolites were significantly up-regulated in the MS30NB group, namely tangetetin and nobiletin. While 30 metabolites were down-regulated, with daidzein decreased 2.35-fold (Fig. 4-B).

A total of 13 and 27 differential metabolites were detected in the SS versus MS60NB and SS versus MS60PB groups, respectively (Appendix B). In the MS60NB group, kaempferol and tangeretin were significantly up-regulated, while 11 metabolites were down-regulated. All 27 differential metabolites in the MS60PB group were down-regulated (Fig. 4-C and D). These compounds primarily belong to the flavonoids, including flavonols, isoflavones, flavanones, and chalcones. Only one differential metabolite, wedelolactone, was observed between MS30PB and MS30NB (Table 2). Additionally, 4 differential metabolites were identified between MS60PB and MS60NB groups (Appendix B), mainly flavonols: morin hydrate, isorhamnetin, kaempferol, and oroxylin A (Table 2).

Further analysis of flavonoid metabolic exudates from maize roots revealed that PB treatment reduced metabolite secretion (Appendix E). In contrast, NB treatment significantly increased diosmin levels in MS30, as well as irisolidone 7-*O*-beta-d-glucoside, limocitrin *O*-hexoside, and apigenin C-pentoside in MS60 (Appendix E).

3.5. Nodule phenotypes and nitrogen fixation after maize root exudates treatment

The nodule number and nodule fresh weight were higher in various soybean genotypes following maize root exudate (RE) treatment compared to distilled water (DW) treatment (Fig. 5-A and B). In NTS, nodule number increased significantly by 23.93 and 29.9%, and nodule fresh weight by 53.05 and 41.8%, at 60 and 90 days, respectively. At 30 day, the *GmENOD40* expression in ND and GX was up-regulated by RE treatment (Fig. 5-C). Similar results were observed for *GmNIN2b* and *GmEXPB2* across all soybean genotypes (Fig. 5-D and E). At 60 day, *GmENOD40* expression was significantly up-regulated in all genotypes under RE treatment (Fig. 5-C). The expression of *GmNIN2b* increased by 2.81-fold in GX and 7.71-fold in NTS relative to DW treatment (Fig. 5-D), while *GmEXPB2* expression in GX increased 4.56-fold (Fig. 5-E).

Nodule size distribution analysis revealed significant differences among soybean varieties (Table 3). RE treatment increased the proportion of large nodules (contain class 1 and class 2), although responses varied across genotypes. Specifically, in GX, the proportion of class 2 nodules significantly increased by 219.59% at 30 day and by 15.00% at 60 day compared to DW treatment. In contrast, ND and NTS exhibited increased the proportion of class 1 nodules.

RE treatment enhanced both nodule nitrogenase activity and whole-plant nitrogen fixation potential (Fig. 5-F and G). Nitrogenase activity in GX significantly increased by 14.15% after RE treatmentat 90 day (Fig. 5-F). Nitrogen fixation potential in NTS significantly increased by 30.30% at 60 day and 48.12% at 90 day compared to DW treatment. Nitrogen fixation potential among different genotypes followed the order NTS>ND>GX (Fig. 5-G). These findings indicate that maize root exudates play a critical role in promoting nodule development and N_2 fixation in soybean.

3.6. Correlation between root exudates and nodules

Tricin 4′-*O*-beta-guaiacylglycerol, apigenin, naringenin chalcone, tricin *O*-sinapoylpentoside, daidzein, eriodictyol and phloretin secreted by soybean roots showed significant

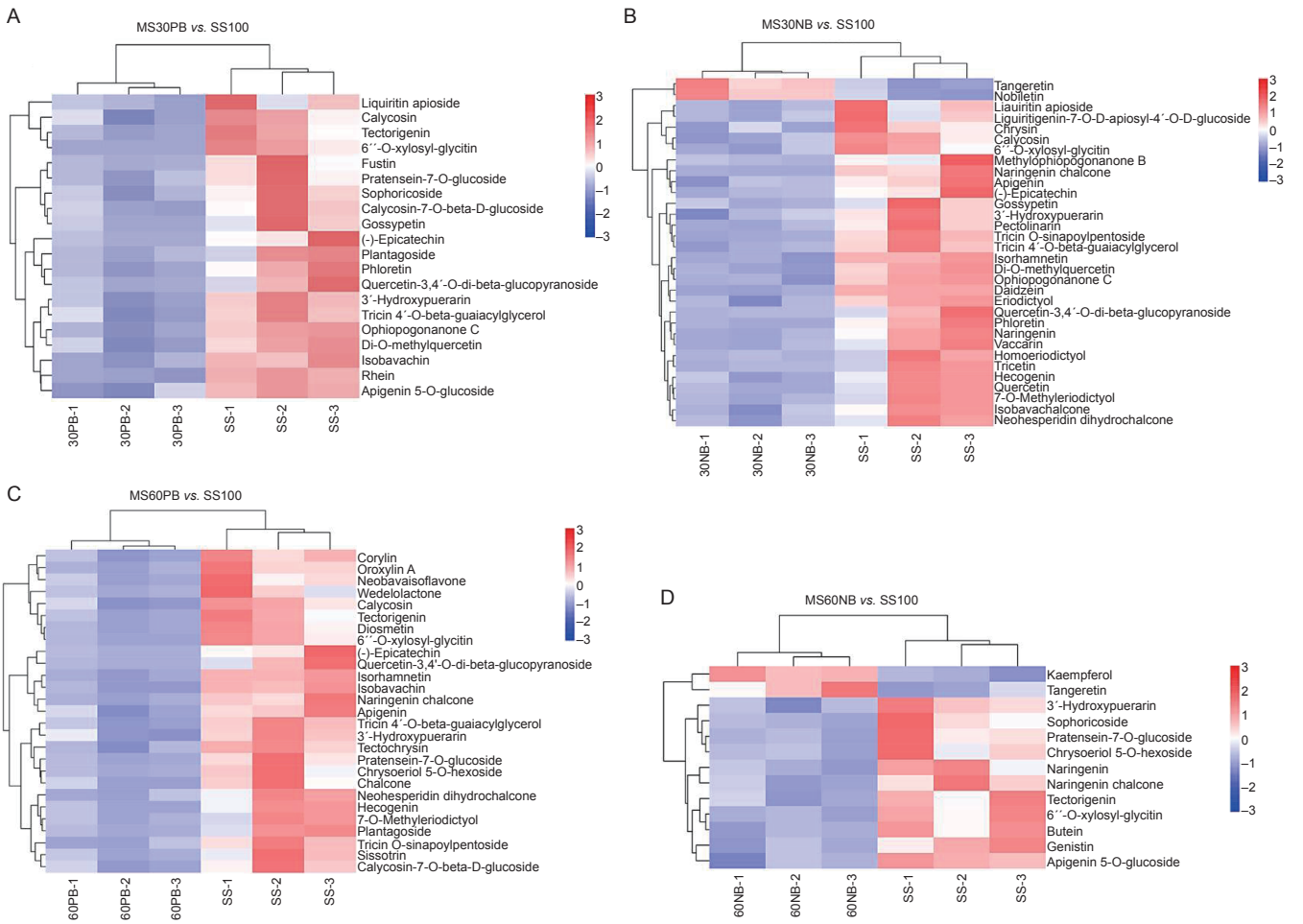


Fig. 4 Differential metabolites of flavonoids in soybean root exudates under maize–soybean strip intercropping. A, MS30PB *vs*. SS100. B, MS30NB *vs*. SS100. C, MS60PB *vs*. SS100. D, MS60NB *vs*. SS100. SS100, soybean monoculture; MS30, MS45, and MS60, space between maize and soybean is 30, 45, and 60 cm, respectively. PB, root separation using a polyethylene plastic barrier; NB, root no barrier. Up-regulated (red) or down-regulated (blue), in the metabolite logarithmic fold change, as indicated in the color index (n=3).

positive correlations with soybean nodule number. Epicatechin and isorhamnetin exhibited significant positive correlations with nodule number, while isorhamnetin further correlated significantly with nodule fresh weight. Nobiletin, tangeretin, kaempferol, and quercetin were positively correlated with nodule diameter (Fig. 6-A). In maize root exudates, isorhamnetin displayed significant positive correlations with both nodule number and fresh weight (Fig. 6-B). Additionally, apigenin C-pentosinoside and limocitrin O-hexoside were significantly positively correlated with soybean nodule diameter (Fig. 6-B). These results indicate that most flavonoids in soybean roots mainly regulate nodule number and weight, while flavonoids and flavonols collectively influence nodule development. Notably, flavonoids capable of promoting soybean nodule development are also present in maize root exudates.

Table 2 Differential metabolites of soybean root exudates after root separation under maize–soybean strip intercropping[1)]

Metabolite	MS30 PB *vs*. MS30 NB		MS60 PB *vs*. MS60 NB	
Coumestanes	$\log_2$(FC)	VIP	$\log_2$(FC)	VIP
Wedelolactone	–1.46	1.43	–	–
Flavonols	$\log_2$(FC)	VIP	$\log_2$(FC)	VIP
Morin hydrate	–	–	–1.46	1.73
Isorhamnetin	–	–	–1.71	1.41
Kaempferol	–	–	–2.30	1.81
Oroxylin A	–	–	–1.82	1.22

[1)] MS30 and MS60, space between maize and soybean is 30 and 60 cm, respectively. PB, root separation using a polyethylene plastic barrier; NB, root no barrier. VIP, variable importance in projection.

4. Discussion

4.1. Positive regulation of soybean nodulation through root interaction under maize and soybean intercropping

Intercropping offers advantages such as resource utilization efficient , enhanced soil fertility, and reduced crop diseases. However, taller crops can shade shorter ones, and impairing photosynthesis in understory crops. In maize–soybean intercropping system, soybean occupies a niche disadvantage, resulting in reduced light energy utilization efficiency, decreased leaf photosynthetic efficiency, and lower biomass accumulation (Raza *et al*. 2021), ultimately

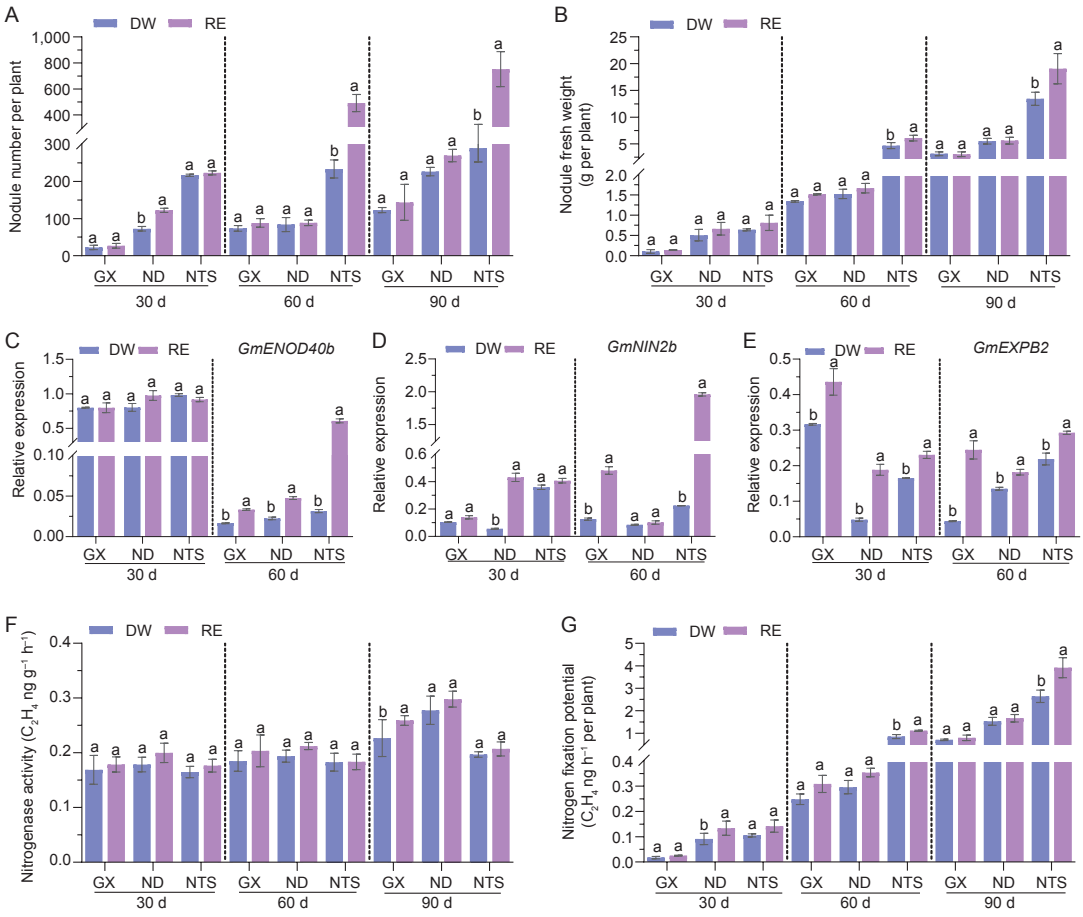


Fig. 5 Nodules phenotype, gene expression and nitrogen fixation after maize root exudate (RE) irrigation. A, nodule number with RE treatment. B, nodule fresh weight with RE treatment. C, the expression of *GmENOD40B* gene. D, the expression of GmNIN2B gene. E, the expression of *GmEXPB2* gene. F, nitrogenase activity for nodule with RE treatment. G, nitrogen fixation potential. Bars mean SE (n=6). GX, Guixia 3; ND, Nandou 12; NTS, NTS1007. RE, maize root exudate; DW, distilled water. Letters represent significant difference between treatments (P<0.05).

Table 3 Effect of maize root exudates (RE) on nodule size grading in different soybean varieties

Variety[1)]	Treatment[2)]	Root nodule diameter								
		30 d			60 d			90 d		
		<0.2 cm (%)	0.2–0.4 cm (%)	>0.4 cm (%)	<0.2 cm (%)	0.2–0.4 cm (%)	>0.4 cm (%)	<0.2 cm (%)	0.2–0.4 cm (%)	>0.4 cm (%)
GX	DW	83.65 a	10.31 b	6.04 a	25.21 a	49.28 b	25.51 a	15.10 a	59.73 a	25.18 a
	RE	58.23 b	32.95 a	8.82 a	15.68 a	56.67 a	27.65 a	8.44 b	66.02 a	25.54 a
ND	DW	41.46 b	53.63 a	4.91 b	18.51 a	55.60 a	25.90 a	15.11 a	59.58 a	25.31 b
	RE	55.20 a	30.06 b	14.75 a	26.87 a	41.72 b	31.41 a	8.83 b	56.63 a	34.55 a
NTS	DW	55.07 a	42.92 b	2.01 a	9.14 a	70.32 a	20.54 a	4.38 a	62.75 a	32.87 a
	RE	43.42 b	51.42 a	5.16 a	11.18 a	62.60 b	26.22 a	4.78 a	56.79 a	38.44 a
Two away ANOVA (F-value)										
Varieties (A)		224.03**	137.86**	9.82**	5.66*	79.99**	0.77	14.83**	1.50	7.78**
Treatment (B)		62.04**	3.62	20.49**	0.01	15.57**	1.62	10.97**	0.15	5.60*
A×B		134.89**	106.46**	3.90	2.61	27.79**	0.11	3.30	2.62	1.45

[1)] GX, Guixia 3; ND, Nandou 12; NTS, NTS1007.
[2)] RE, maize root exudate; DW, distilled water.
Letters represent significant difference between treatments (P<0.05). ** and * mean significant levels at the 0.01 and 0.05 probability levels, respectively.

reducing nodule formation (Zhang *et al*. 2011). This study found fewer nodules in intercropped soybean compared to monoculture, likely due to competitive shading by maize that reduces carbon assimilation. Concurrently, increasing interspecific distance alleviated shading intensity, resulting in higher nodule number and fresh weight, following the trend MS60>MS45>MS30. Legume nodulation is an energy-intensive process: early nodule initiation does not rely on

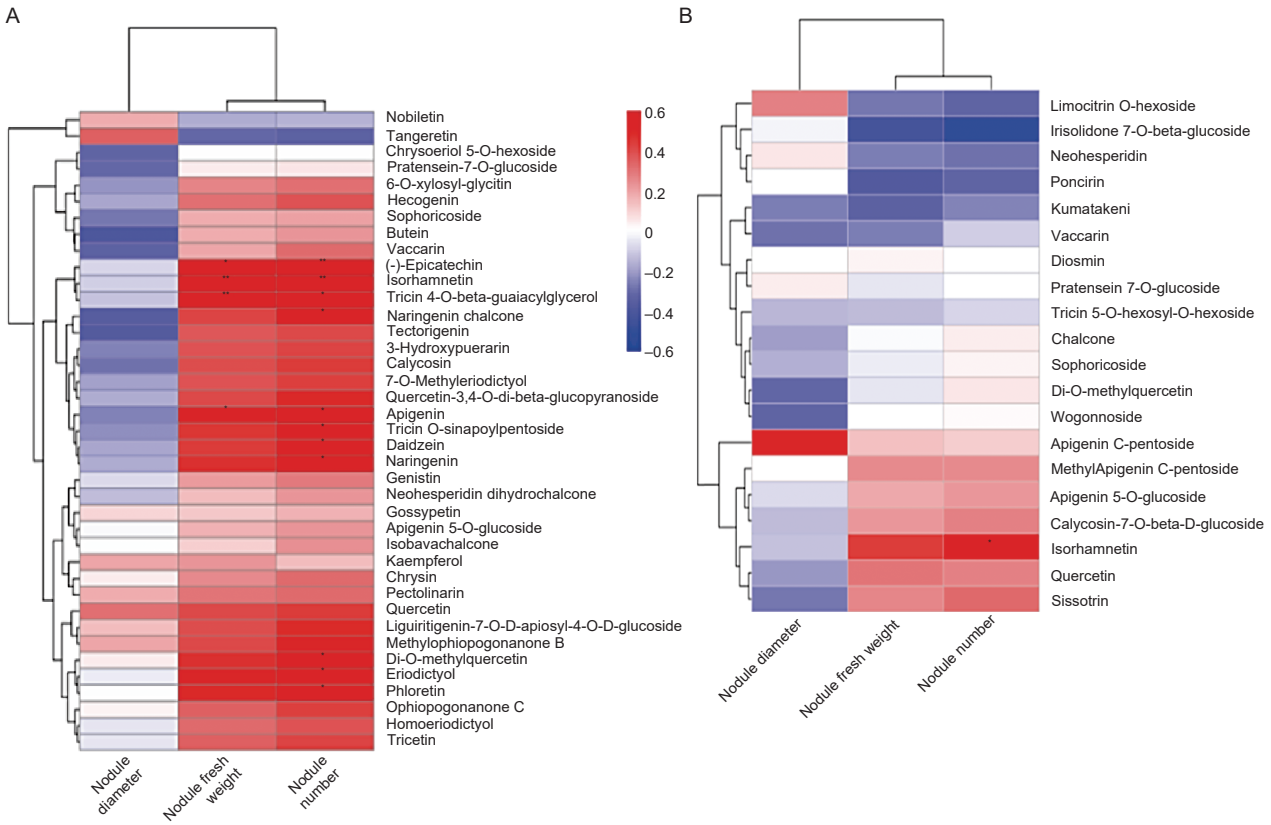


Fig. 6 Correlation analysis of rhizosphere flavonoids and soybean nodule phenotype. A, heatmap of correlation between soybean nodule phenotype and soybean rhizosphere flavonoids. B, heatmap of correlation between soybean nodule phenotype and maize rhizosphere flavonoids. Red indicates a positive correlation, and blue indicates a negative correlation. * and ** indicate significant differences $P<0.05$ and $P<0.01$, respectively.

current photosynthate, but subsequent development depends on allocation of photosynthetic products (Ito *et al.* 2006). Thus, photosynthate supply provides essential energy for the nodule growth and development. Our previous research indicated that intercropped soybean exhibits a prolonged leaf stay-green period (Li *et al.* 2023). After maize harvest, soybean was recovery growth, partially mitigating shade-induced constraints on leaf traits and nodule development (Chen *et al.* 2024). Concurrently, canopy light environment improve in intercropped soybean, enhancing belowground allocation of photosynthates to support nodule development (Zhou *et al.* 2019). We observed that intercropping elevated the expression level of *GmEXPB2* and increased the proportion of large nodules (class 1). This may reflect earlier nodule primordium formation and nodule development relative to monocropped soybean (Li *et al.* 2015), indicating that intercropping compensates for nodule number deficiency by promoting nodule development (Fig. 7).

Notably, root–root interactions between faba bean and maize significantly enhance nodule formation and symbiotic N_2 fixation in intercropped faba bean, and the this promoting effect maize roots on faba bean nodulation is specific to maize (Fan *et al.* 2006; Li *et al.* 2016). Our findings demonstrate that root interactions between maize and soybean across different interspecific distances benefit soybean nodulation, promote the expression of *GmENOD40b* and *GmNIN2b* genes, strongly indicating that maize roots promote nodulation in legumes. The proportion of large nodules (classes 1 and 2) decreased when maize and soybean roots were separated by physical barriers, confirming that root interactions contributes to nodule development. Interspecific competition intensity correlates with planting distance, and closer proximity enhances root system "communication" (Li *et al.* 2009). Nitrogen fixation capacity and nitrogen accumulation were highest under MS60, exceeding those under MS45 and MS30. This result does not reflect significant rhizosphere nitrogen depletion due to maize competition (Yong *et al.* 2015), and this nitrogen limitation may stimulate greater nodulation and N_2 fixation in legumes (Li *et al.* 2009). Furthermore, nitrogen fixation capacity and nitrogen accumulation in soybean were higher under root interaction than under root separation, which possibly because aboveground light dynamics exert stronger regulatory influence than belowground factors. Nevertheless, the rhizosphere secretion environment remains critical for soybean nodulation and nitrogen fixation (Liu *et al.* 2017).

4.2. Effects of rhizosphere secretion environment on the development of intercropping soybean nodules

Plants release substantial amounts of root exudates, which play a role in root-root interactions between plant species (Lian *et al.* 2002; Barcelo and Poschenrieder 2022). Exogenous application of maize root exudates increased

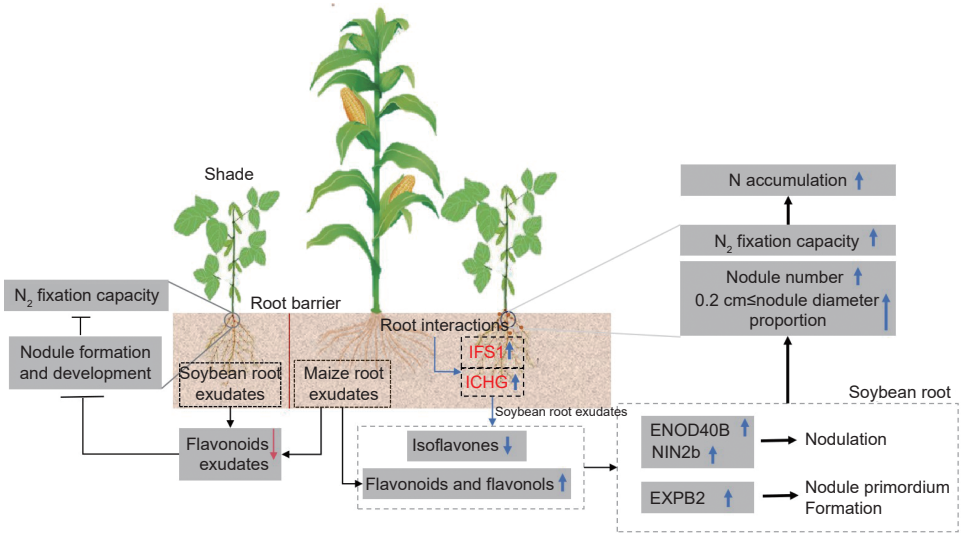


Fig. 7 Root–root interactions stimulate nodulation and nitrogen fixation of soybean in the maize–soybean strip intercropping. Upward arrows indicate increase; downward arrows indicate reduce. IFS, isoflavone synthase; ICHG, isoflavone conjugate-hydrolyzing β-glucosidase.

nodule number and fresh weight in various soybean genotypes, while also increased the proportion of large nodules in the ND genotype (class 1) and medium nodules in the NTS and GX genotypes (class 2). These alters were conducive to enhancing the nitrogen fixation capacity of soybean nodules. Maize root exudates have been shown to induce the production of lipidoligosaccharides in rhizobia, which trigger nodulation in legume roots (Lian *et al.* 2002). Whereas, root exudates collected from maize and faba bean mixtures contain higher concentrations of the nodulation signaling molecule genistein (Li *et al.* 2016). These findings indicate that maize root exudates promote soybean nodule development and mitigate the reduction in nodule number caused by limited shoot energy supply in maize–soybean strip intercropping systems.

Plant root metabolites function as signaling molecules and microbial nutrient sources, thereby regulating microbial community structure and diversity, and mediating plant–microbe interactions, including symbiotic, pathogenic, and fungi (Huang *et al.* 2019; Yan *et al.* 2022). The composition of root exudates depends on crop species and is significantly influenced by environmental conditions, neighboring plants, and microbial symbioses (Yu *et al.* 2021). In this study, intercropping markedly altered the accumulation patterns of flavonoids in maize and soybean root exudates. Specifically, intercropping reduced the secretion of isoflavones while increasing the secretion of flavonoids and flavonols. Light intensity has been shown to alter the flavonoid biosynthesis pathway and enhance root exudation (Silva *et al.* 2022). Expression analysis of key enzyme genes (*GmCHS8* and *GmIFS1*) in the flavonoid biosynthesis pathway revealed that flavonoid synthesis in soybean roots increased with greater interspecific distance. At the R2 stage, *GmICHG*, a key gene regulating isoflavone secretion, was down-regulated under intercropping. However, its expression was higher under root interaction than under root barrier conditions, indicating that maize root exudates influence soybean isoflavone secretion. Thus, the observed decrease in isoflavone secretion in intercropped soybean roots may be partly attributable to shading by maize.

Soybean isoflavones are important flavonoid subclasses that regulate nodulation, but mixtures of flavonoids may play a more significant role in nodule formation and development (Sugiyama *et al.* 2016; White *et al.* 2017), as certain flavonoids inhibit auxin transport and promote nodule occurrence and development (Santelia *et al.* 2008). Correlation analysis showed that most flavonoid and flavonol compounds were positively correlated with nodule diameter. Although intercropping reduced isoflavone secretion in soybean roots and resulted in fewer nodules at the R2 stage compared to monocropping, the proportion of large nodules increased. In contrast, nodule quality under root barrier conditions was poor. These observations suggest that maize and soybean may directly promote nodule development by increasing the secretion of flavonoids and flavonols. There is a “recognition response” that is simply to neighboring plants, alters root exudate composition, and this effect is not species-specific (Hazrati *et al.* 2020). After intercropping with maize, both maize and soybean exhibited increased secretion of flavonoids and flavonols, potentially due to such recognition responses. Flavonoid levels in both species decreased under root barrier conditions, where direct root interactions were prevented.

Therefore, in the maize–soybean strip intercropping system, the low-light environment in the canopy may promote the accumulation of flavonoids in soybean primarily by suppressing isoflavone secretion in the roots. Maize root exudates directly promote the synthesis and secretion of flavonoids and flavonols in soybean roots (Fig. 7).

5. Conclusion

Our study revealed that in the maize–soybean strip

intercropping system, aboveground shading inhibited soybean growth and reduced nodule number, which increased progressively with increasing interspecific distance. The interaction between maize and soybean roots promoted nodule development, increased the proportion of large nodules, and enhanced the nitrogen fixation potential of soybean plants. Maize root exudates promoted nodule formation and development in soybean. In addition, rhizosphere isoflavone secretion decreased in intercropped soybean, whereas flavonoid and flavonol secretion increased in both crops. Under root barrier treatment, the secretion of differential flavonoids metabolites in the rhizosphere of maize and soybean declined. Flavonoids and flavonols were positively correlated with nodule development, suggesting they may play a key role in the nodule development of intercropped soybean.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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

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

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