Effects of Nitrogen Application Rate on Root Exudation of Soybean Isoflavones in Wheat-Broad Bean Intercropping System (Postprint)

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Abstract

Through pot and hydroponic experiments, three planting patterns—wheat//fava bean intercropping, fava bean monoculture, and wheat monoculture—were adopted to investigate changes in soybean isoflavone secretion from roots of intercropped and monoculture wheat and fava bean under different nitrogen levels (low nitrogen, conventional nitrogen application, and high nitrogen) at various growth stages, providing a basis for further elucidating the mechanisms of yield increase and disease control in intercropping systems. The results showed that with increasing nitrogen application, soybean isoflavone secretion from wheat and fava bean roots both decreased. At the wheat jointing stage (60 days after emergence), compared with the low nitrogen treatment, the soybean isoflavone secretion from intercropped wheat and monoculture wheat under conventional nitrogen and high nitrogen treatments decreased by 28.9% and 72.7%, and by 18.9% and 122.1%, respectively. Compared with low nitrogen, under conventional nitrogen and high nitrogen treatments, the soybean isoflavone secretion from intercropped fava bean at the branching stage (60 days after emergence) decreased by 30.7% and 53.5%, respectively, while the difference in monoculture was not significant; at the flowering stage (95 days after emergence), intercropped fava bean decreased by 38.5% and 124.4%, respectively, and monoculture decreased by 43.0% and 67.2%, respectively; at the podding stage (131 days after emergence), intercropped fava bean decreased by 40.2% and 123.5%, respectively, and monoculture decreased by 53.8% and 75.6%, respectively. Intercropping can increase soybean isoflavone secretion from crop roots. Compared with monoculture, under low nitrogen and conventional nitrogen conditions, the soybean isoflavone secretion from intercropped wheat roots increased by 22.5% and 35.6% at the jointing stage (60 days after emergence), and by 28.8% and 7.9% at the flowering stage (95 days after emergence), respectively. Compared with monoculture, under low nitrogen and conventional nitrogen conditions, the soybean isoflavone secretion from intercropped fava bean roots increased by 44.2% and 12.8% at the branching stage (60 days after emergence), by 39.8% and 46.0% at the flowering stage (95 days after emergence), and by 16.0% and 27.0% at the podding stage (131 days after emergence), respectively. In conclusion, both intercropping patterns and nitrogen application rates affect soybean isoflavone secretion from crop roots.

Full Text

Effect of Nitrogen Application Rate on Soy Isoflavone Exudation from Roots in Wheat and Faba Bean Intercropping System

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Abstract

Pot and hydroponic experiments were conducted using three cropping patterns (wheat//faba bean intercropping, faba bean monocropping, and wheat monocropping) to investigate changes in soy isoflavone secretion from wheat and faba bean roots under different nitrogen levels (low nitrogen, conventional nitrogen, and high nitrogen) at various growth stages. The study aimed to provide insights into the mechanisms underlying yield enhancement and disease control in intercropping systems. Results demonstrated that soy isoflavone secretion from both wheat and faba bean roots decreased with increasing nitrogen application. At the wheat stem elongation stage (60 days after emergence), conventional and high nitrogen treatments significantly reduced isoflavone secretion by 28.9% and 72.7% in monocropped wheat, and by 18.9% and 122.1% in intercropped wheat, respectively, compared to low nitrogen treatment. For faba bean at the branching stage (60 days after emergence), intercropped plants showed significant reductions of 30.7% and 53.5% under conventional and high nitrogen, respectively, while monocropped plants showed no significant differences. At flowering (95 days after emergence), intercropped faba bean exhibited decreases of 38.5% and 124.4%, while monocropped faba bean decreased by 43.0% and 67.2%. At podding (131 days after emergence), intercropped faba bean decreased by 40.2% and 123.5%, while monocropped faba bean decreased by 53.8% and 75.6%. Intercropping enhanced soy isoflavone secretion from crop roots. Compared to monocropping, intercropped wheat showed significant increases of 22.5% and 35.6% at stem elongation, and 28.8% and 7.9% at flowering under low and conventional nitrogen, respectively. Similarly, intercropped faba bean exhibited increases of 44.2% and 12.8% at branching, 39.8% and 46.0% at flowering, and 16.0% and 27.0% at podding under low and conventional nitrogen, respectively. In conclusion, both intercropping system and nitrogen application rate significantly influenced soy isoflavone secretion from wheat and faba bean roots.

Keywords: wheat-faba bean intercropping; nitrogen application rate; root exudates; soy isoflavone

Introduction

Plant roots serve as essential organs for nutrient absorption and metabolism, exhibiting high sensitivity to environmental conditions. Numerous studies have demonstrated that plant roots and their secretions play crucial roles in plant nutrition and disease regulation, making root exudates a persistent focus of research attention. In recent years, substantial research has investigated the functions of root exudates in cropping systems such as rotation and intercropping. Flavonoid root exudates act as initial signals perceived by rhizobia and play critical roles in legume nodulation. Discoveries of flavonoids in legume root exudates have primarily focused on monocropped soybean (*Glycine max* (L.) Merr), alfalfa (*Medicago sativa*), pea (*Pisum sativum* Linn), and pigeon pea (*Cajanus cajan*), with identified compounds including naringenin, eriodictyol, apigenin, luteolin, and quercetin. Soy isoflavones, as ubiquitous flavonoid root exudates, reportedly exhibit physiological activities including anti-hemolysis, antioxidant properties, induction of soybean nodulation, and inhibition of pathogen growth, functioning as protective compounds against pests and diseases. Additionally, research indicates that soy isoflavones induce nodulation genes in soybean rhizobia and represent a response to *Phytophthora* root rot infection. However, these findings derive exclusively from monoculture studies, leaving the effects of intercropping, particularly under varying nitrogen levels, poorly understood.

Wheat (*Triticum aestivum* L. cv.) and faba bean (*Vicia faba* L. cv.) intercropping represents an important cropping pattern in Yunnan and southwestern China, offering significant yield advantages and disease control benefits that contribute substantially to farmer income. Previous research has elucidated nutrient uptake patterns, disease occurrence, rhizosphere microbial dynamics, and secretion characteristics of organic acids and phenolic acids in wheat-faba bean intercropping systems, demonstrating that intercropping substantially influences yield increase, disease control, and rhizosphere microbial communities while regulating root exudate composition. However, whether these intercropping effects are modulated by different nitrogen application rates remains unclear. This study investigated wheat-faba bean intercropping systems, employing high-performance liquid chromatography (HPLC) analysis combined with previous research findings to systematically examine the dynamic changes and accumulation patterns of soy isoflavones secreted by intercropped wheat and faba bean roots under different nitrogen levels and growth stages, providing a basis for further elucidating the mechanisms of yield enhancement and disease control in intercropping systems.

1.1 Materials and Crop Varieties

Pot experiments were conducted from October 2014 to May 2015 in the greenhouse of the Plant Nutrition Department at Yunnan Agricultural University. The experimental soil, collected from a lateritic red soil on Yunnan Agricultural University' s campus, had the following basic physicochemical properties: alkaline nitrogen 68 mg·kg ^{1}, available phosphorus 16 mg·kg ^{1}, available potassium 137 mg·kg ^{1}, pH 6.08, and organic matter 28.07 mg·kg ^{1}. Pot dimensions were 238 mm × 320 mm, with each pot containing 10 kg of soil. The hydroponic nutrient solution followed the Morad formula (mol·L ^{1}): K SO 0.75×10 ^{3}, MgSO 0.65×10 ^{3}, KCl 0.1×10 ^{3}, Ca(NO) 2.0×10 ^{3}, KH PO 0.25×10 ^{3}, H BO 1.0×10 , MnSO 1.0×10 , CuSO 1.0×10 , ZnSO 1.0×10 , (NH) Mo O   5.0×10 , and Fe-EDTA 1.0×10 . Hydroponic containers were 3 L PVC buckets measuring 160 mm × 240 mm. The wheat variety was 'Yunmai 42' and the faba bean variety was 'Yuxi Large-Seed' , both provided by the Institute of Food Crops, Yunnan Academy of Agricultural Sciences.

1.2 Experimental Design

The wheat-faba bean intercropping experiment employed both pot and hydroponic culture systems with a two-factor design: Factor A comprised three cropping patterns (wheat//faba bean intercropping, wheat monocropping, and faba bean monocropping), and Factor B comprised three nitrogen supply levels (low nitrogen, conventional nitrogen, and high nitrogen). Low nitrogen treatment represented half the recommended nitrogen rate, while high nitrogen treatment represented 1.5 times the recommended rate. Both pot and hydroponic experiments included nine treatments with three replicates and four sampling events, totaling 108 experimental units. Hydroponic experiments transplanted four faba bean and eight wheat seedlings per pot for intercropping, or eight faba bean and 16 wheat seedlings for monocropping. Pot experiments planted six faba bean and 12 wheat seedlings per pot for intercropping, or 12 faba bean and 24 wheat seedlings for monocropping, arranged in two rows.

Fertilizer applications differed between systems. In pot experiments, nitrogen fertilizer (urea) was applied at 150 mg·kg ^{1} for conventional rate, 75 mg·kg ^{1} for low nitrogen, and 225 mg·kg ^{1} for high nitrogen. Phosphorus (calcium superphosphate) and potassium (potassium sulfate) were both applied at 100 mg·kg ^{1} as basal fertilizers. Nitrogen was split-applied, with half as basal fertilizer and half as topdressing at the wheat stem elongation stage; topdressing was applied only to the wheat side in intercropped treatments, with no nitrogen topdressing for faba bean. In hydroponic experiments, nitrogen was supplied as Ca(NO) solution at 2.0×10 ^{3} mol·L ^{1} concentration, with 50 mL added for conventional nitrogen, 25 mL for low nitrogen, and 75 mL for high nitrogen during the seedling stage, with adjustments throughout the growth period. Uniform water and fertilizer management was maintained for both monocropped and intercropped wheat and faba bean. Pot experiments were watered every seven days to maintain soil moisture at 70% of field capacity, while hydroponic nutrient solutions were replaced every three days with continuous aeration and pH maintained between 6.8 and 6.95.

1.3 Sample Collection

Plant samples were collected at 60, 95, and 131 days after emergence, corresponding to wheat stem elongation, flowering, and grain filling stages, and faba bean branching, flowering, and podding stages, respectively. Root exudates were collected by carefully removing plants from their containers, washing roots repeatedly with tap water followed by three rinses with distilled water, then soaking in 5% thymol solution for three minutes before placing in collection bags containing 500 mL of 0.005 mol · L ^{1} CaCl solution. The bags were returned to the original growth containers, and exudates were collected with aeration for two hours beginning at 10:00 AM. After collection, plants were removed and the exudate solutions were stored at -20°C for later analysis. At maturity, plant samples were collected, dried, and weighed to determine crop yield.

1.4 Sample Processing and Analysis

Collected root exudates were thawed and filtered through filter paper, then extracted three times with ethyl acetate (200 mL, 100 mL, and 50 mL). The combined extracts were evaporated and concentrated using a rotary evaporator, and the concentrate was rinsed with methanol and brought to a final volume of 10 mL. The prepared 10 mL samples were filtered through 0.45 m membranes before HPLC analysis for soy isoflavone content. Chromatographic separation employed a Synergi 4u Hydro-RP 80A column (250 mm × 4.6 mm ID) with mobile phase A (chromatographic grade methanol) and mobile phase B (ultrapure water) under the following gradient elution conditions: A 30%-40% (5 min) → 40%-60% (10 min) → 60%-90% (25 min) → 90% (29 min) → 90%-30% (34 min) → 30% (37 min) → end elution (37 min). HPLC conditions were: detection wavelength 270 nm, column temperature 30°C, and flow rate 0.9 mL · min ^{1}. Under these conditions, chromatograms were obtained for mixed soy isoflavone standards (1000 ng · mL ^{1}) and test samples. The detection limit was 10 ng · mL ^{1}, with target flavonoids identified by retention time and quantified by external standard calibration.

1.5 Data Processing and Analysis

Data were processed and graphed using Microsoft Excel 2010, and statistical analysis was performed using SPSS 19.0 software for significance testing (Duncan’ s method, = 0.05).

Results

2.1 Effects of Nitrogen Application Rate on Grain Yield in Wheat-Faba Bean Intercropping

Experimental results (Table 1) demonstrated that wheat and faba bean grain yields increased significantly with nitrogen application rate. In pot experiments, compared to low nitrogen treatment, conventional and high nitrogen treatments increased wheat grain yield by 29.3% and 50.4%, and faba bean grain yield by 27.2% and 37.2%, respectively. In hydroponic experiments, conventional and high nitrogen treatments increased wheat grain yield by 17.6% and 29.0%, and faba bean grain yield by 15.4% and 21.0%, respectively.

Results also indicated that, under identical nitrogen levels, intercropping significantly promoted grain yield increases, though the magnitude of increase decreased with higher nitrogen application. In pot experiments, intercropped wheat yield increased by 20.9%-43.4% and intercropped faba bean yield increased by 13.5%-32.3% relative to monocropping across nitrogen levels (N/2, N, 3N/2). In hydroponic experiments, intercropped wheat yield increased by 14.3%-17.3% and intercropped faba bean yield increased by 6.7%-13.6% compared to monocropping.

2.2 Effects of Nitrogen Application Rate on Soy Isoflavone Secretion from Wheat Roots

Table 2 reveals that soy isoflavone secretion from wheat roots decreased markedly with advancing growth stages, with no isoflavones detected in root exudates at the grain filling stage (131 days after emergence) under high nitrogen conditions. Within the same growth stage, increasing nitrogen application reduced isoflavone secretion. In pot experiments at wheat stem elongation (60 days after emergence), conventional and high nitrogen treatments reduced isoflavone secretion by 36.6% and 104.8% in intercropped wheat and by 18.1% and 49.6% in monocropped wheat, respectively, compared to low nitrogen. At wheat flowering (95 days after emergence), conventional and high nitrogen treatments reduced isoflavone secretion by 43.4% and 75.9% in intercropped wheat and by 13.1% and 15.5% in monocropped wheat. In hydroponic experiments at stem elongation, conventional and high nitrogen treatments reduced intercropped wheat isoflavone secretion by 1.3% and 139.4%, and monocropped wheat secretion by 39.8% and 95.7%.

Intercropping enhanced soy isoflavone secretion from wheat roots under identical nitrogen levels within the same growth stage. Particularly under low and conventional nitrogen conditions at stem elongation (60 days after emergence), intercropped wheat exhibited 29.6% and 12.0% higher isoflavone secretion in pot experiments and 15.3% and 59.2% higher secretion in hydroponic experiments compared to monocropped wheat. At flowering (95 days after emergence), intercropped wheat showed 71.4% higher isoflavone secretion than monocropped wheat under low nitrogen in pot experiments, though differences were not significant under other nitrogen levels. Under high nitrogen conditions, no significant differences in isoflavone secretion were observed between cropping systems in either experiment.

2.3 Effects of Nitrogen Application Rate on Soy Isoflavone Secretion from Faba Bean Roots

Partial least squares-discriminant analysis (PLS-EDA) effectively groups abstract objects into categories with similar properties, visually displaying relationships among experimental subjects. In pot experiments, PLS-EDA analysis (Figure 1 [Figure 1: see original paper]) showed clear separation among treatments along PC1, with low nitrogen monocropped and intercropped faba bean distributed in the positive direction and conventional and high nitrogen treatments in the negative direction. Along PC2, low nitrogen monocropped faba bean appeared in the negative direction while low nitrogen intercropped faba bean appeared in the positive direction, with no significant differences between conventional and high nitrogen treatments. This indicates that low nitrogen conditions promoted more pronounced soy isoflavone secretion in faba bean root exudates, with significant differences between monocropped and intercropped plants under low nitrogen. In hydroponic experiments, PLS-EDA analysis (Figure 2 [Figure 2: see original paper]) also showed clear separation along PC1, with low nitrogen monocropped faba bean in the positive direction and conventional and high nitrogen monocropped faba bean in the negative direction. Low nitrogen and conventional nitrogen intercropped faba bean appeared in the positive direction while high nitrogen intercropped faba bean appeared in the negative direction, demonstrating clear differences between low and high nitrogen treatments regardless of cropping system. Along PC2, low nitrogen intercropped faba bean appeared in the positive direction while low nitrogen monocropped faba bean appeared in the negative direction, indicating that only low nitrogen treatments showed differences between cropping systems in this dimension.

Further ANOVA results (Figure 3 [Figure 3: see original paper] and Figure 4 [Figure 4: see original paper]) revealed that soy isoflavone secretion from faba bean roots initially increased then decreased with advancing growth stages, reaching maximum secretion at flowering (95 days) in both experiments. However, minimum secretion occurred at branching (60 days) in pot experiments and at podding (131 days) in hydroponic experiments. Within the same growth stage, increasing nitrogen application reduced isoflavone secretion. In pot experiments at branching (60 days), conventional and high nitrogen treatments reduced intercropped faba bean isoflavone secretion by 11% and 40.7% with no significant differences in monocropped faba bean. At flowering (95 days), conventional and high nitrogen reduced intercropped faba bean secretion by 39.5% and 154.4% and monocropped faba bean by 16.3% and 37.4%. At podding (131 days), conventional and high nitrogen reduced intercropped faba bean secretion by 27.6% and 188.4% and monocropped faba bean by 34.9% and 56.8%. In hydroponic experiments at branching (60 days), conventional and high nitrogen reduced intercropped faba bean secretion by 50.3% and 66.3% with no significant monocropped differences. At flowering (95 days), conventional and high nitrogen reduced intercropped faba bean secretion by 37.4% and 94.4% and monocropped faba bean by 69.7% and 97.1%. At podding (131 days), conventional and high nitrogen reduced intercropped faba bean secretion by 52.8% and 58.5% and monocropped faba bean by 72.7% and 94.5%.

Intercropping significantly increased soy isoflavone secretion from faba bean roots under low and conventional nitrogen conditions within the same growth stage. In pot experiments at branching (60 days), intercropped faba bean showed 64.6% and 11.4% higher secretion under low and conventional nitrogen, respectively, compared to monocropping. At flowering (95 days), intercropped faba bean exhibited 29.9% and 9.9% higher secretion, and at podding (131 days), low nitrogen intercropped faba bean showed 18% higher secretion. In hydroponic experiments at branching (60 days), intercropped faba bean showed 23.8% and 14.1% higher secretion under low and conventional nitrogen. At flowering (95 days), intercropped faba bean exhibited 49.6% and 82% higher secretion, and at podding (131 days), intercropped faba bean showed 14% and 54% higher secretion. Under high nitrogen conditions, no significant differences in isoflavone secretion were observed between cropping systems across growth stages.

Discussion

Previous studies have reported that red clover (*Trifolium pratense*) exhibited higher isoflavone content across growth stages when no nitrogen fertilizer was applied or at low application rates (50 kg·hm ^{2}), with isoflavone content decreasing as fertilizer rates increased. Our findings align with these results, showing that soy isoflavone secretion from wheat and faba bean roots decreased significantly with increasing nitrogen application across growth stages, with no isoflavones detected in wheat roots at later growth stages (95 and 131 days) under high nitrogen conditions. This pattern likely reflects the role of soy isoflavones as nodulation gene inducers for rhizobia. Under low nitrogen conditions, crops require substantial nitrogen fixation for normal physiological functioning and growth, stimulating extensive secretion of isoflavone inducers to promote rhizobial nodulation and nitrogen fixation. Under conventional and high nitrogen conditions where nitrogen supply meets crop demands, the secretion of isoflavones—regulatory compounds for nodulation and nitrogen fixation—decreases accordingly.

Research on rice intercropping systems has demonstrated that intercropping hybrid rice 'Hexi-41' with yellow-glumed waxy rice significantly increased flavonoid content in yellow-glumed waxy rice leaves. Lu et al. reported that compared to monocropped yellow-glumed waxy rice, intercropped plants under N300 treatment showed significant increases in leaf flavonoid content at 78, 88, 94, and 108 days after transplanting (increases of 9.5%, 15.9%, 15.9%, and 33.2%, respectively), while N180 intercropped plants showed increases of 8.7%, 4.8%, and 22.5% at 88, 94, and 108 days. Our study similarly demonstrates that intercropping significantly enhanced soy isoflavone secretion, though this advantage was pronounced only under low and conventional nitrogen conditions. Under high nitrogen, no significant differences in isoflavone secretion were observed between monocropped and intercropped wheat and faba bean, and this intercropping effect diminished with advancing growth stages.

Our results indicate that maximum soy isoflavone secretion from wheat roots occurred from tillering to stem elongation (60 days), decreasing progressively thereafter, with minimal secretion at grain filling (131 days) and no detectable isoflavones under high nitrogen conditions. In contrast, faba bean root isoflavone secretion initially increased then decreased with growth stage, reaching maximum secretion at flowering (95 days). These findings partially explain the complementary effects of isoflavone secretion in intercropped wheat and faba bean. During wheat tillering to stem elongation (60 days), wheat roots secrete large quantities of flavonoids including isoflavones. As a cereal crop lacking nodulation capacity, wheat' s root exudates may translocate to stimulate neighboring faba bean, inducing isoflavone secretion and nodulation in faba bean. At faba bean flowering (95 days), increased root isoflavone secretion could activate rhizobia surrounding faba bean roots, promoting nodulation and nitrogen fixation. Meanwhile, adjacent wheat root isoflavone secretion gradually decreased, and by faba bean podding (131 days) when nodulation and nitrogen fixation were complete, isoflavone secretion from both crops decreased, with no detectable isoflavones in wheat roots under high nitrogen conditions.

From a methodological perspective, both soil and hydroponic experiments employed plant removal followed by timed collection in nutrient solution, which may introduce artificial influences during sample collection and contribute to discrepancies between the two experimental systems. Currently, more precise root exudate collection methods remain unavailable, and future rhizosphere research should adopt more accurate sampling techniques to advance understanding. Our findings demonstrate that both intercropping system and nitrogen application rate affect soy isoflavone secretion from wheat and faba bean roots, subsequently influencing nodulation, nitrogen fixation, and ultimately yield. However, the underlying mechanisms of these interactions and their specific effects on faba bean nodulation and nitrogen fixation require further investigation.

Conclusion

Both wheat and faba bean grain yields increased with nitrogen application rate, and intercropping enhanced grain yield compared to monocropping. Intercropping system and nitrogen application rate both influenced the quantity of soy isoflavones secreted by wheat and faba bean roots. Isoflavone secretion from both crops decreased with increasing nitrogen application, with no detectable isoflavones in wheat roots at later growth stages (131 days) under high nitrogen conditions. Under low and conventional nitrogen conditions, intercropped wheat exhibited 22.5% and 35.6% higher isoflavone secretion at stem elongation (60 days after emergence) and 28.8% and 7.9% higher secretion at flowering (95 days) compared to monocropped wheat. Intercropped faba bean showed 44.2% and 12.8% higher secretion at branching (60 days), 39.8% and 46.0% higher secretion at flowering (95 days), and 16.0% and 27.0% higher secretion at podding (131 days) compared to monocropped faba bean. Under high nitrogen conditions, no significant differences were observed between monocropped and intercropped plants, and these intercropping effects became less pronounced with advancing growth stages.

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