Intercropping influences component and content change of flavonoids in root exudates and nodulation of Faba bean

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ABSTRACT
Flavonoids produced by legume roots are signal molecules acting as nod gene inducers for the symbiotic rhizobium partner. Nevertheless, the changes of flavonoids in root exudates in intercropping system are still unknown. Based on pot experiment of faba bean and wheat intercropping, here we showed that faba bean and wheat intercropping increased the nodules number and dry weight, dry weight per nodule of faba bean compared with those found in monocrop, and the increase of faba bean nodulation was likely caused by the enhancement with flavonol, isoflavone, chalcone and hesperetin in its root exudates. It also promoted exudation of five types of flavonoids by wheat compared with monocropping. Our findings suggest that the flavonoids in root exudates have a positive effect on the nodulation and nitrogen fixation of faba bean in faba bean and wheat intercropping.

1. Introduction
Intercropping is advanced as one of the foremost conventional production practices used in agro-ecosystem, a set of investigations have clearly demonstrated that compared with monocropping, it could increase natural resources use efficiency (Gao et al. 2009; Rivest et al. 2010; He et al. 2013), boost crop yield and mineral nutrient accumulation (Nataraj et al. 2010; Miao et al. 2012), enhance biological diversity (Nai et al. 2013; Zhang et al. 2015), and lower disease, insect, and weed pressures (Workayehu & Wortmann 2011; Abdel-Monaima & Abo-Elyour 2012; Boudreau 2013). Recently, it has attracted great attention due to the yield advantage with the serious challenges of resources, environment and food.

Root is the major organ of the plant to uptake nutrients and water, and its root exudates play an important role in rhizosphere processes (Terzano et al. 2015). Flavonoids in root exudates are a class of widely distributed secondary metabolites that play an important function in mediating the interaction of plants with their environment (Pourcel et al. 2007). Studies have demonstrated that it is affected by a range of factors, such as crop species, temperature, UV radiation and nitrogen rates (Coronado et al. 1995; Zhang & Li 2003; Li et al. 2012), and particularly crop species. For instance, the flavonoids in legume root exudates are the inducers of genes responsible for the nodulation process of rhizobium in specific legumes (Broughton et al. 2003; Badri & Vivanco 2009; Paul et al. 2010; Wang et al. 2012). Recently, scientists found that there were different components of flavonoids in legume root exudates in the monocropping system (Miao & Liu 2010; Li 2013), however, when legume is intercropped with cereal does it promote the exudation of the flavonoids by root? How is it promoted? At present, these problems are still not very clear.

Intercropping of wheat (Triticum aestivum L.) with faba bean (Vicia faba) is one of the most popular intercropping systems in Yunnan province, China (Yang et al. 2014; Xiao et al. 2016), has clearly been demonstrated it could make better use of nutrition, increase crop yield and nodule number, lower disease and change the carbon and nitrogen metabolism (Su et al. 2006; Miao et al. 2009; Dong et al. 2013; Xiao et al. 2015), whereas there is lack of information and knowledge about the effect of it on changes of flavonoids in root exudates and its connection with faba bean nodulation.

The major objective of this study was to elucidate how faba bean and wheat intercropping impacts the flavonoids change in root exudates and the potential role of flavonoids in faba bean nodulation, aimed at providing a new perspective for facilitative root–root interactions and developing sustainable agriculture.

2. Materials and methods
2.1. Experiment site
Pot trial was carried out in a greenhouse of Yunnan Agricultural University (YNAU) in 2015, Kunming, Yunnan province, southeast China (25°2’N, 102°42’E). This region has a subtropical monsoon climate with an average annual precipitation of 1035 mm, an average annual temperature of 15°C and an altitude of 1962 m. The soil used was taken from a mountain near YNAU, air-dried and ground and sieved to pass through a 5 mm mesh. The soil was red and its initial properties were as follows: pH 6.08; soil organic matter 28.07 g kg\textsuperscript{-1}; available nitrogen 68 mg kg\textsuperscript{-1}; available phosphorus 16 mg kg\textsuperscript{-1} and available potassium 137 mg kg\textsuperscript{-1}.

Wheat (Triticum aestivum L. cv. Yunmai-42) and Faba bean (Vicia faba L. cv. Yuxidalidou) were chosen for the pot experiment. Both seeds were sterilized in 30% (v/v) H\textsubscript{2}O\textsubscript{2} for 20 min,
washed with distilled water, soaked in saturated CaSO₄ solution for 12 h and then germinated in Petri dishes in the dark for 48 h at 22°C. They were transplanted when the second leaves of wheat and faba bean have expanded to its full size.

2.2. Experiment design

Pot trial was conducted with three planting treatments which consisted of wheat monocropping, wheat and faba bean intercropping, faba bean monocropping and three replications, sampling 3 times and giving a total of 36 pots in a randomized block design. Planting ratio was as follows: four plants of wheat seedlings intercropped with two plants of faba bean seedlings in intercropping pot, and eight plants of wheat seedlings, four plants of faba bean seedlings were planted in each monocropping pot, respectively.

Urea, superphosphate and potassium sulfate were applied as N, P and K fertilizers in the experiment. Fifty percent of N rates (150 mg N kg⁻¹ soil), P and K (100 mg P₂O₅ and 100 mg K₂O kg⁻¹ soil) as basal were incorporated into the soil by mixing with 10 kg soil, and it was then distributed among plastic plant pots (25 cm height × 34 cm diameter), and the remaining 50% of the N dressed in the jointing stage along with irrigation. Soil water content was regularly adjusted during the growth of the plants, and no fungicides or insecticides were applied.

2.3. Sampling

The samplings took place at the tillering stage/branching stage, jointing stage/flowering stage and grain-filling stage/seed-filling stage, respectively. According to the methods conducted by Xiao et al. (2014), crop roots were taken off from the soil, shaken off the loose soil, washed the root with water and rinsed three times with distilled water, soaked in 5% thymol solution for 3 min and transferred into containers with 500 ml CaCl₂ solution for 2 h, and then extracted with 5% thymol solution for 3 min and transferred into containers with 500 ml CaCl₂ solution for 2 h, and then extracted with 200, 100 and 50 ml ethyl acetate, evaporated and concentrated, tiritated to 10 ml with methanol and filtered through 0.45 μm polytetrafluoroethylene filter for high-performance liquid chromatography (HPLC) analysis.

2.4. Measurements

2.4.1. Number of nodulation

The number of nodules were counted and recorded, while the weight of the oven-dried nodules was obtained with the aid of a sensitive weighing balance.

2.4.2. HPLC analysis of flavonoids

An Agilent (Waldbronn, Germany) 1100 HPLC series, which consists of a degasser, binary pump, auto-sampler, thermostat and photodiode array detector, was used to determine the composition and concentration of the flavonoids according to the method reported by Krumbein et al. (2007). A Prodigy column (ODS 3, 150 × 3.0 mm, 5 mm, 100 Å; Phenomenex, Aschaffenburg, Germany) with a security guard C18 column (ODS 3, 4 × 3.0 mm, 5 mm, 100 Å) was used for separating the extracts at 30°C. Solvent A consisted of 99.5% water and 0.5% acetic acid, and solvent B consisted of 100% acetonitrile. The following gradients were used: 30–40% B (5 min), 40–60% B (5 min), 60–90% B (15 min), 90% B (4 min), 90–30% B (5 min) and 30% B (3 min). The chromatogram was monitored at 270 nm with a flow rate of 0.9 ml min⁻¹. Flavonol, isoflavone, flavone, chalcone, quercetin, naringenin and hesperetin were used as external standards for calculating the calibration curve in the range of 0.05–1 mg 100 ml⁻¹.

2.5. Statistical analysis

Data in the tables and figures were reported as the mean (±) standard deviation (SD) of three replicates, while all other data were reported as the mean alone. We used one-way analysis of variance (ANOVA) to identify differences in the responses of faba bean nodulation characteristics and different flavonoids under intercropping using the SPSS 19.0 software, and applied a significance level of P < .05.

3. Results

3.1. Grain yields

In the pot experiment (Table 1), we found that grain yields of faba bean and wheat in intercropping were 8.70%, 14.56% higher than that observed in monocropping, respectively. These results suggest that faba bean intercropped with wheat increased the crop yields compared with monocropping.

3.2. Changes in nodules in faba bean and wheat intercropping system

Nodule is an important condition for symbiotic nitrogen fixation of legume crops, and its number can measure the ability of nitrogen fixation. Significant intercropping effect in faba bean nodules was observed (Table 2). The nodules number and dry weight, dry weight per nodule in intercropped faba bean significantly were 80.23%, 133.33% and 29.62% higher than those found in monocropped at the flowering stage (95d), and 5.80%, 139.58% and 126.11% higher than that observed in monocropped at seed-filling stage (131d), which indicated that faba bean and wheat intercropping likely promote faba bean nodulation.

Additionally, the nodules number increased initially and then decreased with the increase in growth stages, and presented the highest number at the flowering stage (95d) which suggested faba bean root formed lots of mature nodules at the flowering stage (95d), and then it slowly rot off with podding. Thus, we need to focus on the changes in flavonoids in faba bean root exudates during the primary period of nodulation to observe whether it was related to faba bean nodulation.

3.3. Changes in total flavonoids in faba bean and wheat intercropping system

As shown in Figure 1, the concentrations of the flavonol, isoflavone and hesperetin for four treatments were higher than

| Table 1. Grain yields of faba bean and wheat (g/pot). |
|---------------------------------|
| Cropping patterns | Grain yields |
|-------------------|--------------|
| MF                | 22.30        |
| IF                | 24.24        |
| MW                | 21.64        |
| IW                | 24.79        |

Note: MF: Monocropped faba bean; IF: Intercropped faba bean; MW: Monocropped wheat; IW: Intercropped wheat.

*Mean significant difference between monocropping and intercropping pattern (P < .05).
others and the concentration levels varied from 0.0% to 40.79%, 16.98% to 22.49%, 13.26% to 27.01%, and 5.04% to 33%, 14.86% to 25%, 12.67% to 18.38%, respectively, while the flavone concentration was lower.

The intercropping effect on total flavonoids is shown in Figure 2, the total flavonoid concentrations in intercropped faba bean root exudates were 23.72%, 29.21% and 45.24% higher than that found in monocropped at the three growth stages, and a considerable difference was found at the flowering stage (95d). Besides, the total flavonoids concentration in intercropped wheat root exudates were 75.44% and 10.04%, respectively, greater than that of monocropped wheat at tillering (60d) and grain-filling stages (131d), while it was significantly lower than monocropped wheat by 7.81% at the jointing stage (95d).

3.4. Changes in seven types of flavonoids in faba bean and wheat intercropping system

As shown in Figure 3, the intercropping effect on seven types of flavonoids in the faba bean and wheat root exudates was observed. Faba bean intercropped with wheat increased the concentration of flavonol, isoflavone, chalcone and hesperetin in its root exudates in intercropping treatment by 114.33% and 853.50%, 13.77% and 44.26%, 101.84% and 72.09%, and 3.04% and 9.24% compared with that found in monocropping during the nodulation period (95d–131d), while it lowered the concentration of quercetin and naringenin. Also, it enhanced the concentration of flavonol, isoflavone, flavone, chalcone, naringenin and hesperetin in intercropping faba bean root exudates at the branching stage (60d) compared with monocropping. Besides, we also found that the concentrations of isoflavone, flavone, chalcone, quercetin and hesperetin in wheat root exudates in intercropped wheat root exudates at the three stages all were greater than that obtained in monocropped wheat.

3.5. Cluster analysis

Cluster analysis is used to group the abstract objects with similarity into a category that could be more intuitive to reveal the variations among these objects. As shown in Figure 4, the concentration of seven types of flavonoids for four treatments was used for cluster analysis. Result indicated that intercropping and monocropping treatments were obviously divided into two categories that suggested wheat and faba bean intercropping significantly changed the root exudation of seven types of flavonoids.

3.6. Relationship between flavonoids and faba bean nodulation

Recent studies revealed that root exudates played an important role in affecting the legume nodulation and nitrogen

| Table 2. The effects of different cropping system on faba bean nodulation (g·plant⁻¹). |
|---|---|---|---|
| Stages | Cropping patterns | Nodule number | Nodule dry weight | dry weight per nodule |
| 95d | MF | 86 ± 5.57 | 0.48 ± 0.06 | 5.57 ± 0.36 |
| | IF | 155 ± 4.58* | 1.12 ± 0.09* | 7.22 ± 0.42* |
| 131d | MF | 69 ± 5.29 | 0.48 ± 0.05 | 6.97 ± 0.67 |
| | IF | 73 ± 4.58 | 1.15 ± 0.11* | 15.76 ± 1.21* |

Note: MF: Monocropped faba bean; IF: Intercropped faba bean. *Mean significant difference between monocropping and intercropping pattern (P < .05).

Figure 1. Percentage of seven types of flavonoids. MW: monocropped wheat; IW: intercropped wheat; MF: monocropped faba bean and IF: intercropped faba bean.
fixation in the legume and cereal intercropping systems (Paul et al. 2010). As shown in Table 3, the nodules number and dry weight, dry weight per nodule were positively correlated with the flavonol, isoflavone, flavone, chalcone, quercetin, naringenin and hesperetin. Besides, the flavonol, naringenin and hesperetin were highly significantly positively correlated with above parameters of faba bean nodules which suggested that the flavonol, naringenin and hesperetin of flavonoids in root exudates were more sensitive components that relate to the faba bean nodulation.

4. Discussion

4.1. Effects of intercropping on faba bean nodulation

Legumes could form nitrogen-fixing nodules through specific recognition of their roots and rhizobium, and it is an important characteristic distinguishing it from other higher plants.
(Hirsch 1992), so they can reduce nitrogen input through biological nitrogen fixation. Banik and Sharma (2009) found that baby corn and legume intercropping could promote the ability of legume nodulation. Cun et al. (2014) also reported that faba bean intercropped with garlic clearly improved faba bean nodulation and dry weight of nodules. Similarly, faba bean intercropped with wheat could increase the nodule number, nodules dry weight and dry weight per nodule of faba bean compared with that found in monocropping, and these findings indicated faba bean intercropped with wheat could improve the ability of faba bean nodulation which was in agreement with the results of previous studies (Banik & Sharma 2009; Cun et al. 2014). On the one hand, these results may be due to the interspecific facilitation leading to crops releasing richer exudates which could provide more carbon sources for growth and reproduction of specific rhizobium in faba bean rhizosphere, thus promoting faba bean nodulation in the intercropping system. On the other hand, previous study reported that the nodulation level of legumes was negatively correlated with soil nitrogen content, and known as a ‘negative feedback regulation of nitrogen’ (Li et al. 2009). Therefore, when it was intercropped with wheat, rhizosphere N availability was decreased caused by stronger competition of wheat for nitrogen, and this decrease presumably derived greater nodulation of faba bean in the intercropping system.

4.2. Changes in root exudation of flavonoids and its role in faba bean nodulation

Flavonoids are a class of widely distributed secondary metabolites that are exuded by plant root, and it can induce specific rhizobium to synthesize and exude a type of Nod factor (Lipochit oligosaccharide, LCOs) which plays an important role in the symbiotic nodulation of rhizobium and plant (Wang et al. 2012). For instance, some studies have well shown that several flavonoids, including naringenin, hesperetin, genistein and 7, 4-dihydroxyflavone, could induce the expression of Nod gens, and then stimulate the nodulation and nitrogen fixation of legume (Broughtone et al. 2003; Giraud et al. 2007; Maj et al. 2010). In our study, the concentrations of flavonol, isoﬂavone, chalcone and hesperetin in intercropping faba bean root exudates during the nodulation period were increased when compared with monocropping, and we found that flavonol, naringenin and hesperetin of flavonoids in root exudates were more sensitive components relating to faba bean nodulation. Thus, the enhancement of nodulation was likely caused by the enrichment with flavonol, isoﬂavone, chalcone and hesperetin in root exudates when faba bean was intercropped with wheat, which was similar to the result of Li et al. (2012), who found that maize intercropped with faba bean could change the content of quercetin and luteolin that could promote significantly faba bean nodulation. However, legumes nodulation is a complex process which consists of the interaction between legumes root and soil rhizobium, a series of signal transduction and material synthesis, so further investigation is required to evaluate the intrinsic mechanism.

Furthermore, we observed the changes of different flavonoids in wheat and faba bean root exudates at different growth stages, and found that intercropping also increased the concentration of flavonol, isoﬂavone, flavone, chalcone, naringenin and hesperetin in faba bean root exudates at the branching stage (60d) compared with monocropping, these enrichments also likely provide the substrate for initiating early nodulation. At the same time, the concentrations of isoﬂavone, flavone, chalcone, quercetin and hesperetin in intercropping wheat root exudates at the three stages all were higher than that found in monocropping, which suggested faba bean intercropped with wheat also promotes correspondingly the exudation of flavonoids by wheat root through interspecific root interactions. However, the impacts of intercropping on different flavonoids in root exudates were

Table 3. Relationship between seven types of flavonoids and faba bean nodulation.

| Growth stages | Factors          | Flavonol | Isoflavone | Flavone | Chalcone | Quercetin | Naringenin | Hesperetin |
|---------------|------------------|----------|------------|---------|----------|-----------|------------|------------|
|               | Nodule number    | 0.9464** | 0.6502     | 0.0245  | 0.9092** | 0.4700    | 0.9928**   | 0.9928**   |
| 95d           | Dry weight of nodule | 0.9600** | 0.5627     | 0.0608  | 0.9160** | 0.4159    | 0.9822**   | 0.9822**   |
|               | Dry weight per nodule | 0.9256** | 0.4308     | 0.1530  | 0.9112** | 0.4026    | 0.9330**   | 0.9330**   |
|               | Nodule number    | 0.9582** | 0.7174     | 0.5693  | 0.6785   | 0.4640    | 0.9928**   | 0.9928**   |
| 131d          | Dry weight of nodule | 0.9425** | 0.6574     | 0.5249  | 0.6170   | 0.4738    | 0.9822**   | 0.9822**   |
|               | Dry weight per nodule | 0.9726** | 0.6396     | 0.5385  | 0.5778   | 0.4241    | 0.9840**   | 0.9840**   |

Note: * and ** mean the differences are significant at level of 5% and 1%, respectively.
observed only at three primary stages in the present study, so further investigation is necessary to understand the flavonoid changes throughout the entire growth period.

5. Conclusions

Cereal intercropped with legume is advanced and widespread due to the biological nitrogen fixation of the legume which is beneficial for maintaining and improving soil fertility. The effects of wheat and faba bean intercropping on component and content change of flavonoids and their role in faba bean nodulation remain unclear. In our study, the nodules number, nodules dry weight and dry weight per nodule of faba bean in intercropping were increased compared with those found in monocropped faba bean, and the enhancement of faba bean nodulation was likely caused by the enrichment with flavonol, isoflavone, chalcone and hesperetin in its root exudates. Wheat and faba bean intercropping also promoted exudation of five types of flavonoids by wheat compared with monocropping. However, legumes nodulation is a complex process, so further studies are necessary to gain in-depth knowledge about legume and cereal intercropping underlying the component and content change of flavonoids, particularly the role of flavonoids in legumes nodulation and nitrogen fixation.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

We thank to the National Natural Science Foundation of China (NSFC) [31460551, 31260504, and 41361065] for financial support.

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