

# Effect of root interaction on nodulation characteristics and isoflavonoid mechanism of alfalfa in the simulated alfalfa/oat in pots

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## Research Article

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# Effect of root interaction on nodulation characteristics and isoflavonoid mechanism of alfalfa in the simulated alfalfa/oat in pots

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**Abstract:** Rational intercropping is capable of promoting nodulation and facilitating the fixation and utilization of nitrogen (N) of legumes. Isoflavonoid is critical to form root nodules of legumes, whereas the regulation of isoflavonoid over nodulation and N fixation in legume/cereal intercropping remains unclear. In the present study, nutrient solution of different root partitions (e.g., no barrier (A-O), nylon mesh barrier (NA-O), plastic barrier (PA-O) and sole alfalfa (SA)) and nitrogen levels (e.g., 21 and 210 mg L<sup>-1</sup> N) were used to delve into the variations of nodulation, N fixation, isoflavonoid content, isoflavonoid synthase (*IFS*) and nodulation-signaling pathway (*NOD*) genes in alfalfa. As suggested from the results, the parameters of nodulation and N fixation ability with A-O were all evidently higher than those with PA-O and SA. Formononetin and genistein with A-O had noticeably higher contents than those with PA-T and SA. Daidzein and luteolin with A-O had remarkably higher contents than those with NA-O in the root. *IFR* 1, *IFR* 4, *NOD* 1 and *NOD* 2 with A-O and NA-O had noticeably higher relative expressions than those with SA and PA-O. *IFR* 2 and *IFR* 3 with A-T and NA-T had significantly lower relative expressions than those with PA-O and SA. The nodulation and isoflavonoids met significant positive associations. *IFR* and *NOD* genes and TNN and ENN followed significant positive associations. *NOD* genes and formononetin exhibited extremely significant positive associations. The conclusion was drawn that the closer root interaction between alfalfa and oat, the more effective the nodulation, N fixation ability and isoflavoids content of alfalfa will be. Isoflavonoids primarily affected nodulation and N fixation. The mechanism of isoflavonoids on nodulation and N fixation in alfalfa/oat intercropping was explained that root interaction results in the reduction of environmental N, thereby regulating the relative expressions of *IFR* genes, elevating the isoflavonoids contents, and up-regulating the relative expressions of *NOD* genes; as a result, the nodulation and N fixation ability in alfalfa are facilitated.

**Keywords:** alfalfa/oat intercropping; nodulation; isoflavonoid; genes

## Introduction

Facilitative root interactions in mixed cropping systems are critical since they nutritionally improve crops grown in nutrient-poor soils and low-input agro-ecosystems<sup>1</sup>. Among the interactions, legume/cereal intercropping system has been extensively adopted for its ability of nitrogen (N) fixation by legume<sup>2</sup>. The legume may partially transfer symbiotically fixed N to the intercropped cereal<sup>3</sup>, while the cereal is likely to stimulate the N fixation activity of legume<sup>4</sup>. It has been widely evidenced that intercropping can stimulate nodulation growth and N fixation of legume, covering faba bean (*Vicia faba* L.)/maize (*Zea mays* L.) intercropping<sup>2</sup>, pea (*Pisum sativum* L.)/maize intercropping<sup>5</sup> and cowpea (*Vigna unguiculata* L.)/cotton (*Gossypium* spp.) intercropping<sup>6</sup>, etc.

Interspecific competitive and depletion N in legume/cereal intercropping system could down-regulate legume “N suppression” effects and facilitate N fixation<sup>7</sup>. This finding was because that the N content in the soil decreases; as a result, the diffusion of O<sub>2</sub> is promoted in the nodule cortex, the respiration rate of nodule is accelerated, and the nitrogenase activity is enhanced. Moreover, the roots can be stimulated to secrete flavonoids (e.g., isoflavonoids) to further form nodules<sup>8</sup>. Isoflavonoids are critical to various plant–microbe interactions. The first effect is exerted in the rhizosphere, in which plants secrete (iso) flavonoids when N in the soil is scarce<sup>9</sup>. Perception of these compounds by rhizobia could activate the transcription of bacterial nodulation genes, thereby leading to the formation of nodulation factors<sup>10</sup>. The second proposed role of (iso) flavonoids during nodulation also took place inside the root, and it was associated with the notable capacity of (iso) flavonoids to inhibit auxin transport<sup>11</sup>. Kobayashi<sup>12</sup> also considered that daidzein and genistein are the major metabolites of soybean roots, and they could induce soybean nodulation. Several studies clearly suggested that the (ios) flavonoids are crucial for nodulation if the bacteria enter the plant, and exogenous (ios) flavonoids are no longer available<sup>13,14</sup>. Numerous existing studies have been conducted on the relationship between isoflavonoids secreted by roots and nodulation and N fixation in legume/cereal intercropping. However, how isoflavonoids in legume crops have the effect on nodules remains unclear. Isoflavonoid synthase (*IFS*) is a vital enzyme of isoflavone decomposition pathway, critically regulating the content and composition of isoflavonoids. Accordingly, to ascertain the variations of isoflavonoids contents and *IFR* and nodulation-signaling pathway (*NOD*) related genes in legume/cereal intercropping can further explore the regulation of isoflavones on nodulation and N fixation.

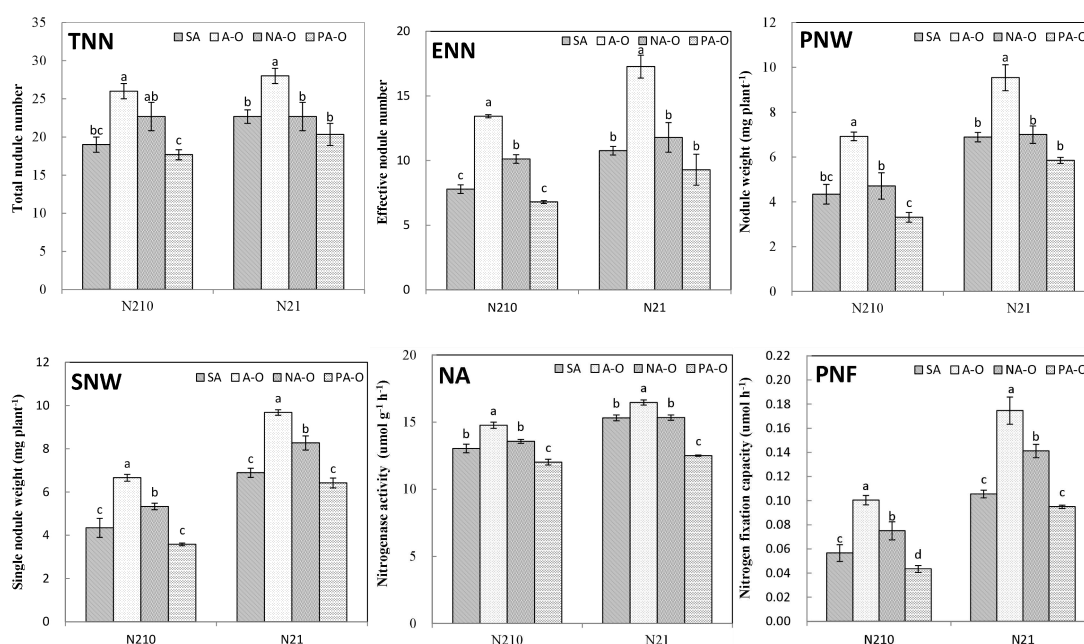
Alfalfa (*Medicago sativa* L.) refers to the crucial perennial legume forage; it can be used to achieve symbiotic N<sub>2</sub> fixation<sup>15</sup>. Alfalfa exhibits the high-yield, rapidly regenerating many new stems after it is harvested; it can also be harvested multiple times in the growing season<sup>16</sup>. For this reason, it is one of the most commonly used species in arid and semi-arid regions. Oat (*Avena sativa* L.) is considered the most extensively used annual cool-season forage worldwide, which is an important source of nutrition for ruminant livestock<sup>17</sup>. It is applied in animal nutrition since it is a fast growing, palatable, succulent and nutritious fodder crop. Alfalfa/oat intercropping

system is capable of enhancing N use efficiency, light use efficiency and land productivity; thus, it has become a major planting pattern in northern China. Nevertheless, the effects of root interaction on nodulation characteristics and isoflavonoids variations of alfalfa in this model remain unclear. Thus, in the present study, the root interaction intensity in alfalfa/oat intercropping was simulated, and sand culture nutrient solution was used to ascertain the nodulation, N fixation capacity, isoflavonoid content, *IFR* and *NOD* genes related expression of alfalfa with the use of different N treatment and planting systems. This study primarily aimed to elucidate how alfalfa/oat intercropping impacts the variation of isoflavonoids contents, as well as the potential role of isoflavonoids on nodulation in alfalfa, as attempt to create a novel perspective for facilitative root interactions and developing sustainable agriculture.

## Results

### Nodulation and N fixation of alfalfa

TNN with A-T was significant higher than that with SA and NA-O ( $P < 0.05$ ). ENN, PNW and SNW with A-O were significant higher than those with SA, NA-O and PA-O ( $P < 0.05$ ) (Fig. 1). ENN and PNW with NA-O were significant higher than that with PA-O under N210. SNW with NA-O were significant higher than that with SA and PA-O ( $P < 0.05$ ). NA with SA and NA-O was significant lower than that with A-O, but NA with SA and NA-O was significant higher than that with PA-O ( $P < 0.05$ ). PNF with A-O was significant higher than that with NA-O; PNF with NA-O was significant higher than that with SA and PA-O ( $P < 0.05$ ).



**Fig. 1 Nodulation and N fixation of alfalfa**

Note: N21 and N210 represent 21 mg L<sup>-1</sup> and 210 mg L<sup>-1</sup> nitrogen treatment. SA, A-T, NA-T and PA-T represent sole alfalfa, no barrier, nylon mesh barrier, plastic barrier. TNN, ENN, PNW, SNW, NA and PNF represent total nodule number, effective nodule number, fresh nodule weight per plant, single nodule weight, nitrogenase activity of nodules and nitrogen fixation capacity per plant.

### Conent of isoflavonoids in alfalfa

Fig. 2 for peak time of daidzein, luteolin, formononetin and genistein. In Table 1, the contents of daidzein and luteolin in stem and leaf, and those with SA and PA-O in root were not detected. The content of daidzein and luteolin with A-O was significant higher than that with NA-O in the root ( $P < 0.05$ ). The content of formononetin with A-O was significantly higher than that with SA and NA-O, and the content of formononetin with NA-O was significantly higher than that with PA-O in stem and leaf ( $P < 0.05$ ). The content of formononetin with A-O was significantly higher than that with PA-O in root ( $P < 0.05$ ). The content of genistein with A-O was significantly higher than that with PA-O ( $P < 0.05$ ).

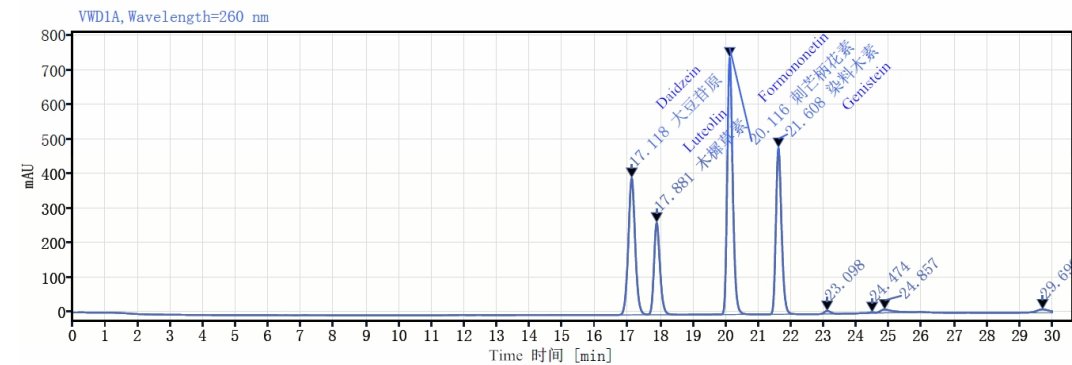


Fig. 2 Peak time of daidzein, luteolin, formononetin and genistein

Table 1 The content of isoflavonoids in the aboveground and underground parts of alfalfa

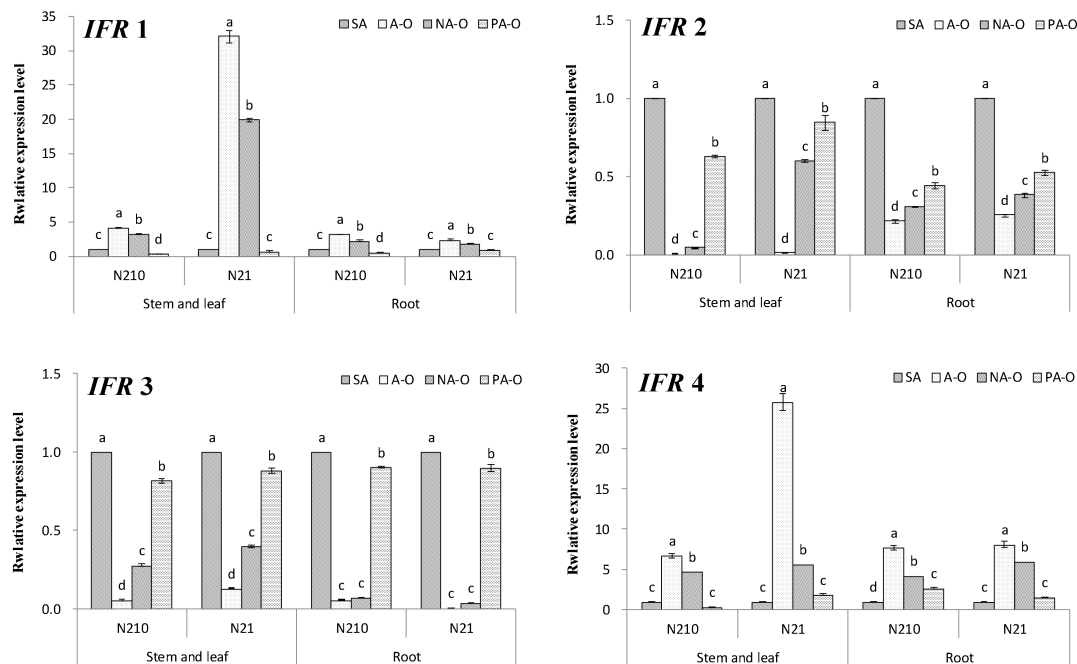
		Stem and leaf				Root			
		Daidzein	Luteolin	Formononetin	Genistein	Daidzein	Luteolin	Formononetin	Genistein
N210	SA	—	—	26.93±1.44bc	2.86±0.23c	—	—	2.81±0.20b	40.41±1.20d
	A-O	—	—	42.67±1.41a	11.57±0.61a	78.40±5.98a	9.14±0.75a	4.48±0.37a	75.05±2.85a
	NA-O	—	—	30.75±0.71b	6.11±0.40b	29.45±4.43b	2.85±0.25b	2.71±0.20b	65.59±1.02b
	PA-O	—	—	24.92±0.99c	2.91±0.16c	—	—	0.48±0.30c	49.04±1.34c
N21	SA	—	—	32.75±0.78bc	4.63±0.50b	—	—	3.79±0.29b	72.26±1.04bc
	A-O	—	—	50.00±0.79a	12.72±1.44a	112.20±4.61a	23.23±0.98a	9.98±0.33a	81.17±2.45a
	NA-O	—	—	34.76±1.00b	6.77±0.44b	75.88±2.50b	12.32±1.22a	9.92±0.38a	74.53±1.11b
	PA-O	—	—	30.32±1.08c	4.06±0.33b	—	—	3.46±0.61b	68.55±1.26c

Note: N21 and N210 represent 21 mg L<sup>-1</sup> and 210 mg L<sup>-1</sup> nitrogen treatment. SA, A-T, NA-T and PA-T represent sole alfalfa, no barrier, nylon mesh barrier, plastic barrier.

### Expression of isoflavonoid and N-fixed related genes in alfalfa

To unravel the underlying molecular mechanism of the stimulatory effects of different alfalfa and oat cropping systems on alfalfa nodulation, we analyzed expression patterns of a series of key genes regulating isoflavonoids (Fig. 3) and nodulation (Fig. 4) in alfalfa. The relative expressions of *IFR 1* and *IFR 4* with A-O were significantly higher than those with NA-O, and the relative expressions of *IFR 1* and *IFR 4* with NA-O were significantly higher than those with SA and PA-O ( $P < 0.05$ ). The relative expression of *IFR 1* with SA was significantly higher than that

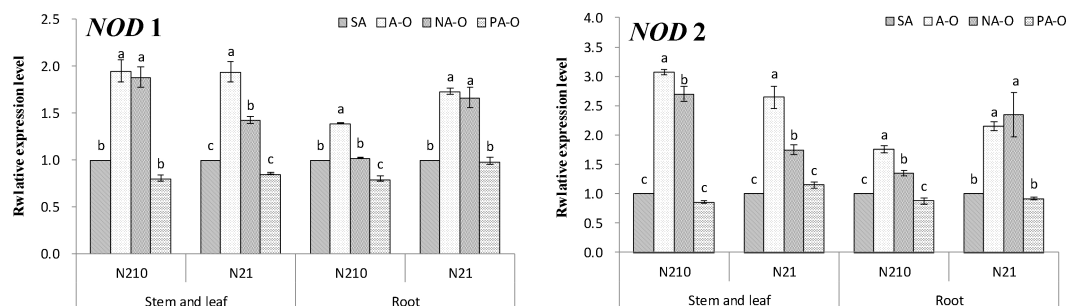
with PA-O under N210 ( $P < 0.05$ ). The relative expression of *IFR 4* with PA-O was significantly higher than that with SA in root under N21 ( $P < 0.05$ ). The relative expressions of *IFR 2* and *IFR 3* with SA were significantly higher than those with PA-O, and the relative expressions of *IFR 2* and *IFR 3* with PA-O were significantly higher than those with NA-O and A-O ( $P < 0.05$ ). The relative expressions of *IFR 2* under N210 and N21 and *IFR 3* under N210 with NA-O were significantly higher than those with SA ( $P < 0.05$ ).



**Fig. 3 Expressions of *IFR 1*, *IFR 2*, *IFR 3* and *IFR 4* in different parts of alfalfa**

Note: N21 and N210 represent 21 mg L<sup>-1</sup> and 210 mg L<sup>-1</sup> nitrogen treatment. SA, A-T, NA-T and PA-T represent sole alfalfa, no barrier, nylon mesh barrier, plastic barrier. Bars represent mean values of 3 independent biological replicates  $\pm$  standard error.

The relative expressions of *NOD 1* and *NOD 2* with A-O and NA-O were significantly higher than those with SA and PA-O ( $P < 0.05$ ). The relative expression of *NOD 2* with A-O was significantly higher than that with NA-O except that in root under N21 ( $P < 0.05$ ).



**Fig. 4 Expressions of *NOD 1* and *NOD 2* in different parts of alfalfa**

Note: N21 and N210 represent 21 mg L<sup>-1</sup> and 210 mg L<sup>-1</sup> nitrogen treatment. SA, A-T, NA-T and PA-T represent sole alfalfa, no barrier,

nylon mesh barrier, plastic barrier. TNN, ENN, PNW, SNW, NA and PNF represent total nodule number, effective nodule number, fresh nodule weight per plant, single nodule weight, nitrogenase activity of nodules and nitrogen fixation capacity per plant. Bars represent mean values of 3 independent biological replicates  $\pm$  standard error.

## Correlation analysis of nodulation, isoflavonoids and genes

The parameters of nodulation (TNN, ENN, PNW, SNW, NA and PNF) and the parameters of isoflavonoids (daidzein, luteolin, formononetin and genistein) had extremely significant positive correlation ( $P < 0.01$ ) except genistein in stem and leaf (Table 2). The correlation coefficient between formononetin in stem and leaf and ENN was the highest (0.904).

*IFR 2* or *IFR 3* had negative correlation with the parameters of nodulation (Table 3). And other parameters were positive correlation. The related genes except *IFR 2* in root and TNN, the all related genes and ENN, the related genes except *IFR 2* and *NOD 2* in stem and leaf and NA or PNF were all extremely significant correlation ( $P < 0.01$ ). The correlation coefficient between *IFR 4* in stem and leaf and ENN, *NOD 1* in root and PNF were the highest (0.83).

*IFR 1* and formononetin, *NOD 1* and formononetin, *NOD 2* and formononetin were all extremely significant positive correlation ( $P < 0.01$ ) (Table 4). *IFR 3* and daidzein, *IFR 3* and luteolin were all extremely significant negative correlation ( $P < 0.01$ ).

**Table 2 Correlation analysis between nodulation and isoflavonoids of alfalfa**

	TNN	ENN	PNW	SNW	NA	PNF
Daidzein - Root	0.755**	0.843**	0.753**	0.543**	0.757**	0.784**
Luteolin - Root	0.719**	0.817**	0.795**	0.611**	0.767**	0.832**
Formononetin - Stem and leaf	0.830**	0.904**	0.868**	0.673**	0.826**	0.884**
Formononetin - Root	0.583**	0.681**	0.798**	0.783**	0.804**	0.820**
Genistein - Stem and leaf	0.826**	0.901**	0.742**	0.476*	0.714**	0.758**
Genistein - Root	0.736**	0.800**	0.803**	0.712**	0.732**	0.785**

Note: TNN, ENN, PNW, SNW, NA and PNF represent total nodule number, effective nodule number, fresh nodule weight per plant, single nodule weight, nitrogenase activity of nodules and nitrogen fixation capacity per plant.

**Table 3 Correlation analysis between nodulation and genes of alfalfa**

	TNN	ENN	PNW	SNW	NA	PNF
<i>IFR 1</i> - Stem and leaf	0.598**	0.731**	0.749**	0.637**	0.740**	0.792**
<i>IFR 1</i> - Root	0.746**	0.808**	0.526**	0.238	0.578**	0.537**
<i>IFR 2</i> - Stem and leaf	-0.632**	-0.744**	-0.368	-0.031	-0.380	-0.393
<i>IFR 2</i> - Root	-0.443*	-0.571**	-0.279	-0.059	-0.184	-0.275
<i>IFR 3</i> - Stem and leaf	-0.692**	-0.801**	-0.507*	-0.223	-0.534**	-0.529**
<i>IFR 3</i> - Root	-0.662**	-0.785**	-0.516**	-0.276	-0.585**	-0.543**
<i>IFR 4</i> - Stem and leaf	0.718**	0.830**	0.773**	0.559**	0.689**	0.805**
<i>IFR 4</i> - Root	0.709**	0.806**	0.614**	0.357	0.624**	0.645**
<i>NOD 1</i> - Stem and leaf	0.677**	0.810**	0.490*	0.215	0.609**	0.528**
<i>NOD 1</i> - Root	0.725**	0.813**	0.807**	0.663**	0.808**	0.830**
<i>NOD 2</i> - Stem and leaf	0.694**	0.777**	0.460*	0.173	0.499*	0.471*
<i>NOD 2</i> - Root	0.639**	0.745**	0.650**	0.486*	0.725**	0.687**

Note: TNN, ENN, PNW, SNW, NA and PNF represent total nodule number, effective nodule number, fresh nodule weight per plant, single nodule weight, nitrogenase activity of nodules and nitrogen fixation capacity per plant.

**Table 4 Correlation analysis between isoflavonoids and genes of alfalfa**

	Daidzein	Luteolin	Formononetin	Genistein
<i>IFR</i> 1	-0.142	-0.130	0.593**	-0.282
<i>IFR</i> 2	-0.344*	-0.306*	-0.217	-0.144
<i>IFR</i> 3	-0.626**	-0.566**	-0.157	-0.276
<i>IFR</i> 4	0.185	0.179	0.460**	-0.022
<i>NOD</i> 1	0.340*	0.360*	0.462**	-0.007
<i>NOD</i> 2	0.290*	0.294*	0.484**	-0.060

## Discussions

Legume/cereal intercropping can enhance N fertilizer utilization efficiency, which is characterized by high yield and low consumption<sup>18</sup>. In legume/cereal intercropping systems, the reliance on symbiotic N<sub>2</sub> fixation is enhanced as a result of a strong competition for soil mineral N by the intercropped cereal. The N competition in intercropping is primarily achieved by root interaction. The root separation method was adopted here to clarify the effect of root interaction on nodulation and N fixation of alfalfa and the regulation of isoflavones on nodulation and N fixation. In this study, the nodulation (TNN, ENN, PNW and SNW) and N fixation (NA and PNF) under normal N level were higher than those under low N level of alfalfa. It was therefore suggested that low N level could promote the nodule and N fixation of alfalfa. It has also been confirmed in other studies that the nodulation and N fixation of legumes were largely affected by the N concentration, namely, the lower N concentration, the higher the nodulation and N fixation ability will be<sup>19</sup>. Compared with NA-O, nodulation and N fixation of alfalfa increased significantly with A-O. When the roots of the two crops with A-O, they can directly compete and exchange environment N through connection of Rhizobia or interception of root surface area. However, the competition and exchange of N with NA-O were achieved primarily through mass flow and diffusion<sup>20</sup>. Accordingly, the N compete ability with A-O was more robust than that with NA-O of oat, and the nodulation and N fixation with A-O was stronger than that with NA-O of alfalfa. Meantime, the nodulation and N fixation with NA-O of alfalfa were more obvious than those with PA-O and SA, since PA-O and SA had no root interaction, and N exchange could not take place. It was therefore indicated that the nodulation and N fixation of alfalfa increased with the rise in root interaction. Some scholars also consider that legume/cereal intercropping cannot up-regulate the amount of N fixation<sup>21</sup>, whereas the majority of scholars consider that legume/cereal intercropping can facilitate the nodulation and N fixation of legume<sup>2,22</sup>. Furthermore, this promotion ability is determined by the combination of crops and cultivation methods<sup>23</sup>.

Isoflavonoids refer to the major signal components of legume root extracts<sup>24</sup>. In the present study, in the roots, daidzein and luteolin contents with A-O were noticeably larger than those with NA-O, and those with no PA-O and SA detected. Formononetin and genistein contents with A-O were obviously larger than those with



PA-O and SA in different parts. All of these findings revealed that alfalfa/oat intercropping gave rise to the differences of isoflavonoids contents in alfalfa; the closer the root interaction, the higher the isoflavonoids contents will be. Meantime, daidzein, luteolin, formononetin and genistein all displayed positive correlations with nodulation and N fixation, suggesting that isoflavonoids contents evidently impacted root nodules in alfalfa. This result was achieved because isoflavonoids participate in the signaling exchange between roots of legumes and N-fixing rhizobia, and they can promote division of cortical cells during nodule formation by hindering auxin transport. Ferguson<sup>25</sup> also reported that genistein application could enhance nitrogenase activity, thus, protein content enhancement could be attributed to hastened N fixation. As revealed from the results of Dolatabadian's experiment, the genistein used elevated nodule number and nodule weight, as well as nitrogenase activity<sup>26</sup>. In brief, root interaction considerably up-regulated the isoflavonoids contents, and isoflavonoids are indispensable for nodulation in alfalfa.

The existing research primarily focuses on the necessity and evidence of isoflavonoids in legume nodulation, whereas isoflavonoids exert less effect in legume, especially in the field of gene research<sup>13</sup>. To delve into the nodulation and N fixation mechanism in alfalfa/oat intercropping, the IFR and NOD related genes were detected. As suggested from the results, the relative expressions of *IFR* 1, *IFR* 4, *NOD* 1 and *NOD* 2 with A-T were evidently higher than those with PA-T and SA, while the relative expressions of *IFR* 2 and *IFR* 3 exhibited opposite performance. It is therefore suggested that root interaction exerted noticeable regulatory effect on *IFR* and *NOD* genes, thereby affecting the isoflavonoids contents and nodulation characteristics in alfalfa. The correlation revealed that *IFR* genes were closely correlated with TNN and ENN, *IFR* 1, *IFR* 3 and *IFR* 4 were tightly associated with NA and PNF. It can also be demonstrated that IFR can significantly regulate the nodules formation of alfalfa, and *IFR* 1, *IFR* 3 and *IFR* 4 can significantly regulate alfalfa N fixation ability. Subramanian<sup>27</sup> also considered that during soybean nodulation, isoflavonoids are necessary as inducers of nodulation genes in roots, and they are critical to determine the degree of nodulation. The relative expressions of *NOD* genes with A-T were obviously higher than those with PA-T and SA. This finding complies with the nodulation, N fixation and isoflavonoids contents, because the N competition in intercropping system is more intense under nutrient stress. To fix more N, the relative expression of *NOD* genes as the signal material of nodulation was up-regulated<sup>28</sup>. Moreover, *NOD* genes in root were positively correlated with nodulation and N fixation, while *NOD* genes displayed positive associations with daidzein, luteolin and formononetin. It is therefore demonstrated that the isoflavonoids in alfalfa are the main inducers of *NOD* genes expression. Subramanian et al.<sup>13</sup> also reported that isoflavonoids are critical to endogenous *NOD* gene induction in the determinate legume. For this reason, the essential effect exerted by isoflavonoids in alfalfa nodulation is associated with their effects in *NOD* gene induction.

## Conclusions

In alfalfa/oat intercropping, the closer root interaction between alfalfa and oat,

the more effective the nodulation, N fixation ability and isoflavonoids content of alfalfa will be. Isoflavonoids primarily affected nodulation and N fixation. The mechanism of isoflavonoids on nodulation and N fixation in alfalfa/oat intercropping was explained that root interaction results in the reduction of environmental N, thereby regulating the relative expressions of *IFR* genes, elevating the isoflavonoids contents, and up-regulating the relative expressions of *NOD* genes; as a result, the nodulation and N fixation ability in alfalfa are facilitated.

## **Materials and Methods**

### **Experimental Materials**

Alfalfa, LW6010, was provided by college of Pratacultural Science, Gansu Agricultural University (GAU), China. Oat, Haywire, was provided by Beijing Clover Company, China. The rhizobium (*Bradyrhizobium japonicum*), *Sinorhizobium meliloti* (12531), was provided by GAU, China.

### **Experimental Design**

In order to prevent the effect of precipitation on the N concentration in the experiment, it was carried out in the growth chamber (light at 28°C/14 h and darkness at 20 °C /10 h, light intensity was 260~350 mol m<sup>-2</sup> s<sup>-1</sup>, and relative humidity was 60%~70%) at Gansu Agricultural University, Lanzhou, China. In order to provide an accurate and stable N environment, and reduce root nodule abscission and RNA degradation though rapid and complete root sampling, nutrient solution sand culture method was used. Nutrient solution sand culture method was conducted with sand culture (25 kg pot<sup>-1</sup> sand washed clean by distilled water) with 5 cropping systems in plastic pots (47 cm diameter, 40 cm height) under 2 N treatments. The 2 N treatments were (1) the low N level, 21 mg N L<sup>-1</sup> as mixed N source of Ca(NO<sub>3</sub>)<sub>2</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (N21), (2) the medium N level (appropriate nitrogen levels of alfalfa), 210 mg N L<sup>-1</sup> as mixed N source of Ca(NO<sub>3</sub>)<sub>2</sub> and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (N210). Hoagland-Arnon solution as the basic nutrient solution was used in the N21 and N210 nutrient solutions, and the ration of NO<sub>3</sub><sup>-</sup> -N: NH<sub>4</sub><sup>+</sup> -N was 1:1. The 5 cropping systems were (1) alfalfa as a monoculture (SA), (2) oat as a monoculture (ST), (3) alfalfa/oat intercropping with no barrier (A-O), (4) alfalfa/oat intercropping with nylon mesh barrier (NA-O), (5) and alfalfa/oat intercropping with plastic barrier (PA-O). Plastic pots were cut in the middle, separated into two compartments and then placed nylon mesh (diameter of 0.2 mm) or plastic film in the middle, and then reconstructed<sup>29</sup>. The A–O planting pattern had no artificial physical barrier between alfalfa and oat, allowing water and nutrients to exchange and possible root interaction between alfalfa and oat. NA–O planting pattern obstructed overlapping of alfalfa roots with oat roots but allowed water and nutrients to exchange through the nylon meshes. The PA–O planting pattern preventing water and nutrients exchanged between alfalfa and oat and with no overlapping of alfalfa roots with oat roots. There were 3 replications in each treatment.

The seeds of alfalfa and oat were chosen and disinfected, and then were planted

in separated one side of plastic pots filled with sand. When the alfalfa and oat grew to 3 cm in height, healthy seedlings were kept in each plastic pot with the rest removed by hand. Sole alfalfa and sole triticale kept 20 seedlings, no barrier, nylon mesh barrier and plastic barrier of alfalfa and triticale kept 10 seedlings, respectively. Seven days after alfalfa seedlings emergence, two levels of N nutrient solution (1000 mL per pot) were added to the pots, and then rhizobium (*Sinorhizobium meliloti*, 12531, provided by College of Pratacultural Science, GAU) liquid was inoculated (50 mL pot<sup>-1</sup>, OD600 between 0.63 to 0.64). Sand was rinsed with distilled water and nutrient solution was replaced once a week. Distilled water (1 L) was slowly added in each pot (prevent salt ion accumulation) in each time. Then pots with plants were rinsed with distilled water for 12 h, and then added nutrient solution (2 L pot<sup>-1</sup>). Distilled water was supplemented every day to the location of the nutrient solution for the first time. Sowing date for alfalfa was 1 March 2018; and sowing date for oat was 23 March 2018; and harvest date for alfalfa and oat was 13 July 2018 (flowering stage of alfalfa). the original source of method descriptions should be clearly acknowledged and cited. The method descriptions were cited from Zhao's research which is our team's preliminary research<sup>30</sup>.

## Measured parameters

### Plant dry matter weight and N accumulate

During the sampling period, the whole 5 plants of alfalfa and oat were selected from each pot for whole plant dry matter. 3 pots were taken for each treatment. Whole plant dry weight (PDW, mg plant<sup>-1</sup>): put fresh samples in 105°C oven for 15 min and then put them in 60~70°C oven until a constant weight.

Plant nitrogen content (PNC) was determined by Kjeldahl procedure after digestion in a mixture of concentrated H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub><sup>31</sup>. Powdered sample was digested in the Kjeldahl digestion flask by boiling with H<sub>2</sub>SO<sub>4</sub>-H<sub>2</sub>O<sub>2</sub> until the mixture became clear. The digested liquid was filtered and volume. Ammonia was steam distilled from the digest to which NaOH solution was added. The distillate was collected in a conical flask containing HCl and red methyl indicator. The ammonia that was distilled into the receiving conical flask reacted with the acid and the excess acid in the flask was estimated by back titration against NaOH with color change from red to yellow (end point). Determinations were made on all reagents alone (blank determinations). N (%) was calculated as  $[(\text{ml standard acid} \times \text{N of acid}) - (\text{ml blank} \times \text{N of base})] \times (\text{ml std base} \times \text{N of base}) \times 1.4007 / \text{Weight of sample in grams} \times 100\%$ . Plant N accumulation (PNA, mg plant<sup>-1</sup>) = PNC × PDW.

### Nodulation

Roots were rinsed with distilled water, and absorbed residual water with absorbent paper, then removed the nodules quickly. All fresh nodules were detached from the roots to collect, and then counted and weighted. Nodulation, including total nodule number (TNN), effective nodule number (ENN), fresh nodule weight per plant

(PNW), single fresh nodule weight (SNW) of alfalfa, was measured. At the time of sampling, some nodules are pink; they can effectively fix N, so they are called “effective nodules”. In addition, some nodules are white or brown, which may be immature or aging, so they are called “non-effective nodules”. The TNN is the sum of ENN and ineffective nodule number. The ratio of ENN to TNN was calculated.

### **Nitrogenase activity**

The nitrogenase activity (NA) was measured by the acetylene reduction assay<sup>32</sup>. Weigh fresh nodules about 0.2 g into a 7 ml glass bottle, seal with a rubber stopper. 10% volume of air was removed and replaced with an equal volume of acetylene. After 30 min at room temperature, duplicate 25  $\mu$ L gas samples were removed and analyzed by gas chromatography for peak of ethylene and acetylene. The standard curve of ethylene was determined and measured under standard conditions using standard ethylene to calculate the nitrogenase activity of the nodule sample. The instrument was the GC-7890F gas chromatograph with column temperature of 180 °C, sampler of 150 °C, and FID detector of 170 °C. Gas pressure: N<sub>2</sub> is 0.3 mPa, H<sub>2</sub> is 0.08 mPa, and air is 0.15 mPa. C<sub>2</sub>H<sub>4</sub> level ( $\mu$ mol g<sup>-1</sup> h<sup>-1</sup>) =  $h_x$  (sample peak area)  $\times$  C (standard C<sub>2</sub>H<sub>4</sub> level,  $\mu$ mol mL<sup>-1</sup>) /  $h_s$  (standard C<sub>2</sub>H<sub>4</sub> peak area)  $\times$  24.9  $\times$  t (C<sub>2</sub>H<sub>2</sub> reaction time, h)  $\times$  m (tumor weight, g). N fixation capacity ( $\mu$ mol h<sup>-1</sup>) = NA  $\times$  SNW.

### **Content of isoflavonoids**

1g of hay sample was accurately weighed (having been powdered and screened for 1mm). Subsequently, 20 ml of methanol solution was added and soaked for 6 h, extracted by ultrasound for 30 min, and then filtered through filter paper. The filter residue was collected again, and 20ml of methanol solution was added. The mixture solution was ultrasonically treated for 20min and filtered with filter paper. The two filter solutions were mixed and spin-dried in vacuum at 30 °C. The crude extract was dissolved in methanol to 10ml and filtered with 0.22  $\mu$  m PTFE membrane for HPLC analysis.

Preparation of standard curve: the quality of standard products (daidzein, luteolin, formononetin, genistein) was accurately weighted, and then the products were dissolved in methanol to prepare standard solution at a certain concentration. The chromatographic peak area of each component was ascertained using the automatic injector. The injection quality of the standard and the corresponding peak area acted as the standard curve.

The column applied in the experiment was Synergi 4U hydro RP 80A (250 mm  $\times$  4.6 mm ID). Mobile phase A was pure methanol, while B was ultra-pure water. The elution conditions included: 30%  $\sim$  40% (5 min)  $\rightarrow$  40%  $\sim$  60% (10 min)  $\rightarrow$  60%  $\sim$  90% (25 min)  $\rightarrow$  90% (29 min)  $\rightarrow$  90%  $\sim$  30% (34 min)  $\rightarrow$  30% (37 min)  $\rightarrow$  stop elution (37 min). The determining conditions of liquid chromatography included: wavelength 270 nm, column temperature 30 °C, flow rate at 0.9 ml min<sup>-1</sup>, and period for 45 min. Detection wavelength: 254 nm. Under the selected chromatographic

conditions, the chromatograms of isoflavonoid (1000 ng ml<sup>-1</sup>) and the sample to be tested were generated. The detection limit reached 10 ng ml<sup>-1</sup>. The target flavonoids were ascertained according to the retention time. The content of isoflavonoids was calculated using external standard method. Ethanol and methanol were of HPLC grade, while all the other chemicals were of analytical grade.

### Quantitative Real-time PCR Analysis of isoflavonoids

For quantitative analysis, 3 independent biological replicates of stem and leaf and root samples of alfalfa from three different pots for each treatment were harvested. The each provided sample obtained from the different alfalfa plants in each pot, frozen in liquid N, and kept at -80 °C for further analyses. Total RNA extraction was isolated using TransZOL method. The concentration of the total RNA was determined by ultra-micro UV spectrophotometer (Quawell-Q5000, USA). Five grams of total RNA were used to synthesize cDNA by reverse transcriptase powerscript™ (Hiscript II Q Select RT SuperMix for qPCR (+ gDNA wiper) test kit; Vazyme; China) following the manufacturer's protocol. The cDNA samples were used as template to quantify the target gene expression levels (quantitative real-time PCR) by ChamQ SYBR Color qPCR Master Mix Test Kit (Vazyme, China) following the manufacturer's protocol.

According to the *Medicago truncatula* gene information (NCBI/GenBank accession NO. MTR\_5g020760 for *IFR 1*; MTR\_5g020800 for *IFR 2*; MTR\_8g071130 for *IFR 3*; MTR\_4g070340 for *IFR 4*; MTR\_8g020840 for *Nod 1*; MTR\_3g072710 for *Nod 2*; MTR\_8g098715 for 18s), we designed the primers for each gene, where *IFR 1*, *IFR 2*, *IFR 3* and *IFR 4* are isoflavonoids reeducate genes, *NOD 1* and *NOD 2* are nodulation genes (Table 5).

**Table 5 The specific primers for the genes**

Name	Accession NO.	Sequence
<i>IFR 1</i>	MTR_5g020760	F: 5'-CACGAGGCAGTTGAGCCAGTTAG-3'
		R: 5'-ACGTCGAGTTGAGCCAAGTTACG-3'
<i>IFR 2</i>	MTR_5g020800	F: 5'-CACGAGGCAGTTGAGCCAGTTAG-3'
		R: 5'-ACGTCGAGTTGAGCCAAGTTACG-3'
<i>IFR 3</i>	MTR_8g071130	F: 5'-ATGGTTGTTCACGAGACCTCAGTG-3'
		R: 5'-ACGGCAATGCACTAGGCTTAAGAG-3'
<i>IFR 4</i>	MTR_4g070340	F: 5'-TCGATGTTTCAGTGTCCGTGT-3'
		R: 5'-CAGCCAGACAAATTGCAAGTCC-3'
<i>NOD 1</i>	MTR_8g020840	F: 5'-TGTCTGTGCTCAGTTCAGGTTGC-3'
		R: 5'-GCTCCATGCCTCTCAACACCTTC-3'
<i>NOD 2</i>	MTR_3g072710	F: 5'-TCACAGCCAATCAAGCCATCATCG-3'
		R: 5'-TGTGGACCGTTGTTGTTGAAGC-3'

### Statistical analysis

Data were analyzed using Statistical Analysis Software (SPSS software, 17.0, SPSS Institute Ltd, USA) with the standard split-plot design analysis method to test

for significance of treatments, and means were compared by least significance difference (LSD). Where indicated, the results are expressed as mean values ( $\pm$ SE) from three independent experiments. Harvest period, N treatment and cropping system were considered as fixed effects and replication as random effects. All significances were declared at the probability level of 0.05.

## Guidelines statement

I statement that our experimental research and field studies on plants are complied with relevant institutional, national, and international guidelines and legislation.

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## Author Contributions

XL took part in designing the experiment and participated in drafting the work; YZ conceived and designed the experiment, conducted it and acquired the data, analyzed the data and wrote the manuscript; CT and YW helped experiment test.

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### **Additional Information**

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Figures

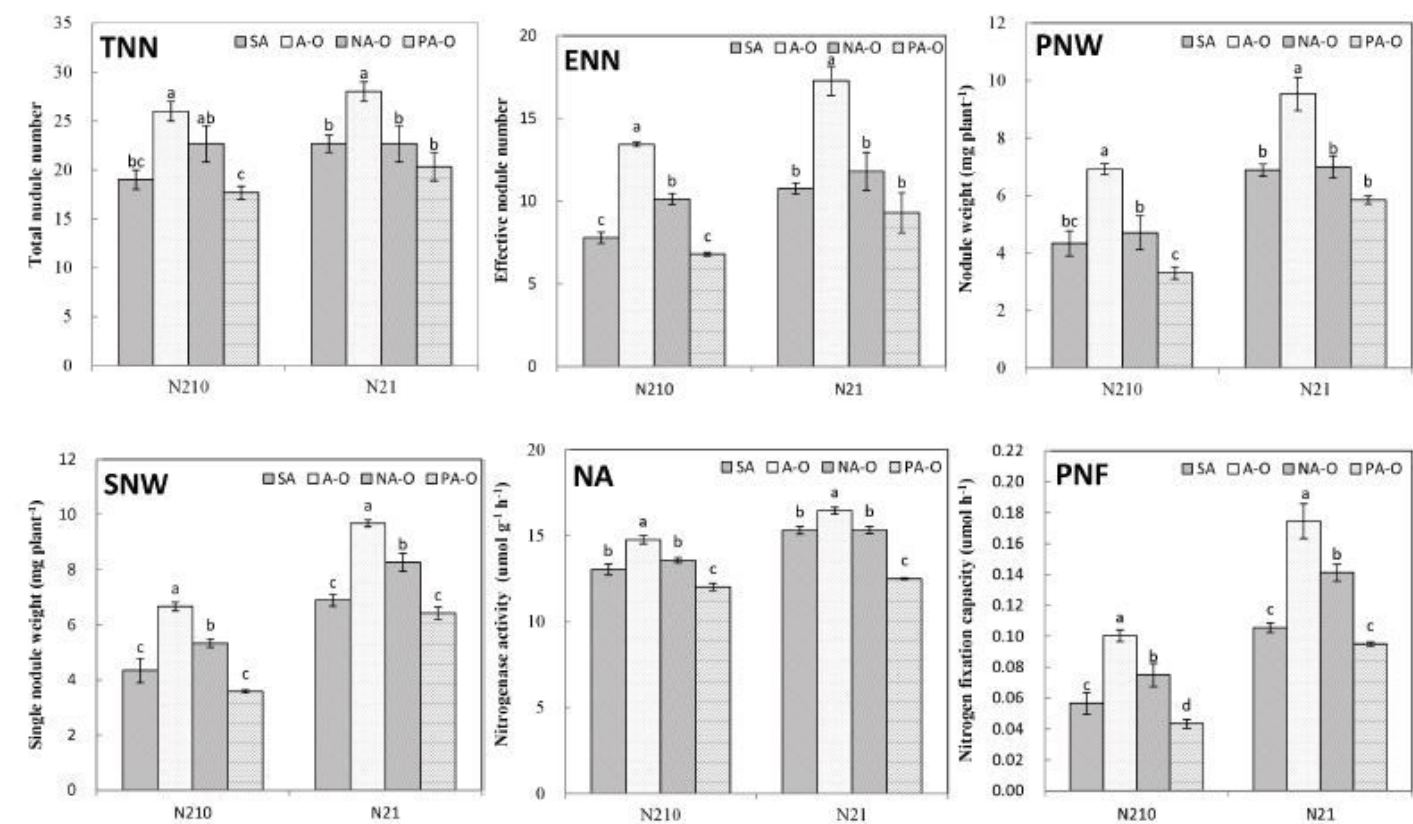


Figure 1

Nodulation and N fixation of alfalfa Note: N21 and N210 represent 21 mg L<sup>-1</sup> and 210 mg L<sup>-1</sup> nitrogen treatment. SA, A-T, NA-T and PA-T represent sole alfalfa, no barrier, nylon mesh barrier, plastic barrier. TNN, ENN, PNW, SNW, NA and PNF represent total nodule number, effective nodule number, fresh nodule weight per plant, single nodule weight, nitrogenase activity of nodules and nitrogen fixation capacity per plant.

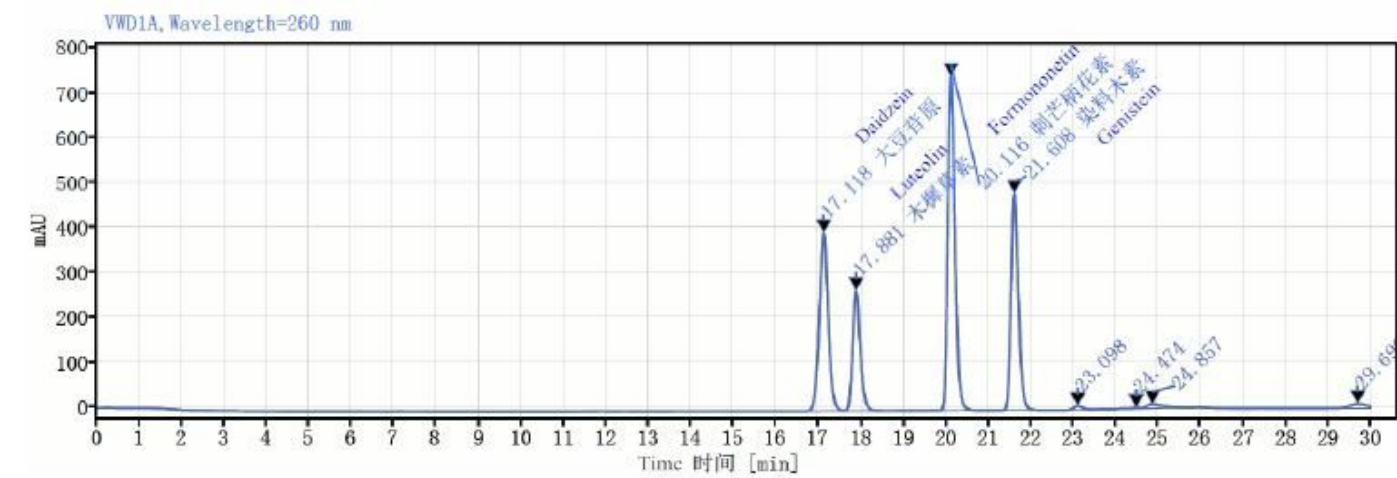


Figure 2

Peak time of daidzein, luteolin, formononetin and genistein

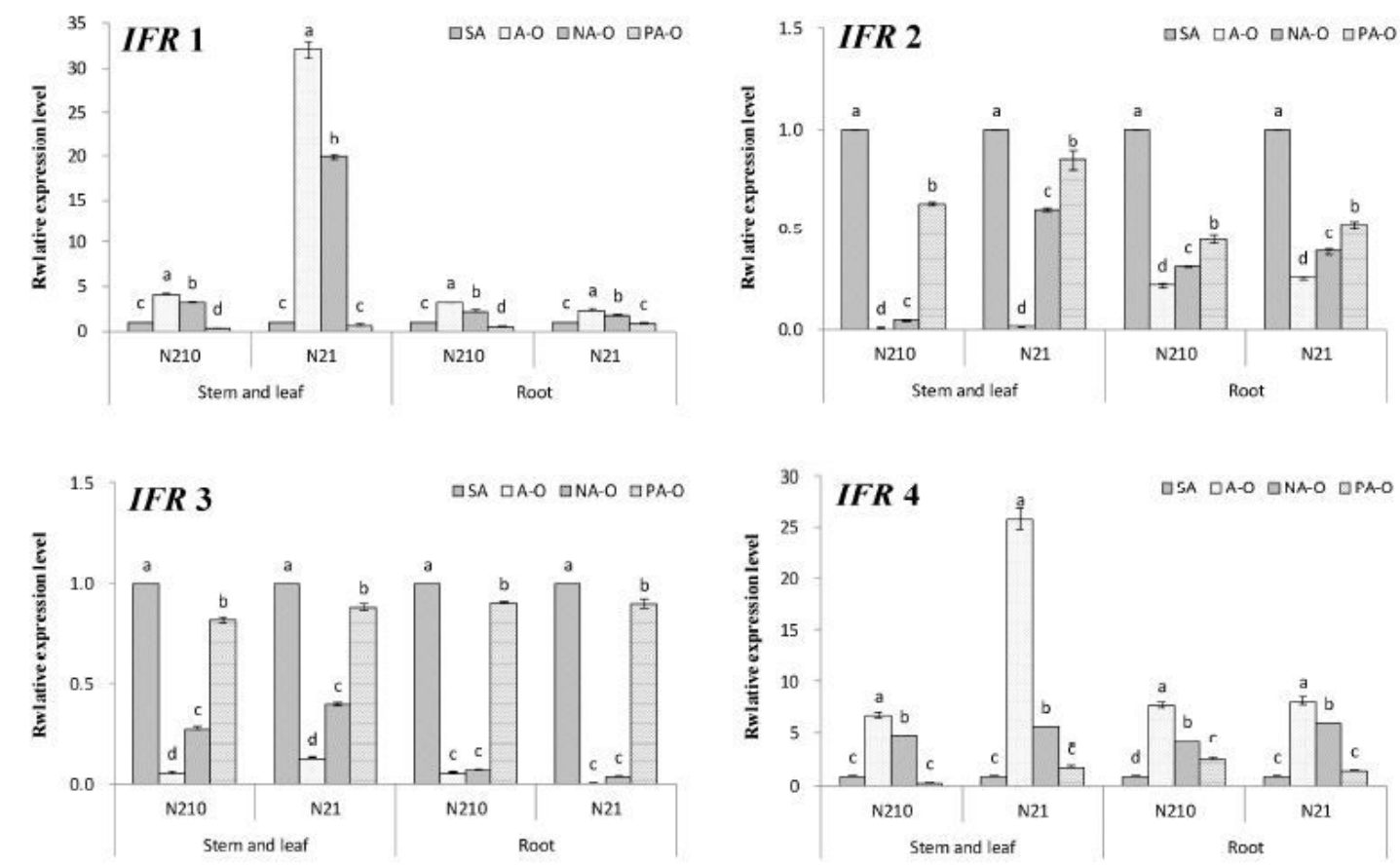


Figure 3

Expressions of IFR 1, IFR 2, IFR 3 and IFR 4 in different parts of alfalfa Note: N21 and N210 represent 21 mg L-1 and 210 mg L-1 nitrogen treatment. SA, A-T, NA-T and PA-T represent sole alfalfa, no barrier, nylon mesh barrier, plastic barrier. Bars represent mean values of 3 independent biological replicates  $\pm$  standard error.

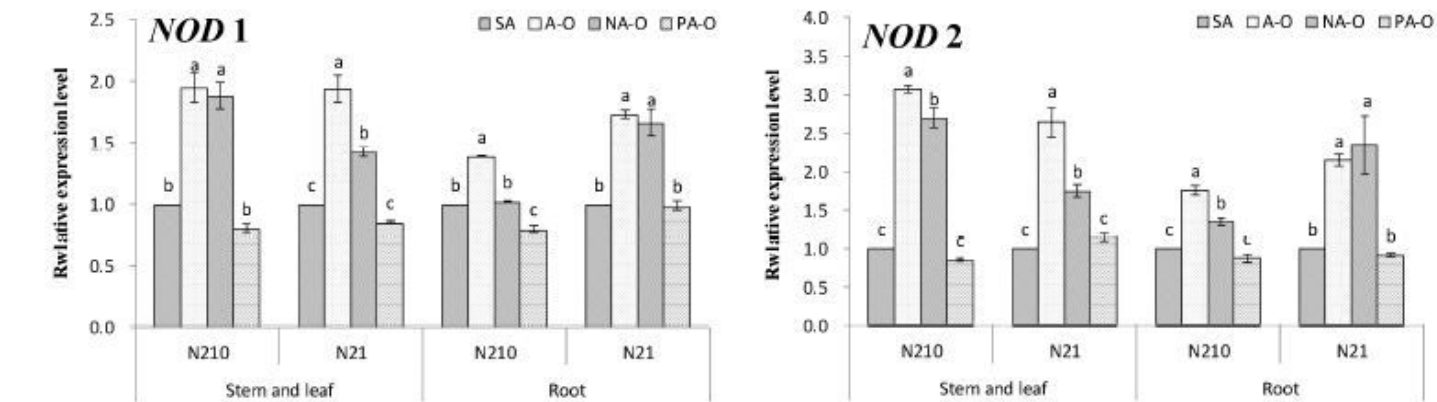


Figure 4

Expressions of NOD 1 and NOD 2 in different parts of alfalfa Note: N21 and N210 represent 21 mg L<sup>-1</sup> and 210 mg L<sup>-1</sup> nitrogen treatment. SA, A-T, NA-T and PA-T represent sole alfalfa, no barrier, nylon mesh barrier, plastic barrier. TNN, ENN, PNW, SNW, NA and PNF represent total nodule number, effective nodule number, fresh nodule weight per plant, single nodule weight, nitrogenase activity of nodules and nitrogen fixation capacity per plant. Bars represent mean values of 3 independent biological replicates  $\pm$  standard error.