

Article

Regulation of Phosphorus Supply on Nodulation and Nitrogen Fixation in Soybean Plants with Dual-Root Systems

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Abstract: Phosphorus (P) is an important nutrient affecting nodulation and nitrogen fixation in soybeans. To further investigate the relationship of phosphorus with soybean nodulation and nitrogen fixation, the seedling grafting technique was applied in this study to prepare dual-root soybean systems for a sand culture experiment. From the unfolded cotyledon stage to the initial flowering stage, one side of each dual-root soybean system was irrigated with nutrient solution containing 1 mg/L, 31 mg/L, or 61 mg/L of phosphorus (phosphorus-application side), and the other side was irrigated with a phosphorus-free nutrient solution (phosphorus-free side), to study the effect of local phosphorus supply on nodulation and nitrogen fixation in soybean. The results are described as follows: (1) Increasing the phosphorus supply increased the nodules weight, nitrogenase activity, ureide content, number of bacteroids, number of infected cells, and relative expression levels of nodule nitrogen fixation key genes (*GmEXPB2*, *GmSPX5*, *nifH*, *nifD*, *nifK*, *GmALN1*, *GmACP1*, *GmUR5*, *GmPUR5*, and *GmHIUH5*) in root nodules on the phosphorus-application side. Although the phosphorus-application and phosphorus-free sides demonstrated similar changing trends, the phosphorus-induced increases were more prominent on the phosphorus-application side, which indicated that phosphorus supply systematically regulates nodulation and nitrogen fixation in soybean. (2) When the level of phosphorus supply was increased from 1 mg/L to 31 mg/L, the increase on the P- side root was significant, and nodule phosphorus content increased by 57.14–85.71% and 68.75–75.00%, respectively; ARA and SNA were 218.64–383.33% and 11.41–16.11%, respectively, while ureide content was 118.18–156.44%. When the level of phosphorus supply was increased from 31mg/L to 61mg/L, the increase in the regulation ability of root and nodule phosphorus content, ARA, SNA, and ureide content were low for roots, and the value for nodules was lower than when the phosphorus level increased from 1 mg/L to 31 mg/L. (3) A high-concentration phosphorus supply on one side of a dual-root soybean plant significantly increased the phosphorus content in the aboveground tissues, as well as the roots and nodules on both sides. In the roots on the phosphorus-free side, the nodules were prioritized for receiving the phosphorus transported from the aboveground tissues to maintain their phosphorus content and functionality.

Keywords: dual-root soybean; phosphorus; nodulation; nitrogen fixation; ultrastructure



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1. Introduction

Phosphorus (P) is one of the three essential elements for plants and is the second most limiting element for plant growth. Currently, 40% of the world's cropland lacks phosphorus, resulting in lower yields [1]. Phosphorus is a very important nutritional element that affects soybean growth and nodule nitrogen fixation. Proper application of phosphorus fertilizer can regulate root nodules growth, nitrogenase activity, and metabolic pathways, as well as enhance the capacity of nitrogen-fixing root nodules -promoting nitrogen by phosphorus [2–9].

The effect of phosphorus on nitrogen fixation mainly includes soybean plant growth [4,10], nodule formation [6,9], and nodule metabolism [3]. The promotion of phosphorus in nitrogen fixation is achieved by stimulating the growth of the host plant rather than by promoting the growth of rhizobia or the formation and function of nodules [11–14]. Additionally, the optimal phosphorus environment for host plant growth and the phosphorus requirements for symbiotic nitrogen fixation are determined by the development and function of the root nodules [15]. Current studies on phosphorus metabolism in nodules of leguminous crops are mainly focused on the analysis of compounds that affect nodule nitrogen fixation and indirectly explain the mechanism of phosphorus inhibition of nodule nitrogen fixation [3]. Several studies have suggested that low phosphorus stress inhibits nitrogen-fixing enzyme activity in legume nodules resulting from reduced nodule ATP energy [16], leghemoglobin content [13], Fe ion content [17], and excessive secretion of organic acids [18]. Other studies found that legume crops can adapt to low phosphorus stress by increasing root nodule phytase and phosphatase activities [19,20].

To better understand the phosphorus regulation of growth and phosphorus uptake in leguminous plants, split-root, and dual-root experiments were performed. In Shu et al. [21], the growth and P uptake of the root system significantly increased on the phosphorus-application side and remained stable on the phosphorus-free side after P ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, FePO_4 , $\text{C}_6\text{H}_6\text{O}_{24}\text{P}_6\text{Na}_{12}$, or KH_2PO_4) was supplied to one side of the root system of *Lupinus albus*. In Scott and Robson [22], supplying KH_2PO_4 to one side of the root system of subterranean clover significantly promoted P uptake by roots on the phosphorus-application side but not by roots on the phosphorus-free side and the shoots. However, Snapp and Lynch [23] found that a high-concentration of $\text{NH}_4\text{H}_2\text{PO}_4$ supply to the roots on one side of *Phaseolus vulgaris* L. promoted P uptake on both the phosphorus-application side and phosphorus-free side.

In existing studies, the uneven distribution of phosphorus fertilizer in the soil due to the application method results in a wide variation in the fertilizer available to the root system, with some roots receiving phosphorus fertilizer to meet their needs, while others do not. The existing studies on the regulatory effect of P supply on nodulation and nitrogen fixation in soybeans have focused mainly on its local effect under single-root conditions through direct contact and seldom on its systemic effect. Most of the research has mainly focused on the study of phosphorus in nodule-regulating substances but less on the mode of regulation. Split-root and dual-root experiments are effective methods to study the mode of regulation. In the above-described method of rooting, the root system of the legume crop plant was divided into two parts so that neither subsystem remained intact, which may affect the accuracy of the experimental results. In this study, a grafting approach of soybean seedlings [24] was used to prepare a dual-root system in soybean plants. Soybean seedlings were grafted to obtain a double-rooted soybean plant with complete roots on both sides, and the test results were more accurate. The dual-root system of soybean plants received different concentrations of P nutrient solution in the root system on one side and phosphorus-free nutrient solution on the other side [21,25]. Nodule weight, nitrogenase activity, ureide concentration, and the expression levels of key genes related to nodulation and nitrogen fixation were measured, and the nodule ultrastructure was observed. This study systematically investigated the effects of P supply on soybean nodulation and nitrogen fixation, providing a reference that clarifies the regulatory mechanisms of nodulation and nitrogen fixation in soybean.

2. Materials and Methods

This study was conducted at the Experimental Base of Northeast Agricultural University, Harbin city, Heilongjiang Province, China (126°43' E, 45°44' N) from 10 June to 25 July 2020. Sand-cultured dual-root soybean systems were used. The tested soybean variety was Kenfeng 16 (*Glycine max* L. cv.) obtained from Heilongjiang Academy of Land Agricultural Reclamation Science, Heilongjiang, China).

2.1. Experimental Treatments

Dual-root soybean plants were prepared according to the grafting method described by Xia et al. [24] and they were potted with river sand. The method is described in detail in S1. Figure 1 shows the root morphology of a dual-root soybean plant under P31/0 treatment at the initial flowering stage, with the phosphorus-application side on the left and the phosphorus-free side on the right.

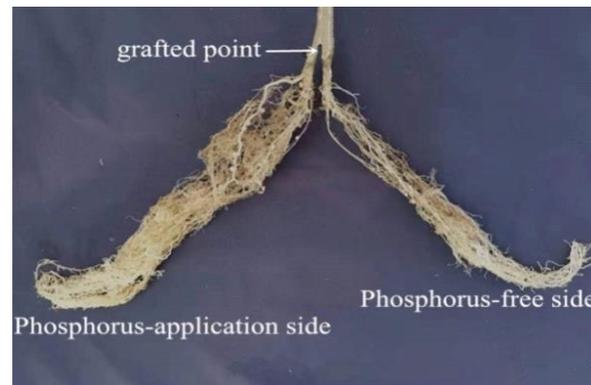


Figure 1. Roots of dual-root soybeans.

From the VC stage (unfolded cotyledon stage) to the R1 stage (initial flowering stage), the dual-root soybean plants were irrigated with a P-containing nutrient solution on one side (P+ side) and a P-free nutrient solution on the other side (P– side). On the P+ side, the nutrient solution applied contained P at three different concentrations, 1 mg/L (P1), 31 mg/L (P31), and 61 mg/L (P61), which were denoted as the P1/0, P31/0, and P61/0 treatments, respectively. Using KH_2PO_4 as the P source, the nutrient solution was prepared as proposed by Hoagland and Arnon [26] Yao et al. [27]. The potassium level in the P1/0 and P31/0 treatments was adjusted to the same level as that of P61/0. The types and concentrations of nutrient solution (mg/L) are shown in Tables S1 and S2.

From the VE stage (emergence stage) to the VC stage, the plants were irrigated with distilled water once a day at 250 mL on each side. Then, 250 mL of P-containing nutrient solution was applied on the P+ side, and 250 mL of P-free nutrient solution was applied to the P– side, once a day at 7:00–8:00 during the VC stage to V4 stage (fourth trifoliolate leaf stage) and twice a day at 7:00–8:00 and 17:00–18:00 from the V4 stage to the R1 stage. Starting from the VC stage, field-grown soybean nodules cryopreserved from the previous year were ground and added to the nutrient solution (approximately 5 g/L), which was inoculated in the plants continuously for 5 days. The reproductive stage was recognized as described by Fehr et al. [28].

2.2. Sampling and Measurement

Samples were taken once in the V4 and R1 stages. The shoots were cut along the grafting site between 8:00 and 10:00 on a sunny day. The underground roots on both sides were washed with distilled water to remove the sand. For the V4 stage, the nodules of average size within 5 cm of the root base were harvested and fixed in formalin and then dehydrated with alcohol and glacial acetic acid (FFA) solution for ultrastructural observation. For the R1 stage, all the root nodules were harvested and stored in a freezer at -80°C to measure the relative gene expression levels of *GmEXPB2*, *nifHDK*, *GmALN1*, etc. The intact roots in the V4 and R1 stages were used to measure the nitrogenase activity of the nodules. Subsequently, the nodules were removed and dried at 65°C to measure the dry weight of the P content of each part, as well as the nodule ureide content. Each measurement was repeated 4 times, 2 biological replicates, for a total of 8 plants.

Nodule nitrogenase activity was measured using the acetylene reduction activity (ARA) assay described by Gremaud and Harper [29].

For the ultrastructural observation of nodules, the removed nodules were halved longitudinally, and these halves were cut into 1×3 mm rectangular slices longitudinally with a blade, followed by FAA fixation and dehydration. Finally, ultrathin sections were cut with an LKB-V microtome. A Hitachi-600 transmission electron microscope was used for observation and imaging. The fixation and slide preparation procedures followed Goodchild and Bergersen) [30].

The P contents in plants were measured by the molybdenum antimony anti-colorimetric method with CuSO_4 and K_2SO_4 as catalysts and concentrated H_2SO_4 for digestion.

The nodule ureide content was measured following the method of Trijbels and Vogels [31].

For nodule RNA extraction and quantitative real-time reverse transcription-PCR (qRT-PCR) analysis, total RNA was extracted from root nodules using TRIzol reagent (Servicebio, Wuhan, China), and cDNA was synthesized using a RevertAid reverse transcription kit (Servicebio, Wuhan, China). The qRT-PCR analysis was performed by SYBR Green monitored qPCR (Servicebio, Wuhan, China), with the following reaction conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 30 s. The primer sequences used for qRT-PCR amplification are shown in Table S3. Three biological replications were included. In this study, 16S rRNA was used as the reference for *nifD*, *nifH*, and *nifK* genes in the calculation of the qRT-PCR data, as previously described by Sulieman et al. [32], while 18S rRNA was used as the reference for *GmSPX5*, *GmACP1*, *GmUR5*, *GmPUR5*, and *GmHIUH5* genes in the calculation of the qRT-PCR data, as previously described by Carter et al. [33].

2.3. Statistical Analyses

All statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, Illinois). Before performing a one-way analysis of variance (ANOVA) on the data, all data were tested for normality, and Duncan's multirange test was used with a significance level of $p < 0.05$.

3. Results

3.1. Effects of P Supply on P Content and Dry Matter Accumulation in Soybean Plants with Dual-Root Systems

Table 1 shows that the P content was significantly higher in nodules than in the roots and shoots and higher on the P+ side than on the P− side at V4 and R1 for all treatments. At the V4 and R1 stages, the P content in the roots and shoots was similar between the P31/0 and P61/0 groups, but both groups had significantly higher P than the P1/0 group. At the V4 stage, the nodule P content increased significantly with the P concentration supplied. At the R1 stage, the nodule P content on the P+ side was significantly higher after the P31/0 and P61/0 treatments than after the P1/0 treatment but did not show any significant differences between P31/0 and P61/0 treatments. On the P− side, the nodule P content was similar between all treatments. At the V4 and R1 stage, the nodule P content on the P− side increased by 85.71% and 57.14%, respectively, under the P31/0 treatment when compared with P1/0, and by 0% and 9.09%, respectively, and under the P61/0 treatment when compared with P31/0. The root P content on the P− side increased by 68.75% and 75.00%, respectively, under the P31/0 treatment when compared with P1/0, and by 29.63% and 25.00%, respectively, under the P61/0 treatment when compared with P31/0. Under a low P supply (P1/0), the P contents in the roots and nodules were similar between the P+ side and P− side in the R1 stage, during which the difference in the nodule P content between the two sides reached an extremely significant level. Under the P31/0 and P61/0 treatments, the P content in the roots and nodules was higher on the P+ side than on the P− side, and all differences reached extremely significant levels at the V4 stage. In the R1 stage, the P content difference between the two sides was extremely significant in the roots under both the P31/0 and P61/0 treatments and was significant in the nodules under P31/0, but it was nonsignificant in the nodules of the P61/0-treated plants. The root P

content ratio between the two sides (P^-/P^+ ratio) was 1.00 under P1/0; the P^-/P^+ ratio under the P31/0 and P61 treatments was 0.62 and 0.54 in the V4 stage and 0.55 and 0.63 in the R1 stage, respectively. The P^-/P^+ ratio of the nodule P content was greater than 0.80 under all treatments. This finding indicated that the root system prioritized the P^- side nodules for distribution of P transported from the shoots to ensure normal functioning of the nodules.

Table 1. Effect of P supply on the P content of dual-root soybeans.

Treatments	Roots				Nodules				Shoot(%)	
	P+(%)	P-(%)	IP-(%)	P-/P+	P+(%)	P-(%)	IP-(%)	P-/P+		
V4	P1/0	0.07 ± 0.00 ^b	0.07 ± 0.00 ^b	85.71	1.00	0.20 ± 0.01 ^{c**}	0.16 ± 0.01 ^c	68.75	0.80	0.07 ± 0.01 ^b
	P31/0	0.21 ± 0.01 ^{a**}	0.13 ± 0.01 ^a	0.00	0.62	0.32 ± 0.01 ^{b**}	0.27 ± 0.01 ^b		0.84	0.16 ± 0.01 ^a
	P61/0	0.24 ± 0.02 ^{a**}	0.13 ± 0.01 ^a		0.54	0.44 ± 0.01 ^{a**}	0.35 ± 0.01 ^a	29.63	0.80	0.18 ± 0.01 ^a
R1	P1/0	0.07 ± 0.01 ^b	0.07 ± 0.00 ^b	57.14	1.00	0.16 ± 0.01 ^b	0.16 ± 0.01 ^c	75.00	1.00	0.09 ± 0.00 ^b
	P31/0	0.20 ± 0.01 ^{a**}	0.11 ± 0.01 ^a	9.09	0.55	0.34 ± 0.01 ^{a*}	0.28 ± 0.01 ^b		0.82	0.21 ± 0.01 ^a
	P61/0	0.19 ± 0.01 ^{a**}	0.12 ± 0.01 ^a		0.63	0.36 ± 0.01 ^a	0.35 ± 0.02 ^a	25.00	0.97	0.22 ± 0.01 ^a

Note: All data in the table are expressed as the mean ± standard error ($n = 4$). Different lowercase letters indicate the differences between treatments at a significance level of 5%. Longitudinal comparison. * and ** denote a significant difference between the P^+ side and the P^- side at the 5% level and 1% level, respectively. IP- is the increment of P^- .

Table 2 reveals that the dry weight showed an increasing trend in all parts of the soybean plants with increasing P supply on the P^+ side. At the V4 stage, the dry weight of the shoots was significantly different between all treatments; at the R1 stage, although the difference was nonsignificant between P31/0 and P61/0, the dry weight of the aboveground tissues under these two treatments was significantly higher than that under the P1/0 treatment. On the P^+ side, the dry weights of roots and nodules showed marked differences between treatments; on the P^- side, the differences were nonsignificant between the P31/0 and P61/0 treatments. The root and nodule dry weights showed no significant differences between the P^+ side and the P^- side under a low P supply (P1/0); however, they were higher on the P^+ side than on the P^- side under the P31/0 and P61/0 treatments, demonstrating extreme differences in the plants supplied with higher concentrations of P. The P^-/P^+ ratio of root dry weight at V4 the stage was 0.91 under the P1/0 treatment, 0.49 under the P31/0 treatment, and 0.50 under the P61/0 treatment, and 0.41 under both the P31/0 and P61/0 treatment at R1ste stage. The P^-/P^+ ratio of nodule dry weight at the V4 stage was 1.00 under P1/0 and 0.22 under both P31/0 and P61/0, and it was 0.27 and 0.23 under the P31/0 and P61/0 treatments at the R1. These results show that the higher P supply increased the dry weights of all parts of the soybeans, and the increase was more prominent in the roots and nodules that directly contacted the supplied P. Combined with the data in Table 2, the distribution of P to the P^- side was prioritized by absorption and transport from the P^+ side, to support the growth of the root system, and the root P content was maintained at a reduced level to ensure root development; moreover, nodule growth was suppressed to reserve a certain level of P for nodule development and normal physiological functioning. The effect of phosphorus supply on the number of root nodules in double-rooted soybeans is shown in Table S4.

Table 2. Effect of P supply on the dry weight of dual-root soybeans.

Treatments	Roots			Nodules			Shoots (g/Plant)	
	P+(g/Plant)	P–(g/Plant)	P–/P+	P+(g/Plant)	P–(g/Plant)	P–/P+		
V4	P1/0	0.78 ± 0.03 ^c	0.71 ± 0.01 ^b	0.91	0.01 ± 0.000 ^c	0.01 ± 0.001 ^b	1.00	1.78 ± 0.11 ^c
	P31/0	1.72 ± 0.05 ^{b**}	0.84 ± 0.05 ^a	0.49	0.18 ± 0.006 ^{b**}	0.04 ± 0.001 ^a	0.22	3.99 ± 0.52 ^b
	P61/0	1.87 ± 0.01 ^{a**}	0.93 ± 0.03 ^a	0.50	0.23 ± 0.011 ^{a**}	0.05 ± 0.001 ^a	0.22	5.39 ± 0.40 ^a
R1	P1/0	0.92 ± 0.04 ^c	0.80 ± 0.03 ^b	0.87	0.02 ± 0.003 ^c	0.02 ± 0.003 ^b	1.00	2.09 ± 0.04 ^b
	P31/0	2.61 ± 0.01 ^{b**}	1.07 ± 0.03 ^a	0.41	0.22 ± 0.003 ^{b**}	0.06 ± 0.003 ^a	0.27	6.49 ± 0.71 ^a
	P61/0	2.73 ± 0.03 ^{a**}	1.11 ± 0.04 ^a	0.41	0.31 ± 0.012 ^{a**}	0.07 ± 0.003 ^a	0.23	7.20 ± 0.26 ^a

Note: All data in the table are expressed as the mean ± standard error ($n = 4$). Different lowercase letters indicate the differences between treatments at a significance level of 5%. Longitudinal comparison. ** denotes a significant difference between the P+ side and the P– side at the 1% level, respectively.

3.2. Effect of P Supply on the Activity of Nitrogenase and the Nodule Ureide Content in Soybean Plants with Dual-Root Systems

Table 3 shows the specific nitrogenase activity (SNA) and acetylene reduction activity (ARA) on both sides of the dual-root soybean plants under different levels of P supply at the V4 and R1 stages. As Table 3 shows, both ARA and SNA increased with the P supply and showed significant differences between treatments on both sides of the dual-root system of soybean plants at the V4 and R1 stages. At the V4 stage, ARA and SNA on the P+ side increased by 2288.00% and 29.00%, respectively, under the P31/0 treatment when compared with P1/0, and by 52.43% and 19.44%, respectively, under the P61/0 treatment when compared with P31/0. On the P– side, P31/0 increased ARA and SNA by 383.33% and 16.11%, respectively, compared with P1/0, and P61/0 increased them by 36.21% and 10.46%, respectively, when compared with P31/0. At the R1 stage, ARA and SNA on the P+ side increased by 1568.33% and 56.12%, respectively, under P31/0 treatment, compared with P1/0, and by 60.64% and 10.23%, respectively, under P61/0, compared with P31/0. On the P– side, P31/0 increased ARA and SNA by 218.64% and 11.44%, respectively, compared with P1/0, and P61/0 increased them by 50.00% and 15.78%, respectively, compared with P31/0. The change in SNA with the enhancement in P supply was less prominent than that of ARA, suggesting that the decrease in ARA mainly resulted from the decreases in nodule dry weight and number. In addition, ARA and SNA did not show significant differences between the P+ and P– sides under the P1/0 treatment, but they were markedly higher on the P+ side than on the P– side, and the differences were extremely significant under the P31/0 and P61/0 treatments. The P–/P+ ratio of ARA was 0.96–0.98 under the P1/0 treatment and only 0.17–0.19 under the P31/0 and P61/0 treatments. The P–/P+ ratio of SNA was greater than 0.75 under all treatments. These results indicate that P deficiency could significantly inhibit ARA and suppress SNA less markedly in soybean.

Table 3. Effects of P supply on ARA and SNA in dual-root soybeans.

Treatments	ARA				SNA				
	P+(C ₂ H ₄ μmol ⁻¹ h ⁻¹ Plant ⁻¹)	P-(C ₂ H ₄ μmol ⁻¹ h ⁻¹ Plant ⁻¹)	IP–	P–/P+	P+(C ₂ H ₄ μmol ⁻¹ g ⁻¹ h ⁻¹)	P-(C ₂ H ₄ μmol ⁻¹ g ⁻¹ h ⁻¹)	IP–	P–/P+	
V4	P1/0	0.25 ± 0.01 ^c	0.24 ± 0.01 ^c	383.33	0.96	25.76 ± 0.87 ^c	24.96 ± 0.47 ^c	16.11	0.97
	P31/0	5.97 ± 0.04 ^{b**}	1.16 ± 0.06 ^b		0.19	33.23 ± 0.31 ^{b**}	28.98 ± 0.54 ^b		0.87
	P61/0	9.10 ± 0.35 ^{a**}	1.58 ± 0.01 ^a	36.21	0.17	39.69 ± 1.27 ^{a**}	32.01 ± 0.17 ^a	10.46	0.81
R1	P1/0	0.60 ± 0.01 ^c	0.59 ± 0.02 ^c	218.64	0.98	29.81 ± 0.49 ^c	29.81 ± 1.18 ^c	11.41	1.00
	P31/0	10.01 ± 0.17 ^{b**}	1.88 ± 0.07 ^b		0.19	46.54 ± 0.34 ^{b**}	33.21 ± 0.82 ^b		0.75
	P61/0	16.08 ± 0.76 ^{a**}	2.82 ± 0.16 ^a	50.00	0.18	51.30 ± 0.90 ^{a**}	38.45 ± 0.65 ^a	15.78	0.75

Note: All data in the table are expressed as the mean ± standard error ($n = 4$). Different lowercase letters indicate the differences between treatments at a significance level of 5%. Longitudinal comparison. ** denotes a significant difference between the P+ side and the P– side at the 1% level, respectively. IP– is the increment of P–.

Table 4 shows the ureide content of root nodules at the V4 and R1 stages in soybean plants supplied with different levels of P. Table 4 also reveals that nodule ureide content at the V4 and R1 stages showed a changing trend similar to those of SNA and ARA, shown in Table 3—namely, they increased with P supply and varied significantly between treatments on both sides. At the V4 and R1 stages, the ureide content on the P+ side increased by 234.31% and 281.03%, respectively, when the P supply was increased from P1/0 to P31/0. The ureide content on the P+ side increased by 7.92% from P31/0 to P61/0 at the V4 stage and decreased by 0.90% at the R1 stage. On the P− side, the ureide content at the V4 and R1 stages increased by 118.18% and 156.44% from P1/0 to P31/0 and by 6.02% and 9.27% from P31/0 to P61/0, respectively. In addition, the ureide content did not show any significant difference between the P+ and P− sides under the P1/0 treatment, but it was extremely significantly higher on the P+ side than on the P− side under the P31/0 and P61/0 treatments. The P−/P+ ratio of the ureide content was 0.97–0.87 under the P1/0 treatment and only 0.59–0.65 under the P31/0 and P61 treatments. The results indicate that P deficiency can severely inhibit the synthesis of ureides in soybean and more markedly increase the number of nodules that directly contact P.

Table 4. Effect of P supply on the ureide content in nodules of dual-root soybeans.

Treatments	Ureide Content (mg/g DW)		IP-(%)	P−/P+	
	P+	P−			
V4	P1/0	1.02 ± 0.02 ^c	0.99 ± 0.04 ^b	118.18	0.97
	P31/0	3.41 ± 0.08 ^{b**}	2.16 ± 0.08 ^a	6.02	0.63
	P61/0	3.68 ± 0.11 ^{a**}	2.29 ± 0.05 ^a		0.62
R1	P1/0	1.16 ± 0.01 ^b	1.01 ± 0.01 ^b	156.44	0.87
	P31/0	4.42 ± 0.09 ^{a**}	2.59 ± 0.13 ^a	9.27	0.59
	P61/0	4.38 ± 0.03 ^{a**}	2.83 ± 0.11 ^a		0.65

Note: All data in the table are expressed as the mean ± standard error ($n = 4$). Different lowercase letters indicate the differences between treatments at a significance level of 5%. Longitudinal comparison. ** denotes a significant difference between the P+ side and the P− side at the 1% level, respectively. IP− is the increment of P−.

3.3. Effect of P Supply on the Ultrastructure of Soybean Plants with Dual-Root Systems Root Nodules

Rhizobial infection of host cells is the first step in nodule development. Host cells can be classified as infected cells (ICs) and uninfected cells (UCs). Figure 2A shows a cross-section of the nodule ultrastructure under a transmission electron microscope (4000×) at the V4 stage on both sides of the dual-root soybean plants with different levels of P supply. A treatment comparison revealed that the number of ICs was largest under the P31/0 treatment and smallest under P1/0. There was a very slight difference in the area of nodule infection between P31/0 and P61/0. In contrast, the number of UCs showed the opposite changing pattern with the increase in P supply: it was largest under P1/0 and smallest under P31/0, and the number was slightly larger under P61/0 than under P31/0. In addition, there were more ICs on the P+ side than on the P− side. Rhizobia enter the host cell through an infection thread to form a bacteroid (Bt), and the rhizobia that have successfully invaded the host cells can divide to form multiple new Bts. Figure 2B shows that the number of Bts at the V4 stage was counted on the cross sections of nodules under a transmission electron microscope (20,000×) on both sides of the dual-root soybean plants with different levels of P supply. The number of Bts in root nodules was smallest under the P1/0 treatment and largest under the P31/0 treatment, and it was slightly smaller under P61/0 than under P31/0. In addition, the nodules on the P+ side had more Bts than those on the P− side. The results show that the numbers of ICs and Bts on both sides of the soybean root systems can be significantly affected by supplying P to only one side, and the

changes increase with the increase in P supply. The effect of P supply on the number of IC, UC, and Bt nodules in double-root soybeans is shown in Table S5.

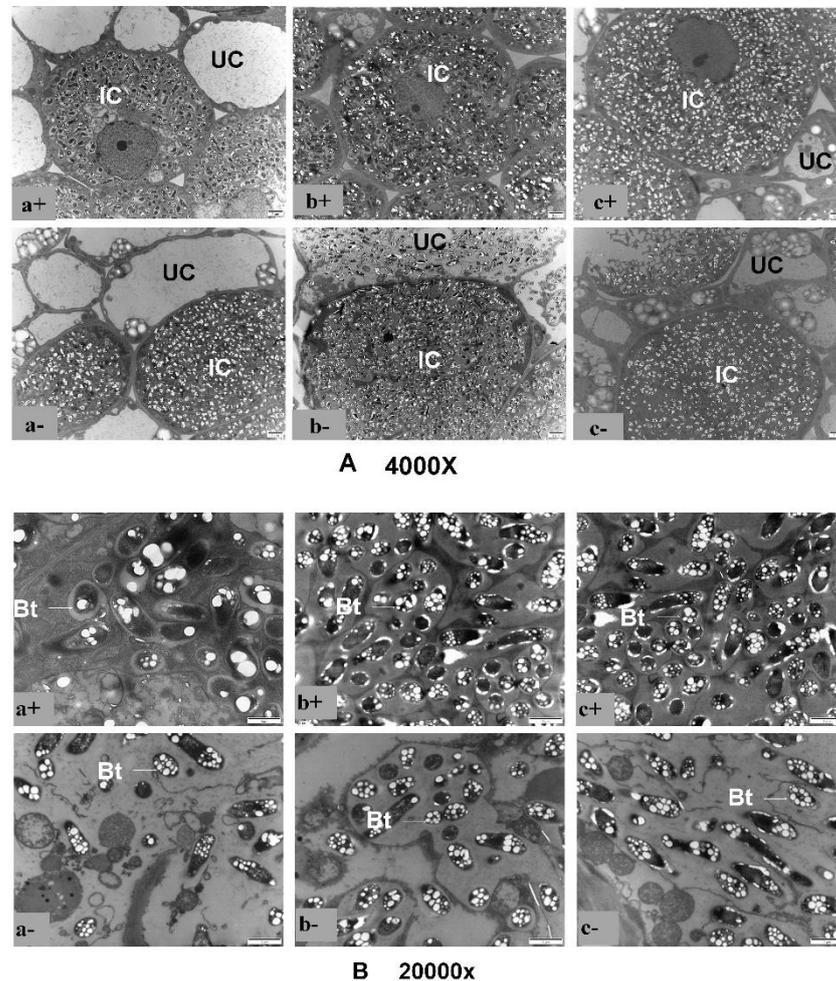
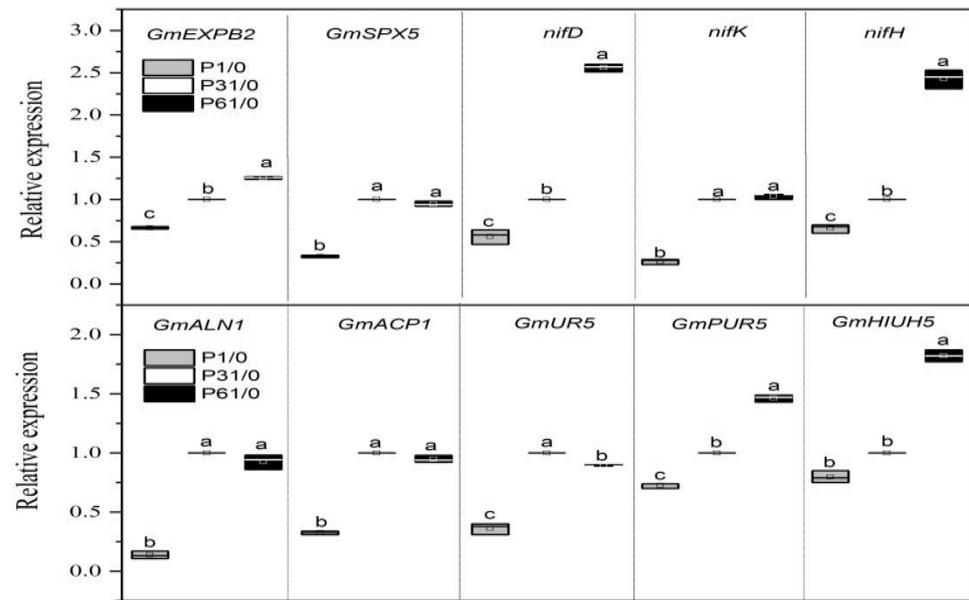


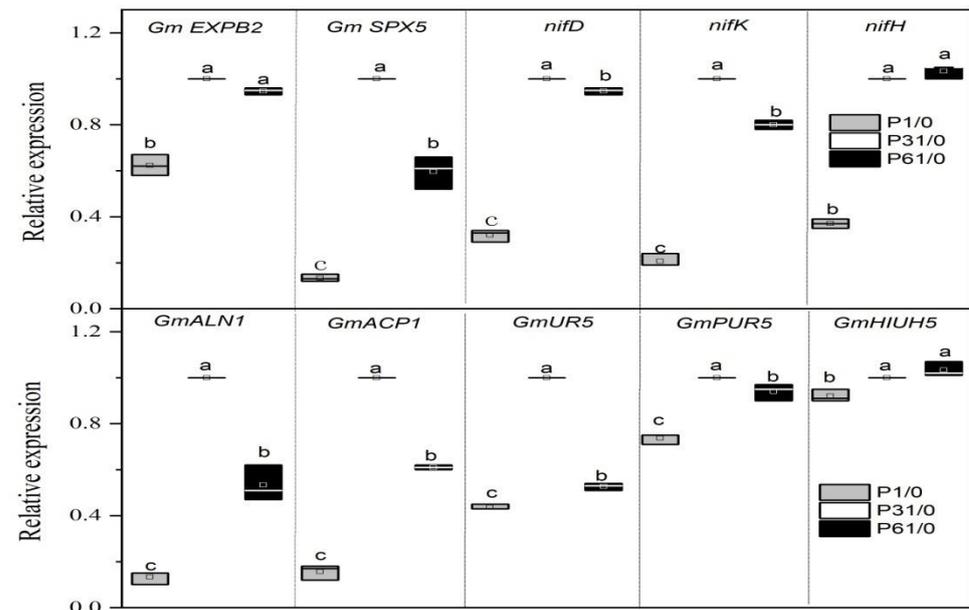
Figure 2. Ultrastructure of nodules on both sides of a dual-root soybean plant at V4 stage: (A) is under a transmission electron microscope 4000 \times , (B) is under a transmission electron microscope 20,000 \times ; a+: P1/0 P+; a-: P1/0 P-; b+: P31/0 P+; b-: P31/0 P-; c+: P61/0 P+; c-: P61/0 P-; IC: infected cell; UC: uninfected cell; Bt: bacteroid.

3.4. Effect of P Supply on the Expression of Key Genes Related to Nodulation and Nitrogen Fixation in Soybean Plants with Dual-Root Systems

Figure 3 shows the relative expression levels of key genes related to nodulation and nitrogen fixation in nodules on both sides of the dual-root soybean plants with different levels of P supply. Figure 3A shows that with P31/0 as a control, the expression levels of *GmEXPB2*, *nifD*, *nifH*, and *GmPUR5* on the P+ side increased significantly with an enhanced P supply; *GmSPX5nifK*, *GmALN1*, and *GmACP1* expressions were higher under P31/0 than under P1/0, and the difference in P31/0 vs. P61/0 was not significant. *GmHIUH5* expression was significantly higher for P61/0 than P31/0, and the difference between P31/0 and P1/0 was not significant; *GmUR5* was significantly higher for P31 than P61/0, and P61/0 was significantly higher than P1/0. In Figure 3B, with P31/0 as a control, the gene expression levels are presented.

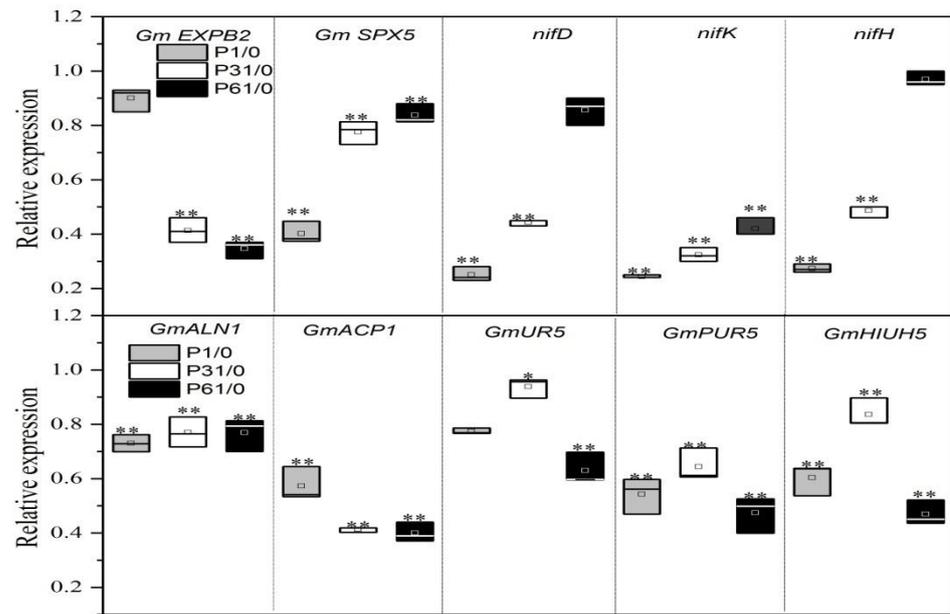


(A)



(B)

Figure 3. Cont.



(C)

Figure 3. (A) Relative gene expression on the phosphorus-application side (the P31/0 as control). (B) Relative gene expression on the phosphorus-free side (the P31/0 as control). (C) Relative expression of phosphorus-free genes side (phosphorus-application side as a control). Effects of P supply on the relative expression levels of key genes related to nodulation and nitrogen fixation on both sides of the dual-root soybean. In (A,B), different lowercase letters indicate the significant differences between treatments at the 5% level. In (C), *,** denotes a significant difference between the P+ side and the P− side, with P** indicating a significant difference at the 1% level; *GmEXPB2* is nodulation-related key gene; *nifD*, *nifH*, and *nifK* are regulatory key genes of nitrogen fixation; *GmALN1*, *GmACP1*, *GmUR5*, *GmPUR5*, *GmHIUH5*.

The expression levels of *GmEXPB2*, *GmSPX5*, *nifD*, *nifK*, *GmALN1*, *GmACP1*, *GmUR5*, and *GmPUR5* on the P− side showed a trend of first increasing and then decreasing with the increase in P supply. All three gene expression levels increased significantly when the P supply increased from 1 mg/L to 31 mg/L; however, after the P supply reached 31 mg/L, the *GmEXPB2*, *nifH*, and *GmHIUH5* expression levels changed nonsignificantly, and *GmSPX5*, *nifD*, *nifK*, *GmALN1*, *GmACP1*, *GmUR5*, and *GmPUR5* expression levels markedly decreased with the increase in P supply. In Figure 3C, compared with the P+ side (1.0), gene expression levels on the P− side were all low. In addition, the relative *GmEXPB2* and *GmACP1* expression decreased with the increase in P supply, showing an extremely significant difference between the two sides under P31/0 and P61/0 and no significant difference under P1/0. *GmACP1* showed highly significant differences at all phosphorus supply levels. All expression levels of *GmSPX5*, *nifD*, *nifK*, and *nifH* increased with the increase in P supply. *nifD* and *nifH* showed extremely significant differences between the two sides under P1/0 and P31/0 but no significant difference under P61/0, while *GmSPX5* and *nifK* showed an extremely significant difference between the two sides under all levels of P supply. Gene expressions of *GmUR5*, *GmPUR5*, and *GmHIUH5* tended to increase and then decrease with increasing phosphorus supply levels. *GmEXPB2* showed highly significant differences at the phosphorus supply levels of P31/0 and P61/0, and no significant differences at P1/0, while *GmPUR5* and *GmHIUH5* showed highly significant differences at all phosphorus supply levels. One-sided P supply significantly affected the relative expression levels of genes regulating nodulation and nitrogen fixation in soybean, and the demand for P to meet the needs of gene expression on the P+ side was greater than that on the P− side.

4. Discussion

4.1. Effects of P Supply on Nodulation and Nitrogen Fixation in Soybean Plants

Increasing a soybean's P supply can significantly increase its dry weight, the number of nodules [10,13,34], and nodule nitrogenase activity [5,35,36]. In this study, we applied the grafting approach for soybean seedlings developed by Xia et al. [24] to prepare a dual-root system of soybean plants, which received different concentrations of P nutrient solution at the root system on the phosphorus-application side and phosphorus-free nutrient solution on the phosphorus-free side. We found that the increase in P supply significantly enhanced the dry weight, ARA, and SNA of nodules on the phosphorus-application side and indirectly affected these parameters on the phosphorus-free side of the dual-root soybeans, and the increases were more marked in the roots and nodules on the side in direct contact with a P supply. Le Roux et al. [18] found that low P treatment significantly reduced the content of ureides in soybean nodules. Magadlala et al. [37] found that the ureide content decreased in the nodules but increased in the roots of the legume tree after low P treatment. The present study found that increasing the P supply on only one side increased the ureide content in nodules on both sides of the dual-root soybeans, further confirming that P supply systemically affects nitrogen fixation in soybean nodules.

Isidra-Arellano et al. [38] reported that low P treatment reduced the number of curled root hairs and reduced the relative gene expression levels of *PvNSP2*, *PvNIN*, and *PvFLOT2*, which control the formation of infection threads in common beans. Gentili et al. [39] found that P at a moderate concentration most significantly stimulated cell divisions in the cortex, nodule primordia emergence, and initial nodule emergence in the roots of *Alnusincana*. In the present study, by observing the nodule ultrastructure of dual-root soybeans at the V4 stage, we found that as the P supply increased, the formation of nodule Bts and the number of ICs both increased. *GmEXPB2* and *GmSPX5* are associated with the formation and extension of cell walls during nodule formation and development in soybean [5,40]. Li et al. [40] found that high P treatment increased the relative expression of the *GmEXPB2* gene in the nodules, but this effect was reversed after 14 days of treatment. Zhou et al. [41] found that P supply increased the relative gene expression level of *GmEXPB2* in roots and leaves of transgenic soybean. *GmSPX1* has been documented to exhibit no response to Pi starvation in soybean roots [42]. In the present study, the relative *GmEXPB2* and *GmSPX5* expression levels in soybean nodules were increased by a high P supply and decreased by a low P supply, which is consistent with previous studies. Nitrogenase is encoded by the nitrogen fixation gene *nif*. In a nitrogenase system, *nifD* and *nifK* are the structural genes encoding the MoFe protein subunit of the nitrogenase complex, and *nifH* encodes ferritin [43,44]. Nasr Esfahani et al. (2016) [45] stated that low P treatment reduces the relative expression levels of *nifH* and *nifK* and suppresses nitrogenase activity in nodules. Sulieman et al. [32] reported that low P treatment reduced the number and dry weight of root nodules and increased the relative expression levels of *nifH*, *nifD*, and *nifK* in soybean nodules. Our finding that low P supply reduced the relative expression levels of *nifH*, *nifD*, and *nifK* in soybean nodules contrasts with the findings of Sulieman et al. [32]. The inconsistency might be attributed to the fact that in this study, low P supply decreased ARA and SNA and, thus, suppressed the expression of genes regulating nodulation and nitrogen fixation in nodules. *GmALN1* (Allantoinase 1), *GmACP1* (Acid Phosphatase), *GmUR5* (AIRS synthetase), *GmPUR5* (AIRS synthetase), and *GmHIUH5* (hydroxyisourate hydrolase) are the key genes for ureide synthesis, and they are significantly expressed in soybean nodules [33]. Alamillo et al. [46] found that ALN gene expression also increased in *Phaseolus vulgaris* L. roots and in shoots in response to drought. Díaz-Leal et al. [47] found that nitrate stress reduced the ALN gene expression in roots, stems, and leaves of *Phaseolus vulgaris* L. The present study found that increasing the P supply on only one side increased the key genes for ureide synthesis in nodules on both sides of dual-root soybeans. It is worth mentioning that increasing the P supplied to only one side of the dual-root soybeans promoted the formation of nodule Bts, increased the number of ICs, and elevated the relative expression of regulatory genes for nodulation and nitrogen fixation (*GmEXPB2*, *GmSPX5*, *nifH*, *nifD*,

nifK, *GmALN1*, *GmACP1*, *GmUR5*, *GmPUR5*, and *GmHIIUH5*) on the phosphorus-free side in our study. From the perspectives of nodule structure and relative key gene expression, our findings further confirm that the local P supply can systemically regulate nodulation and nitrogen fixation in soybean.

4.2. Effects of P Supply Level on P Absorption and Transport in Soybean Plants

The level of P supply can affect the P absorption of soybean plants [35,48,49]. The P contents in the aboveground tissues and nodules of soybean increase with the increase in P supply [50]. In a study that supplied soybean plants with 0.005 and 0.025 mM KH_2PO_4 , Georgiev and Tsvetkova [51] found that treatment with 0.025 mM KH_2PO_4 increased the P content in the root system but reduced the P content in the shoots of the plants. In the present study, applying high concentrations of P to only one side significantly increased the P content in the shoots, roots, and nodules on both sides of dual-root soybean plants. Under low P stress, nodules are prioritized for receiving P to ensure their growth [4,52,53]. In a study conducted by Qin et al. [8], where 5 or 250 μM KH_2PO_4 was applied to soybean roots under hydroponic conditions and ^{33}P -labeled KH_2PO_4 was applied to the bottom of the root area without nodules, 5 μM KH_2PO_4 treatment resulted in a significantly higher ^{33}P content in soybean nodules than 250 μM KH_2PO_4 treatment. Al-Niemi et al. [54] applied 0 and 5 mM KH_2PO_4 to common bean plants that had been supplied with 0.75 mM KH_2PO_4 until the V2 stage and then applied ^{33}P -labeled KH_2PO_4 to the bottom area of the roots without nodules or to the entire root system. They found that P-free treatment promoted the transport of ^{33}P from the roots and nodular surface into the nodules. In the present study, we also found that P transported from the aboveground tissues to the root system on the P– side might be prioritized for root nodules to maintain their function.

Using the root separation method to apply P to only one side of subterranean clover and *Lupinus albus*, Scott et al. [22] Shu et al. [21] found that P uptake in the roots was enhanced on only the P-supply side and remained stable on the non-P-supply side. Burleigh et al. [55], Shen et al. [25], and Wouterlood et al. [56] applied high concentrations of P to one side and a low concentration or no P to the other side of chickpea, *Lupinus albus* and *truncatula* plants, respectively, using the root separation method, and they found that the P uptake by the roots was enhanced on both sides. Compared with both sides of the roots supplied with phosphorus, the treatment with no phosphorus supply on one side of the roots inhibited *Phaseolus vulgaris* root growth on the phosphorus supply side (Bonser et al. [57]), while low levels of phosphorus on one side of the roots and normal levels of phosphorus on the other side inhibited normal phosphorus supply soybean root nodule growth on the low-level phosphorus supply side; however, the relative expression levels of genes PvNIN, PvRIC1, and PvRIC2, which regulate the formation of nodules by rhizobial symbiosis, were only upregulated on the low-phosphorus side, according to Isidra-Arellano et al. [58]. In our study, after supplying different concentrations of P for a long time on one side of the dual-root soybeans, the P content in the roots and nodules on the P– side significantly increased with the increase in P supply to the P+ side, which is different from the results of Scoot et al. [22] and Shu et al. [21]. Our experiment was conducted under sand culture conditions to eliminate soil phosphorus interference, which may account for the differences in the results.

5. Conclusions

1. In the dual-root soybean plants supplied with P on only one side, the nodule weight, ARA, SNA, ureide content, the number of Bts, the number of ICs, and relative expression levels of key genes related to nodulation and nitrogen fixation (*GmEXPB2*, *GmSPX5*, *nifH*, *nifD*, *nifK*, *GmALN1*, *GmACP1*, *GmUR5*, *GmPUR5*, and *GmHIIUH5*) on the P+ side increased with the increase in P supply during the VC-R1 period. Those on the P– side showed the same pattern as those on the P+ side but less prominently. Therefore, P supply regulates soybean nodulation and nitrogen fixation.

2. When the level of phosphorus supply was increased from 1 mg/L to 31 mg/L, the increase on the P⁻ side root was significant, and nodule phosphorus content increased by 57.14–85.71% and 68.75–75.00%; ARA and SNA were 218.64–383.33% and 11.41–16.11%, respectively, and ureide content was 118.18–156.44%. When the level of phosphorus supply was increased from 31 mg/L to 61 mg/L, the increases in the regulation ability of the root and nodule phosphorus content were only 0.00–9.09% and 25.00–29.63%, respectively; ARA and SNA were 36.21–50.00% and 10.46–15.78%, respectively, and ureide content was 6.02–9.27%.
3. When the dual-root soybean plants were supplied with different concentrations of P on one side, the P content in the roots and nodules on the P⁻ side increased with the increase in P supply. These findings show that P can be transported to the roots and nodules on the non-supplied side through the shoots, and the nodules are prioritized to receive P.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11112354/s1>, Table S1: The elements of P or K in different P level treatments, Table S2: Concentrations of elements in nutrient medium of the sand culture, Table S3: Primer sequences of key genes for nodulation and nitrogen fixation in soybean, Table S4: Effect of phosphorus supply on the number of nodules in double-root soybeans, Table S5: Effect of P supply on the number of IC, UC, and Bt in nodules of double-root soybeans.

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