Hanguk Nonghwahak Hoechi
(J. Korean Agric. Chem. Soc.)
35(6), 449~456(1992)

The effect of phosphorus stress on the energy status and bacteroid content in soybean nodules

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Abstract : Expermient were conducted to determine the effect of phosphorus stress on bacteroid content and energy status of soybean (*Glycine max* [L.] Merr.) nodules. Plants inoculated with *Bradyrhizobium japonicum* MN 110 were grown with P-stressed (0.05 mM-P) and control (1 mM-P) treatment in the greenhouse. Phosphorus stress decreased nodule mass per plant and nodule mass to whole plant mass ratio. Phosphorus concentration in leaf, stem and root tissues were reduced by 75% but in nodule tissue was reduced only by 40% under phosphorus stress during 3 week experimental period. The bacteroid content per unit nodule mass and the distribution of total nitrogen and total phosphorus among the bacteroid and plant cell fractions of nodule were not affected significantly by phosphorus stress. Regardless of phosphorus treatment, 22% of the nitrogen and 27% of the phosphorus in whole nodules were associated with the bacteroid fraction. The ATP and total adenylate concentrations in and energy charge of whole nodule were decreased 77%, 46% and 37%, respectively, by phosphorus stress. The ATP concentration in and energy charge of the host plant cell fraction of nodules were decreased 86% and 59%, respectively, but these parametres in bacteroid in nodules were not affected by phosphorus stress. These results indicated that nodule is a strong phosphorus sink and that nodule growth and development are regulated to maintain a high phosphorus and energy content in bacteroid even when the host plant is subjected to phosphorus deficiency(Received August 11, 1992, accepted October 22, 1992).

Phosphorus has an important role in nodule growth and function as well as host plant growth. An improvement in phosphorus status of several legumes including soybeans, has been reported to increase nitrogen concentration in plant tissues as well as overall plant growth.[1,2] Increasing the phosphorus supply available to stylosanthes and soybean plants from deficient to sufficient levels has been shown to increase nodule number, mass and relative growth rate.[3,4] These responses are consequences of nodule growth, development and functioning being stimulated to a greater degree by improvement in phosphorus nutrition than host plant growth. Phosphorus deficiency has also been shown to affect nodule function. Gates[4] observed that increasing phosphorus supply from deficient to sufficient levels increased whole plant nitrogen accumulation rate of *Stylosanthes humilis* plant dependent on symbiotic dinitrogen fixation from 17 to 53 mg N per gram nodule dry weight per day during the 23 to 26 day interval after inoculation. Bethlenfalvay and Yoder[5] reported that increasing available phosphorus concentration from 20 to 500 μM resulted in a 5.6 fold increase in the specific acetylene re-

Key words : Phosphorus, bacteroid, energy charge
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duction activity of soybean nodules.

The amount of metabolically available energy in the adenylate pool is expressed by the energy charge concept.[6,7] This parameter ([ATP]+1/2[ADP]/[ATP]+[ADP]+[AMP]) is useful in determining the effect of energy state of the cell on metabolic regulation. Oxygen enrichment[8] stimulated the nitrogenase activity, ATP concentration and energy charge of nodule, whereas, subjecting the host plant to water stress[9] and dark treatment[10] decreased these parameters of the nodules.

Nitrogen fixation in the bacteroids and ammonia assimilation in the host plant cell fraction of nodules are both energy consuming processes. Therefore, if phosphorus deficiency altered the energy status of nodules, it could also alter the distribution of energy between the bacteroid and host plant cell fraction. Hence, the objective of experiments described here was to test these predictions by examining the effect of phosphorus deficiency on adenylate concentrations and energy charge of whole soybean nodules and the distribution of nitrogen, phosphorus and adenylate among bacteroid and host plant cell fractions of soybean nodules.

Materials and Methods

Plant culture and inoculation

Soybean (*Glycine max* [L.] Merr.) plants were grown in greenhouse pot culture. Nutrient solutions were prepared in tap water and the composition of nutrient solution was same as described by Israel.[1] Nutrient solution used for the deficient treatment contained 0.05 mM-P and that for the control treatment contained 1.0 mM-P. Roots of seedlings (3 day old) were dipped in the fresh inoculum (10^9 colony forming units/m*l*) of *Bradyrhizobium Japonicum* MN 110 just before transplanting. Immediately after transplanting, 0.5 m*l* of inoculum was applied at the base of each seedling. Other growth conditions were the same as deseribed before.[1] Plant material was harvested at the 28, 35, 42 and 49 days after transplanting (DAT). Plants were separated into leaflet, stem plus petiole, root and nodule fractions. Nodules were detached from roots as rapidly as possible (15 to 30 min) and frozen in liquid nitrogen and stored at −80℃. All plant material was dried at 65℃ for 72 h, weighed and ground to pass through a 1 mm screen.

A second group of plants with the same phosphorus treatments (0.05 mM-P and 1.0 mM-P) was grown to obtain nodule tissue for measurement of adenylate concentrations in whole nodules and isolated bacteroids. Nodules were detached at 40 DAT and frozen in liquid notrogen and stored at −80℃ until extraction and analysis of adenylates could be accomplished.

Adenylate extraction and quantitation

Adenylates were extracted from nodules using the methanol/trichloroacetic acid (TCA) method of Khym.[11] Nodules were weighed (0.8～1.2 g) and placed into a 30 m*l* centrifuge tube containing a cold (4℃) solution of 30% (v/v) methanol and 8% (w/v) trichloroacetic acid. After removing TCA from the supernatant by partitioning into tri-*n*-octylamine/Freon-TF solution, ATP was determined by the luciferin-luciferase assay system.[9] The luciferin-luciferase system was also used to measure ADP and AMP in the extract following enzymatic conversion to ATP. Pyruvate kinase was used to catalyze the phospho-enol-pyruvate dependent conversion of ADP to ATP. Myokinase, which utilized endogenous ATP to convert AMP to ADP, and pyruvate kinase were used to convert ADP to ATP. Samples subjected to enzymatic conversion reactions were incubated at 35℃ for 30 min.

Bacteroid isolation

Nodules were surface sterilized in 80% ethanol for 15 seconds and rinsed with cold redistilled water. Nodules were homogenized in Polytron for 30 seconds in 15 m*l* of 20 mM HEPES buffer (pH 6.9) containing 5% (v/v) polyvinyl pyrollidone (PVP), 10 mM sodium ascorbate, 10 mM glucose and 100 μg/m*l* chloramphenicol. This suspension was filtered through four layers of cheezecloth of remove nodule cortex tissue and the filtrate was centribuged at 12,000 g for 10 min, at 4℃. The pellet of bacteroids was resuspended and centrifuged again

in the same buffer (10 m*l*) lacking the PVP. To measure bacteroid adenylate concentration, 2.0 m*l* of bacteroid suspension was mixed with 10 m*l* methanol/TCA solution to extract adenylates. All other steps were the same as those described for adenylate measurement of whole nodules. The adenylate concentration in nodule host cells were estimated by measuring adenylates in a subsample of whole nodules and in bacteroid isolated from a second subsample of nodules from the same plant.

Nitrogen and phosphorus determination

The total nitrogen concentration of nodule samples (100~200 mg) was determined by Kjeldahl procedure that included a salicylic acid predigestion step to convert nitrate to ammonia.[12] After alkalinization of digests, ammonia was steam distilled into boric acid and quantified by titration with potassium biiodates. Appropriate aliquots of diluted Kjeldahl digest were analyzed for total phosphorus by the ammonium molybdate method of Murphy and Riley.[13]

Total nitrogen and phosphorus in isolated bacteroids were determined by the same procedures. The nitrogen and phosphorus concentration in the host plant cell fraction on a dry weight basis was then calculated in same manner as described for adenylate concentration.

Experimental Design and Statistical Analysis

A randomized complete block design with 3 replications was used. All combinations of treatment and time were assigned randomly within each block. The data were analyzed by the ANOVA procedure of Statistical Analysis System.

Results

Total dry mass at 28 DAT was not significantly affected by phosphorus deficiency but total dry mass of phosphorus deficient plants was only 30% of that of control plants at 49 DAT (Table 1). The total dry mass of control plants increased 8 fold between 28 and 49 DAT, but it increased only 2.5 fold in phosphorus-stressed plants during same period. Nodule dry mass per plant was decreased 60% and 83% by phosphorus deficiency at 28 and 49 DAT, respectively. Over the experminental period the ratio of nodule mass to whole plant mass ranged from 0.052 to 0.074 for control plants and 0.028 to 0.038 for phosphorus-deficient plants. This indicated that phosphorus deficiency restrict nodule growth to a greater degree than host plant growth.

Phosphorus deficiency decreased phosphorus concentrations in leaf, stem and root tissue by about 75% during the experimental period, but it reduced phosphorus concentration in nodules by

Table 1. The effect of phosphorus deficiency on whole plant dry mass, nodule dry mass and nodule mass to plant mass ratio in vegetative soybean plants

DAT	Whole plant mass (g/plant)		Nodule mass (g/plant)		Nodule mass / Whole plant mass	
	1.0 mM-P	0.05 mM-P	1.0 mM-P	0.05 mM-P	1.0 mM-P	0.05 mM-P
28	1.98	1.66	0.15	0.06	0.074	0.038
	(0.01)	(0.12)	(0.02)	(0.01)	(0.010)	(0.006)
35	5.63	2.63	0.33	0.08	0.060	0.029
	(1.24)	(0.37)	(0.05)	(0.01)	(0.007)	(0.006)
42	8.86	3.92	0.46	0.11	0.052	0.028
	(0.47)	(0.80)	(0.05)	(0.01)	(0.003)	(0.006)
49	16.87	5.36	0.89	0.15	0.055	0.028
	(1.47)	(0.47)	(0.10)	(0.05)	(0.002)	(0.006)
$LSD_{0.05}$	1.08		0.038		0.006	

( ): Represent standard error (n=3)

only 40% (Fig. 1). Phosphorus concentrations in nodules were constant in each treatment at 7 mg P /GDW in nodules of control plants and 4 mg P/ GDW in nodules of phosphorus-deficient plants, and these values were higher than those in any other tissues (Fig. 1).

The proportion of whole plant phosphorus in leaf and stem tissues of control plants were higher than that in phosphorus-deficient plants, whereas, roots and nodules of phosphorus-deficient plants contained a higher proportion of whole plant phosphorus than those of control plants (Fig. 2).

The effects of phosphorus deficiency on the bacteroid content of nodules and phosphorus and nitrogen concentrations of bacteroid and nodule host cell fractions are illustrated in Table 2. Phosphorus deficiency had no significant effect on the bacteroid content of nodules, as bacteroid contents ranged from 150~163 mg/GDW in nodules of control plants and from 116~139 mg/GDW in nodules of phosphorus-deficient plants over the experimental period. Phosphorus concentrations in bacteroids in each treatment were constant throughout the experimental period. Averaging over sampling dates indicates that phosphorus deficiency reduced total phosphorus concentration in bacteroids by 27% relative to the control.

The nitrogen concentration in the bacteroids at 28 DAT was not affected by phosphorus deficiency, but phosphorus deficiency reduced nitrogen concentration in bacteroids by 18 to 25% from 35 to 49 DAT. The phosphorus and nitrogen concentrations in the host plant cell fraction of nodules were 49% and 56% of the respective concentrations in the bacteroids fraction of nodules of control plants, and 38% and 57% of the respective concentrations in the bacteroid fraction of nodules of phosphorus-deficient plants.

The phosphorus concentrations were always higher in host plant cell of the nodules than in

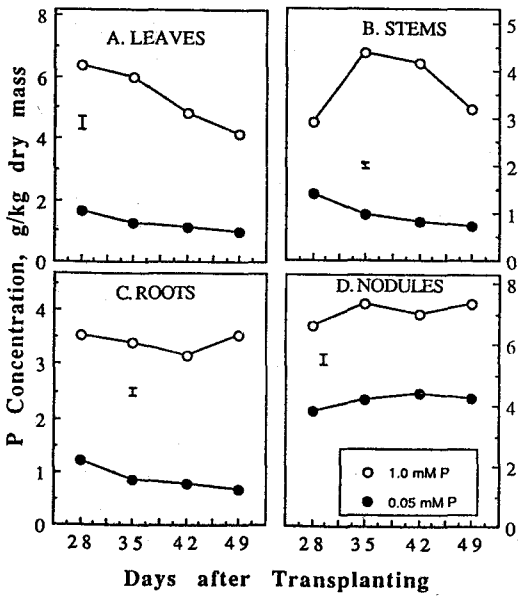

Fig. 1. The effect of phosphorus deficiency on phosphorus concentration in leaf, stem, root and nodule tissues of soybean plant.
Data points represent means of 3 replications. The treatment effects for A, B, C and D were significant ($p=0.0001$). The bars indicate $LSD_{0.05}$ values calculated on the basis of significant treatment effects and can be used to compare means at same sampling data.

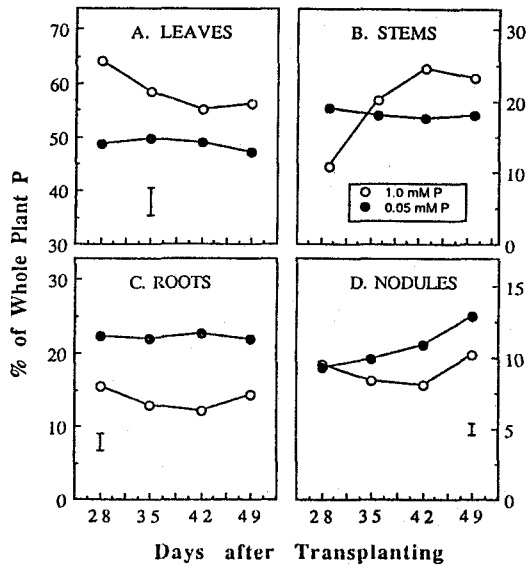

Fig. 2. The effect of phosphorus deficiency on the distribution of phosphorus among leaf, stem, root and nodule tissues.
Data points represent means of 3 replications. Treatment effects for A and C significant at $p=0.001$, and for D at $p<0.001$, but not significant for B ($p>0.05$). The bars indicate $LSD_{0.05}$ values calculated on the basis of significant treatment effects and can be used to compare means at the same sampling data.

Table 2. The effect of phosphorus deficiency on bacteroid content and on phosphorus and nitrogen concentrations in bacteroid and host plant cell fraction

DAT	Bacteroid content		Phosphorus concentration				Nitrogen concentration			
	mg-Bacteroid/ GDW nodule		Bacteroid mg-P/ GDW bacteroid		Plant cell mg-P/ GDW plant cell		Bacteroid mg-N/ GDW bacteroid		Plant cell mg-N/ GDW plant cell	
mM-P	1.0	0.05	1.0	0.05	1.0	0.05	1.0	0.05	1.0	0.05
28	149.7	115.5	12.36	9.28	5.56	3.07	100.2	100.5	61.2	48.2
35	152.0	119.2	11.94	8.67	6.48	3.62	102.0	79.2	51.6	53.5
42	152.4	138.6	12.50	8.94	5.93	3.69	103.3	80.0	62.4	49.3
49	163.3	133.2	13.07	9.45	6.22	3.47	110.7	84.6	60.0	46.3
Mean	154.4	126.6	12.46	9.09	6.05	3.46	104.1	86.1	58.8	49.3
$LSD_{0.05}$	NS		0.95		0.44		5.28		3.80	

Values represent means of 3 replications

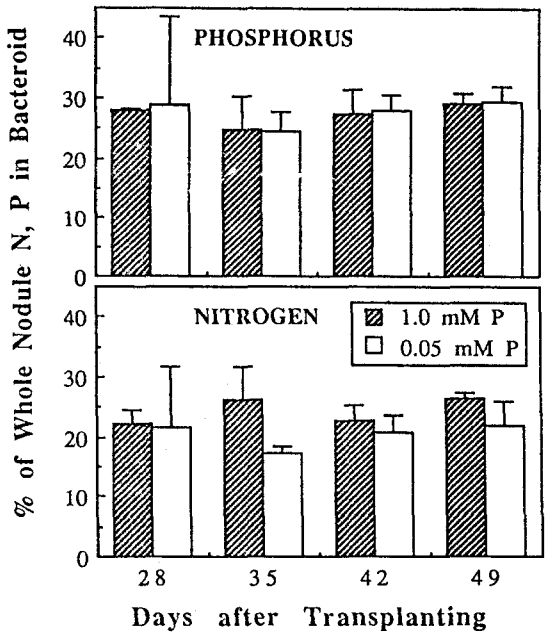

Fig. 3. The effect of phosphorus deficiency on the distribution of P and N in the whole nodule.
The effect of time, treatment and time by treatment interaction were not significant ($p<0.05$) for A or B.

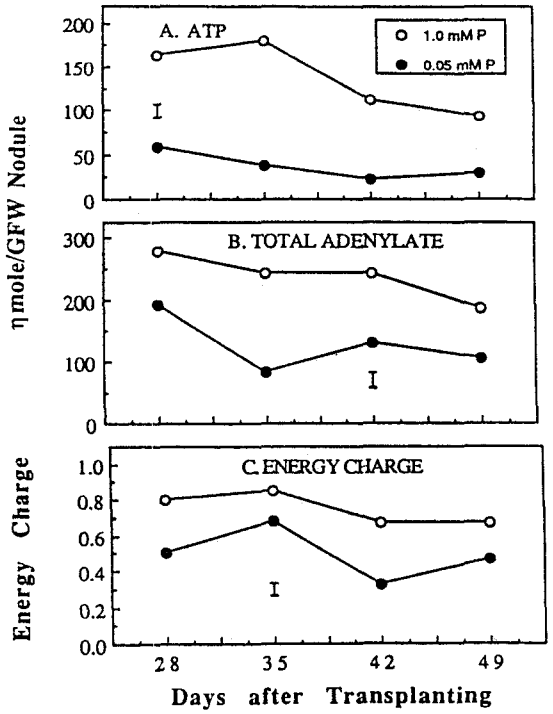

Fig. 4. The effect of phosphorus deficiency on the ATP and total adenylate concentration and energy charge of whole nodules.
Data points represent means of 3 replications. Treatment effects were significant for A, B and C ($p=0.0001$). The bars indicate $LSD_{0.05}$ values calculated on the basis of significant treatment effects and can be used to compare means at the same sampling date.

other plant tissues regardless of phosphorus treatment (Fig. 1 and Table 2). Phosphorus deficiency decreased phosphorus and nitrogen concentrations in host plant cells by an average of 43% and 16%, respectively, during the experimental period (Table 2). In both phosphorus treatments, 22% of the nitrogen and 27% of the phosphorus in whole nodules was associated with the bacteroid fraction during the 3 week experimental period (Fig. 3).

Table 3. The effect of phosphorus deficiency on distribution of adenylates in soybean nodules at 40 DAT

	1.0 mM-P			0.05 mM-P		
	Whole nodule	Bacteroid	Plant cell	Whole nodule	Bacteroid	Plant cell
	ηmole/GFW Nodule					
ATP	148.1	44.6	103.5	45.7	39.3	6.4
	(19.4)	(9.5)	(19.8)	(2.9)	(1.3)	(4.1)
ADP	102.0	15.3	86.7	48.4	7.7	40.7
	(2.7)	(2.8)	(5.3)	(4.2)	(3.5)	(2.3)
AMP	14.4	6.3	8.1	28.2	2.8	25.4
	(6.3)	(3.6)	(3.4)	7.2)	(1.2)	(5.6)
Total	264.7	66.4	198.3	122.3	49.8	72.5
	(16.2)	(5.8)	(22.6)	(1.5)	(2.7)	(4.2)
Energy	0.75	0.82	0.74	0.57	0.86	0.37
Charge	(0.03)	(0.10)	(0.03)	(0.03)	(0.03)	(0.06)

( ): Represents standard error (n=3)

Phosphorus deficiency significantly decreased ATP and total adenylate concentration in and energy charge of whole nodules throughout the experimental period (Fig. 4). The maximum values of total adenylate concentration and energy charge occurred at 28 DAT and 35 DAT, respectively. The effect of phosphorus deficiency on the distribution of adenylate in the bacteroid and host plant cell fraction of soybean nodules at 40 DAT is illustrated in Table 3. The bacteroid fraction of nodules from control plants contained 30% and 25% of the whole nodule ATP and total adenylate, respectively. In contrast, the bacteroid fraction of nodules from phosphorus-deficient plants contained 85% and 40% of the whole nodule ATP and total adenylate, respectvely. Phosphorus-deficiency reduced energy charge of whole nodules and the host plant cell fraction of nodules by 30% and 50%, respectively, but the energy charge in the bacteroid fraction was not altered by phosphorus deficiency.

Discussion

Nutrient concentration in an organ depends on its sink strength for a given nutrient. Nodules have typically been shown to be very strong sinks for phosphorus when symbiotic plants are grown under phosphorus-limited conditions.[1,5,14] In this study, phosphorus concentration of nodules was effected less by phosphorus deficiency than that of any other organ (Fig. 1). The proportion of whole plant phosphorus in nodules was higher for phosphorus-deficient plant than for non-stressed control plant (Fig. 2). These results indicate that the nodule is a strong sink for phosphorus. Jacobson[15] suggested that the high sink strength of nodules for phosphorus is associated with nitrogenase having a very high demand for ATP and with nodules having a high microbial biomass content. Microbial cells typically have a higher phosphorus concentration than plant cells.

In this study, the phosphorus concentration in the bacteroid fraction was 2~3 times higher than that in host plant cell fraction (Table 2). Even though the phosphorus concentration of the host plant cell fraction of nodules was lower than that of the bacteroid fraction, it was always higher than in any other plant organ. Phosphorus concentrations in the host plant cell fraction of nodules were less affected than any other plant tissue by phosphorus deficiency. Thus both bacteroid and plant cell fractions of nodules are strong phosphorus sinks. It is apparent that allocation of phosphorus within the whole soybean plant is regulated to insure sufficient phosphorus concentrations in the nodules to sustain a significant though diminished level of nitrogen fixation and associated metabolic functions.

Phosphorus deficiency decreased the ratio of no-

dule mass to whole plant mass by approximately 50% over the 3 week experimental period (Table 1). This indicate that the more severe restriction in nodule growth and development than host plant growth also contributed to the maintenance of relatively high phosphorus concentrations in nodules even when growth of the host plant was limited by phosphorus deficiency.

The small change in bacteroid content and phosphorus concentration in bacteroid in comparison to change in dry mass and phosphorus concentration of other tissues in response to phosphorus deficiency indicated that host cell fraction of nodule contained enough inorganic phosphate for bacteroid development and proliferation even when the host plant suffered phosphorus deficiency. Phosphate storage may be important in rhizobial ecology, allowing them to multiply and colonize in soils under low phosphorus conditions and phosphate depleted cells have been shown to increase their internal phosphate concentration above a certain critical level before resuming growth.[3] Sa and Israel[16] reported that the bacteroid number ranged from 0.87 to 1.30×10^{11} per gram fresh weigh nodule and that the P-uptake rates were the same for bacteroids isolated from nodules of plants receiving sufficient or deficient external-P supply.

Although ATP concentration, total adenylate concentration and energy charge of whole nodules were decreased significantly by phosphorus stress, these parameters were not affected in the bacteroid fraction. This indicates that the energy status of host plant cell fraction is more sensitive to phosphorus stress than energy status of bacteroids.

Phosphorus deficiency is expected to impose limitations on glycolytic flux in plant cells of nodules to the ATP, Pi and ADP substrate dependence of fructose 6-phosphate 1-phosphotransferase, NAD-glyceraldehyde 3-phosphate dehydrogenase and 3-phospoglycerate kinase, respectively. Duff *et al.*[17] reported that phosphate starvation induces the bypass of nucleotide phosphate of Pi dependent glycolytic reactions, but ATP generating efficiency was less than with the conventional pathway. Tsai *et al.*[18] proposed that phosphate deficiency inhibited ATP regeneration and that the resulting low energy charge inhibited biosynthetic reactions after acetyl-CoA formation.

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인산결핍이 대두근류의 bacteroid 함량과 energy 상태에 미치는 영향

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초록 : 인산처리가 근류의 bacteroid 함량과 energy 상태에 미치는 영향을 살펴보기 위하여 *Bradyrhizobium japonicum* MN10을 접종한 대두식물(*Glycine max* [L.] Merr.)에 저해농도(0.05 mM-P)와 정상농도(1.0 mM-P)를 처리하여 온실에서 재배하였다. 인산결핍은 식물체의 근류량과 근류량 식물체량의 비에 유의성있는 감소를 보였다. 잎, 줄기, 뿌리의 인산농도는 인산결핍에 의하여 75%가 감소하였으나 근류의 인산농도는 40%만 감소하였다. 근류의 bacteroid 함량과 근류에서 전인산, 전질소의 bacteroid와 식물세포로의 분배는 인산결핍의 영향을 받지 않았으며 전질소의 22%, 전인산의 27%가 bacteroid에 존재하였다. 근류의 ATP농도, 전 adenylate 농도와 energy charge는 인산결핍에 의하여 각각 77%, 46%, 37%가 감소되었다. 인산결핍은 근류내 식물세포의 ATP 농도와 energy charge를 각각 86%, 59%를 감소시켰으나 bacteroid의 ATP농도와 energy charge에는 영향을 미치지 않았다. 이러한 결과는 근류가 인산의 강한 보유원이며, 기주식물에 대한 인산결핍조건하에서 bacteroid는 정상적인 인산농도와 energy양을 함유한다는 것을 나타낸다.