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Effect of external-phosphorus supply on the phosphorus status of soybean nodules and the P-uptake system of isolated bacteroids

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Abstract : Soybean plants inoculated with *Bradyrhizobium japonicum* MN 110 were supplied with nutrient solutions containing 1.0, 0.25 and 0.05 mM-P to characterize the effect of external-P supply on the phosphorus status of nodules and on the P-uptake system of isolated bacteroids from nodules. After 48 days of growth, whole plant dry mass in the 0.25 and 0.05 mM-P treatments decreased significantly. The Pi concentrations in nodules were 4.1, 2.5 and 2.0 mM for 1.0, 0.25 and 0.05 mM-P treatments, respectively. The external-P supply did not significantly affect the distribution of phosphorus among inorganic phosphate(Pi), soluble organic-phosphorus(SOP) and insoluble organic-phosphorus(IOP) fractions in nodules. The Pi concentrations in young leaves of 0.25 and 0.05 mM-P plants were 33% and 20%, respectively, of those in young leaves of 1.0 mM-P plants and Pi concentrations in old leaves were only 16% and 7%, respectively, of those in old leaves of 1.0 mM-P plants. Phosphorus deficiency decreased the percentage of total leaf phosphorus in the Pi fraction and increased the percentage of total leaf phosphorus in the IOP fraction. The bacteroid number ranged from 0.87 to 1.30×10^{11} per GFW nodule regardless of external-P supply to the host plants and plant age. The P-uptake rates were the same (15-16 pmoles /min./10^8 bacteroids) for the bacteroids isolated from nodules of 1.0 mM-P and 0.05 mM-P plants. These results indicate that Pi concentrations in nodules of phosphorus-deficient plants are sufficient for proliferation of bacteroids and that the P-uptake system of bacteroids is in a repressed state even when host plant growth is severely restricted by phosphorus-deficiency stress.

The phosphorus present in plants can be broadly separated into organic-P and inorganic-P fractions[1]. When plants are grown under phosphorus-deficiency conditions, concentrations of both fractions decrease, but the greatest decreases occur in inorganic-P fraction[2,3,4]. When phosphorus is absorbed in excess of the growth requirement the excess is accumulated mainly as inorganic-P[5]. A slow turnover time indicates that the vacuolar Pi pool is relatively unavailable and may have a storage function[6] Bieleski[7] reported that when plants were switched from sufficient to deficient-P conditions, the non-metabolic(vacuole)-Pi pool was progressively utilized but the metabolic(cytosol)-Pi concentration decreased very slowly , and when non-metabolic-Pi was depleted growth ceased because the matabolic-Pi rapidly decreased to the minimum concentration required for maintenance.

Appearance of ^{32}P label in nodules after feeding to leaves of lotus plants revealed movement of phosphorus from leaves to nodules in the phloem[8]. The pathway by which phosphorus moves from the external solution to the nodules presumably involves transport from roots to leaves in the xylem, exchange into the phloem either in inorganic- or organic-phosphate

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form, and transport to nodules in the phloem along with organic carbon compounds.

The phosphorus requirement for optimal growth of *Rhizobium* and *Bradyrhizobium japonicum* in pure culture with glutamate as a nitrogen source has been shown to be strain dependent. Strain 110 exhibited optimal growth at 0.06μM-Pi when the concentration was maintained at this level with an iron oxide dialysis system[9]. Smart et al[10]demonstrated that, when *Rhizobium trifolii* WU 95 and *Rhizobium* NGR 234 were grown under phosphorus-sufficient conditions in chemostats elevated concentrations of cellular phosphorus and the storage compound, polyphosphate, accumulated in the cells. However, phosphorus-limited cultures of these strains also contained detectable levels of polyphosphate. These observations indicate that growth of free-living rhizobia and bradyrhizobia is controlled by attainment of a critical internal phosphorus concentration. Short term ^{32}P uptake experiments have shown that the P-uptake system in free-living rhizobia and bradyrhizobia is a single, feedback repressible-inhibitable, unidirectional and energy dependent system[11]. The P-uptake system of bacteroids isolated from nodules of both phosphorus-deficient and phosphorus-sufficient snakebean plants has been reported to be in a repressed state[11].

The bacteroid content of nodules and phosphorus concentration in bacteroids isolated from nodules of soybean plants were less affected by phosphorus deficiency than plant growth and phosphorus concentration in other plant parts including the plant cell fraction of nodules[12]. These results support the hypothesis that the P-uptake system of bacteroids isolated from nodules of phosphorus-deficient soybean plants may be in a repressed state.

Therefore, the objectives of the study described in this report were to evaluate this hypothesis by examining the effect of phosphorus deficiency on leaf and nodule phosphorus status and the relation between nodule phosphorus status and P uptake rate of bacteroids isolated from soybean nodules.

Materials and Methods

Plant culture

Seeds of soybean[*Glycine max* [L.] Merr. IRansom'[were germinated in germination paper saturated with 0.5mM $CaSO_4$ in a dark germination chamber and kept moist by capilary action from a 0.5mM $CaSO_4$ solution. After 4 days, seedlings were transplanted into 6 L pots (three for 1.0 and 0.25mM-P treatment and four for 0.05mM-P treatment) containing Perlite amended with 300g of crushed oyster shells to control rhizosphere acidification[13]. Roots of seedlings wee dipped into an inoculum of *B. japonicum* MN 110 (10^9 CFU/mL) before transplanting. After transplanting, 0.5mL of inoculum was applied to the Perlite at the base of each seedlings. Plants were grown from mid October to November, 1989. The greenhouse temperature was mintained below 35℃ during daylight hours with evaporative cooling and the photoperiod was extended to 17 hrs. by using metal halide lamps from 6 a.m. to 11p.m. each day. There P treatments were used : 0.05 mM-P(low-P), 0.25mM-P(mid-P) and 1.0 mM-P(high-P). Other cultural procedures were the same as described before[12].

Sampling Procedures

Plants were harvested at 35 and 48 days after transplanting(DAT). Trifoliolate leaves at the 2 highest node positions on the main stem were designated as young leaves and trifoliolate leaves at the 2 lowest node positions on the main stem were designated as old leaves. Plants were separated into leaves, stems, roots and nodules after removing young and old leaves. Nodules(*ca. 1g*) were kept on ice after detaching until bacteroid isolation. The remander of nodules and young and old leaves were frozen in liquid N_2 until extraction and assays could be performed. The other plant parts were freeze-dried and weighed.

Fractionation and analysis of phosphorus

Fresh nodule and leaf tissues (*ca.* 1g) were homog e- nized in 15 mL ice-cold 8% (W/V) trichloroacetic acid, 20% (V/V) methanol mixture using a Polytron at full speed for 15 sec. After homogenates were centrifuged at 6,000 rpm for 20 min. at 4℃, aliquots (2 mL) of the supernatants were transferred to vials for inorganic phosphate measurement. For soluble organic phosphorus determination, aliquots (5 mL) of the supernatant were transferred to digestion tubes.

The pellets containing insoluble organic phosphorus were transferred to digestion tubes using the TCA /methanol mixture (15-20 mL). The samples in digestion tubes were heated at 97℃ with aeration until all liquid had evaporated. The samples were digested by a Kjeldahl procedure[14] that employed a zirconium-copper catalyst[15]. The samples were diluted with 20 mL redistilled water after digestion. Aliquots were taken from the diluted digests and were analyzed for phosphorus by the ammonium molybdate method of Murphy and Riley[16].

Bacteroid isolation and counting

Nodules(*ca.* 1g) were surface sterilzed in 80% ethanol for 15 sec. and rinsed with cold redistilled water. All buffers, the cheesecloth and all containers that came in contact with the bacteroid fraction were steam sterilized. The homogenizer probe was rinsed thoroughly with 80% ethanol. Nodules were homogenized in the Polytron for 30 sec. in 15 mL of 20 mM HEPES buffer (pH 6.9) containing 5% (W/V) polyvinyl pyrollidone (pVP), 10 mM sodium ascorbate, and 10 mM glucose. The homogenates were filtered through four layers of cheesecloth and the filtrates were centrifuged at 12,000g for 10 min. at 4℃. The pellet of bacteroids was washed twice in the samc buffer without PVP and washed again with minus-P galactose-arabinoseglutamate(GAG) medium. The bacteroid suspension (1 mL) was diluted (10^5-10^7) and planted on GAG plates (pH 6.0) containing 1 mM-P and colonies were counted after incubation at 28℃ and 2% atmospheric CO_2 for 72hrs.

P-uptake assay

Stock solutions of ^{32}P were filter sterilized through 0.45-um sterile, prepacked filter units(Nalgene, Sybron Corp., Rochester, NY) prior to use. The specific activity of the solution was 4cpm/pmol-Pi. Bacteroids isolated from nodules of 1.0 mM-P and 0.05 mM-P plants were used for P-uptake assay with 3 replications. Bacteroid suspension (0.2 mL) was added to minus-P GAG medium (8.8 mL) and agitated in water bath at 28℃. Two minutes after adding bacteroid suspension. ^{32}P solution (1 mL) was added, resulting in a final concentration of 50 nmoles Pi/mL. Samples (0.5 mL, ca 10^8 cells) were taken at 20 second intervals for the initial 3 minutes and then at 1 minute intervals until 10 minutes. After filtering samples through 0.22-um Millipore membrane filters, filters were washed with 5 mL of 5 mL KH_2PO_4 /MES buffer (pH 5.6). The filters were soaked in the buffer prior to use to minimize adsorption of label. The filters were transferred to 20 mL scintillation vial and 10 mL aliquots of 2.5 mM ANDA(wavelength shifter) were added. Cerenkov radiation emitted from each vial was measured for 20 min. using a Packard liquid scintillation counter[17].

Experimental design and statistical analysis

All combinations of three phosphorus treatments by two sampling dates were replicated three times and arranged in a randomized complete block design. The data were analyzed using the ANOVA procedure of the Statistical Analysis System[18].

Table 1. The effect of external phosphorus supply on dry mass yield of soybean plants

Treatment (mM-P)	DAT	Leaflets	Stems	Roots	Nodules	Whole Plant
				g/plant		
1.0	35	1.29	0.69	0.61	0.20	2.79
0.25	35	1.18	0.75	0.72	0.15	2.79
0.05	35	0.68	0.46	0.63	0.05	1.82
1.0	48	4.40	3.01	2.43	0.75	10.60
0.25	48	3.27	2.37	2.27	0.34	8.24
0.05	48	1.26	0.72	1.16	0.09	3.05
$LSD_{0.05}$		0.49	0.42	0.26	0.03	1.01

Values represent means of 3 replications.

Table 2. The effect of external phosphorus supply on inorganic phosphate(Pi), soluble organic phosphorus(SOP), insoluble organic phosphorus(IOP) and total phosphorus concentrations in nodules

Treatment (mM-P)	DAT	Inorganic P	Soluble organic-P	Insoluble organic-P	Total P
		ug-P/GFW			
1.0	35	115	160	947	1,222
	48	95	184	831	1,124
0.25	35	70	117	771	957
	48	51	163	735	995
0.05	35	57	76	641	774
	48	42	117	635	794
$LSD_{0.05}$		11	37	295	309

Values represent means of 3 replications.

Results

At 35 DAT, the whole plant dry mass of the 0.05 mM phosphorus treatments was significantly lower than that of the 0.25 mM and 1.0mM phosphorus treatment(Table 1). The latter two treatments had similar whole plant dry mass after 35 days of growth. A significantly lower whole plant dry mass at 48 DAT for the 0.25 mM-phosphorus treatment compared to the 1.0 mM-phosphorus treatment indicates that phosphorus eventually became growth limiting in the 0.25 mM-phosphorus treatment (Table 1).

Inorganic phosphate concentrations in the nodules of 0.25 and 0.05 mM-phosphorus plants were 58% and 48% of that of 1.0 mM-phosphorus plants when averaging over 2 sampling dates(Table 2). Inorganic phosphate concentrations in nodules from plants in all external phosphorus treatments decreased between 35 and 48 DAT (Table 2). Assuming that fresh nodules contained 80% water, the nodule Pi concentrations were 4.1, 2.5 and 2.0mM, respectively, in nodules of 1.0, 0.25 and 0.05 mM-phosphorus plants. Soluble organic-P(SOP) and insoluble organic-P (IOP) concentrations in nodules of 0.25 mM-phosphorus plants were 23% and 18%, respectively, less than those in nodules of 1.0 mM-phosphorus plants and 30% and 15%, respectively, higher than those in nodules of 0.05 mM-phosphorus plants (Table 2). The percentage of total phosphorus as Pi, SOP and IOP in nodules is illustrated in Fig. 1. The external phosphorus concentration did not significantly affect the distribution of phosphorus among Pi, SOP and IOP fractions of nodules (Fig. 1).

The inorganic phosphate concentrations in young

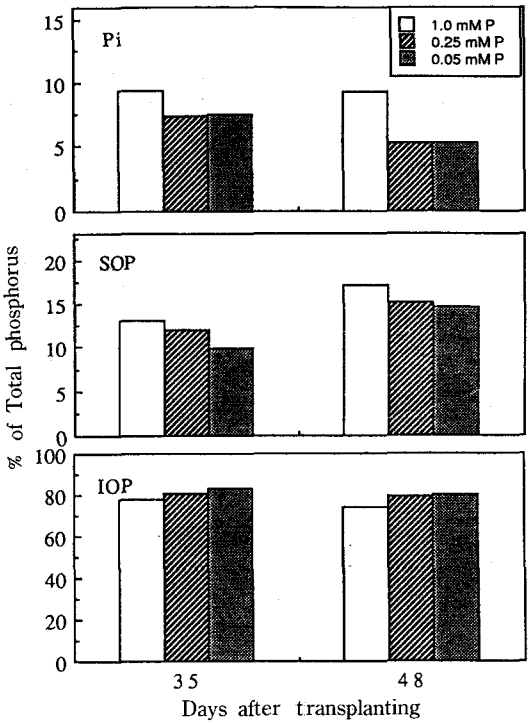

Fig. 1. The effect of external phosphorus supply on distribution of total-P among inorganic-P(Pi), soluble organic-P(SOP) and insoluble organic-P (IOP) in nodules.

Values represented by height of bars are means of 3 replications.

Table 3. The effect of external phosphorus supply on inorganic phosphate(Pi), soluble organic phosphorus(SOP), insoluble organic phosphorus(IOP) and total phosphorus concentrations in soybean leaves at 48 DAT

Treatment (mM-P)	Relative leaf age	Inorganic P	Soluble organic-P	Insoluble organic-P	Total P
		ug-P/GFW			
1.0	Young	332	164	462	959
	Old	548	113	598	1,259
0.25	Young	112	38	303	453
	Old	87	43	259	389
0.05	Young	65	31	184	280
	Old	40	31	170	245
$LSD_{0.05}$		58	41	16	17

Values represent means of 3 replications.

leaves of the 0.05 and 0.25 mM-phosphorus plants were 33% and 20%, respectively, of that in young leaves of 1.0 mM-phosphorus plants, and only 16% and 7%, respectively, of that in old leaves of 1.0 mM-phosphorus plants. The Pi concentration was lower in young leaves than in old leaves of 1.0 mM-phosphorus plants, but young leaves of 0.05 and 0.25 mM-phosphorus plants contained higher Pi concentrations than old leaves (Table 3). The SOP concentrations in young and old leaves of 0.05 mM and 0.25 mM-phosphorus plants were similar and significantly lower than those of 1.0 mM-phosphorus plants. The IOP concentrations were higher in young leaves than in old leaves of 0.25 and 1.0 mM-phosphorus plants, but the IOP concentration was 30% higher in old leaves than in young leaves of 0.05 mM-phosphorus plants.

Inorganic phosphate as a percentage of total-P in both young and old leaves decreased with decreases in external phosphorus concentration(Fig. 2). The IOP as a percentage of total-P in both young and old leaves was significantly lower in 1.0 mM-phosphorus treatment than in the 0.05 and 0.25 mM phosphorus treatments. In the two lower external phosphorus treatments, IOP contained 60% of the total-P in young and old leaves, whereas, this fraction contained less than 50% of the total-P in these leaves of plants in the high external phosphorus treatment.

The effect of host plant phosphorus nutrition on the bacteroid number (colony forming units) in nodules is illustrated in Table 4. The bacteroid number

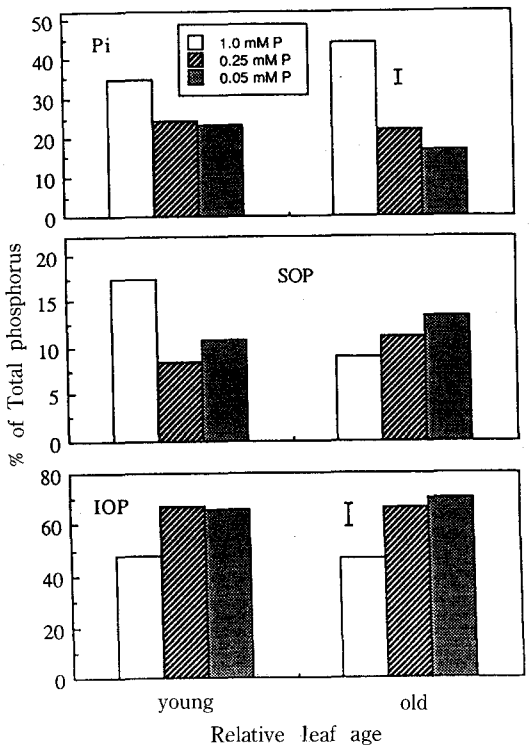

Fig. 2. The effect of external phosphorus supply on distribution of total-P among inorganic-P(Pi), soluble organic-P(SOP) and insoluble organic-P (IOP) in leaves at 48 DAT.

Values represent by height of large bars are means of 3 replications. The small bars repressent $LSD_{0.05}$ values calculated on basis of significant P treatment effects.

Table 4. The effect of external phosphorus supply on bacteroid number of soybean nodules

	1.0 mM-P	0.05 mM-P
	($\times 10^{11}$CFU/GFW NODULE)	
35 DAT	1.27(0.15)	1.22(0.05)
38 DAT	1.30(0.17)	0.87(0.26)

Values represent means of 3 replications.
( ) represents standard error.

ranged from 0.87 to 1.30×10^{11} per GFW nodule. Bacteroid number per unit nodule weight was not significantly affected by external phosphorus supply or plant age.

Bacteroids isolated from nodules of both 1.0 and 0.05 mM-phosphorus plants exhibited a linear accumulation of labelled-P for 10 min. The P-uptake rate was the same (15-16 pmoles/min./10^8 bacteroids) for the bacteroids isolated from nodules of 0.05 mM and 1.0 mM-phosphorus plants (Fig. 3).

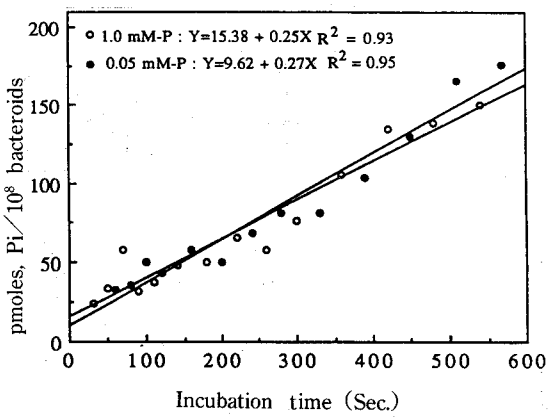

Fig. 3. The effect of external phosphorus supply on P-uptake rate of bacteroid isolated from nodules.
Data points represent means of 3 replications.

Discussion

The Pi concentrations in leaves of 1.0 mM-phosphorus plants were much higher than those of 0.05 mM-phosphorus plants. Old leaves contained a higher Pi concentration than young leaves of 1.0mM-phosphorus plants, but the Pi concentration in young leaves was higher than that in old leaves of 0.05 mM-phosphorus plants. These results suggested that the Pi can be retranslocated from old leaves to any other metabolically active sites. Higher Pi concentration in old leaves of 1.0 mM-phosphorus plants was probably due to a large storage capactity of Pi in vacuoles rather than due to a decrease in the amount of Pi retranslocated from old to young leaves, because Pi concentration in young leaves of 1.0 mM-phosphorus plants was much higher than that of 0.05 mM-phosphorus plants. Foyer and Spencer[19] demonstrated that cytoplasmic Pi concentration was similar in the leaves of plants grown at 1 mmol P/L and 25 mmol P/L, while the vacuolar Pi pool fluctuated in order to buffer the Pi concentration in the cytoplasm. However, when plants were grown without external phosphorus for an extended time period, the vacuolar Pi was depleted and the Pi concentration in cytoplasm could not be maintained. When white clover plants were supplied with an adequate external phosphorus concentration, a large proportion of Pi accumulated in old leaves was retranslocated to young leaves, whereas, a much smaller proportion of Pi was retranslocated from old to young leaves when plants were grown under phosphorus-deficient conditions[20]. Inorganic phosphate was the phosphorus fraction in nodules affected most severely by phosphorus deficiency, however, the decrease in Pi concentration in nodules caused by phosphorus-deficiency was less than the decrease observed for young and old leaves (Table 5). Nodule growth is inhibited to a greater degree by phosphorus deficiency than host plant growth[21]. Therefore, maintenance of relatively high Pi concentration in nodules of phosphorus-deficient plants is associated with a high sink strength for phosphorus[12] and with differential restriction of nodule growth[21].

Inorganic phosphate concentration in nodules of phosphorus-deficient plants was 2 mM. Assuming that 50% of the Pi is localized in bacteroid fraction, that bacteroids comprise 10% of the nodule dry weight and that fresh nodules contain 80% water, the Pi concentration in the plant cell fraction would be 1.1 mM. This concentration is much higher than that required for optimal growth of bradyrhizobia in chemostat culture[10] or that in soil solution of typically productive soils[22].

The same Pi-uptake rate by bacteroids isolated from nodules of phosphorus-sufficient and phosphorus-deficient plants indicated that the P-uptake sys-

Table 5. Comparison of the relative effects of phosphorus deficiency on concentrations of different phosphorus fractions in nodules and leaves at 48 DAT

Tissue	Treatment (mM-P)	Inorganic P	Soluble organic-P	Insoluble organic-P	Total P
		% of 1.0 mM-P plants			
Nodule	0.25	53.7	88.6	88.4	85.5
	0.05	44.2	64.0	76.4	71.5
Young Leaves	0.25	33.7	23.2	65.6	47.3
	0.05	19.6	18.9	39.8	29.2
Old Leaves	0.25	15.9	38.1	43.3	30.9
	0.05	7.3	27.4	28.4	19.5

tem of bacteroids isolated from nodules of phosphorus-deficient plants was in a repressed state. The phosphate-uptake rate of bacteroids isolated from phosphorus deficient snakebean plants was about 4-fold higher than that of bacteroids isolated from phosphorus-sufficient plants but this value was only 5% of the rate measured for free-living cells grown in phosphorus-limited chemostat culture and only slightly higher than the repressed levels of the free living-cells [11].

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인산공급이 대두근류의 인산형태와 bacteroid의 인산흡수에 미치는 영향

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초록 : 기주 식물에 대한 인산공급이 근류의 인산 형태와 bacteroid의 인산흡수에 미치는 영향을 살펴보고저 *B. japonicum* MN 110을 접종한 대두식물을 1.0, 0.25, 0.05mM-P 조건에서 재배하였다. 1.0, 0.25, 0.05mM-P 조건에서 재배한 대두식물의 근류 Pi 농도는 각각 4.1, 2.5, 2.0mM이었으며 인산공급이 근류의 Pi, SOP, IOP의 분포에는 유의성있는 영향을 미치지 못하였다. 0.25, 0.05mM-P 조건에서 재배한 대두식물의 유엽 Pi 농도는 대조구(1.0mM-P)의 각각 33%, 20%이었으며 노엽 Pi 농도는 대조구의 각각 16%, 7%였다. 잎에서는 인산 결핍이 전인산에 대한 Pi의 분포를 감소시켰으나 IOP의 분포는 증가시켰다. 근류내의 bacteroid의 수는 0.87-1.30×10^{11} /GFW nodule이었으며, 인산흡수도는 15-16 pmoles /min/10^{8} bacteroid로 기주식물의 인산공급에는 영향을 받지 않았다. 이러한 결과는 인산결핍 대두식물의 근류내 Pi 농도는 bacteroid의 생육과 번식에 충분하며 인산결핍에 의하여 기주식물의 생육이 저해될 때에도 bacteroid의 인산흡수는 억제상태에 있음을 나타낸다.