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## Phosphate Role in the *Rhizobium*-Legume Symbiosis- A Review

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### Abstract

The international emphasis on maintaining farming systems productivity and sustainability is focusing on the use of renewable plant nutrients resources. In case of nitrogen (N), biological N<sub>2</sub> fixation via symbiosis is the most important input for agricultural systems sustainability. Research studying the association between rhizobia and the host legume plants has enhanced our knowledge and understanding of this symbiosis process. Our current knowledge establishes that any interruption in the flow of nutrients between symbionts affects this association. Since phosphorus (P) is one of the essential nutrients in *Rhizobium*-legume symbiosis, and its limitation affects every aspect of the symbiosis, it is important to understand its function in the symbiosis process. This review will emphasis on the role of P in nodulation and in the functional symbiosis between rhizobia and legumes.

**Key Words:** Legumes; Nitrogen fixation; Phosphorus (P); *Rhizobium*; Symbiosis.

### 1. Introduction

The rhizobium-legume symbiotic association is built, basically, on the exchange of carbon (C) for nitrogen (N) in a reduced form. In the last thirty years, the accumulated research of the relationship between plant and bacteroid concerning the C-N association established a wide knowledge foundation of this subject.

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This knowledge enabled us to know a great deal, about how these nutrients are shared between the symbionts. From the studies conducted on the different rhizobium-legumes so far, the model of uptake and oxidation of a dicarboxylic acid will explain the substantial amount of C flow to the bacteroid.

It is not surprising, though, the exchange of nutrients between symbionts is not limited to carbon and nitrogen, furthermore, it is clear that nodule and the symbiotic function encompasses balanced flow of other nutrients [1]. A prominent example of that is phosphorus (P). Restriction to biomass production (ecosystem assessment) or crop production (agronomic viewpoint) is expected to be encountered much more frequently with P than any other nutrient (including N in many cases). Virtually every aspect of the Rhizobium-legume symbiosis is affected, and eventually limited, by P availability. This review will emphasis on the role of P in nodulation and in the functional symbiosis between rhizobium and legumes.

## **2. Importance of phosphorus research to Society**

Estimates show that the Earth's population will reach 10 billion by the year 2040 [2,3]. Feeding the world's population, while minimizing the impact of agriculture on land resources, will demand that crop production and fertilizer use efficiency be at a complete optimum. There is an immediate urgency to optimize P use efficiency in crop plants, especially in legumes such as beans, which are a major source of dietary protein for humankind. When taking this into consideration, along with projections that easy-access rock phosphate reserves will be depleted within 60-90 years [3], it becomes apparent that there is an immediate need to investigate P-based crop production problems. There are also grave ecological concerns regarding uncontrolled introduction of P into the environment. Inefficient use of P fertilizers and mismanagement of manure P has led to significant environmental problems such as the "dead zone" in the Gulf of Mexico [4], and has been described as "a potential phosphate crisis" [5].

## **3. Overall Effects of Phosphorus Limitation on Symbiosis**

Every aspect of symbiosis, from infection to nodule function, is sensitive to P limitation. Alfalfa [6,7], soybean [8,9,10], clover [11], common bean [12,13], pigeon pea [14], and cowpea [8] show extremely significant positive responses to P application. While it depends on how the plants were cultured, responses to added P generally include increased whole plant N concentration, plant dry matter, nodule number, nodule mass, and whole plant nitrogenase activity. Studies on nitrogen (supplied as  $\text{NO}_3^-$  or from symbiosis) and P interactions in soybean indicate that this specific legume has a higher P requirement when it gets its nitrogen from symbiosis as compared to mineral nitrogen [9]. It is possible and likely that multiple other legumes are similar in this regard.

### **3.1 Nodulation**

The fundamental foundations for P influence on nodulation is not well understood. Root-tip marking experiments demonstrate that nodulation effectiveness by *Bradyrhizobium japonicum* is reduced when cultures are P-stressed prior to inoculation onto soybean roots [15]. P stress severely limits Nod factor production and

excretion [16], suggesting that rhizobia infection activities will be inhibited by low P conditions. This is not unexpected, but acquiring a full knowledgebase of P influences on nodulation may be difficult in some cases, as nodulation responses will also vary between legumes depending on how P availability influences plant participation in the infection process.

### **3.2 Nodule function**

One sequelae of P limitation to plants is reduced photosynthesis [17,18,19] and therefore one can speculate that P effects on nodule function might be tied to photosynthesis efficiency. Any decrease in photosynthesis could be expected to negatively affect C flow to the nodule and thus decrease nitrogen fixation. Nodules on P-limited soybeans and alfalfa appear to have increased oxygen permeability [20,21,22]. A portion of this change in O<sub>2</sub> flux may be due to decreased nodule size (increased surface-to-volume ratio) that is common with P-limited plants. However, Drevon and Hartwig (1997) describe that this would not account for all of the increase in oxygen permeability and that other factors to explain this might include the activation of alternative respiratory pathways that increase the amount of O<sub>2</sub> consumed [20]. Effects of P limitation on specific nodule nitrogenase activity vary [23,24] compare to [7,21,25,26,27]. Some of the variation is partly due to differences between legumes, method of plant growth, or method used for assessing nitrogen fixation. There is also evidence connecting soybean nodule nitrogenase activity with P availability and nodule energy state [28].

### **4. Flow of P within the symbionts**

The flow of P within the symbiosis is not well-understood. From the viewpoint of P use efficiency, it is at least possible that excess P transfer to the nodule may happen, with the surplus P being stored in either vacuoles or sequestered by bacteroids. It is unknown if Pi taken up by the nodule from the soil is ever released to the host plant, or if P translocated via the phloem to the nodule is ever recycled to the shoot. The literature does suggest that legume nodules are strong sink holders for P, with possible net P cycling in and out of the nodule resulting in P accumulation [13,29]. <sup>32</sup>Pi feeding studies demonstrated that bean nodules would quickly take up Pi from the external nutrient solution [30], with the rate of nodule uptake and translocation to bacteroids within being significantly greater in Pi-stressed plants. Al-Niemi and his colleagues (1998) conducted short term <sup>32</sup>Pi labeling experiments where nodulated root systems of Pi-stressed intact bean plants were either partially submerged so that the majority of the nodules did not contact the label, or fully submerged such that all nodules contacted the labeled nutrient solution [30]. In these studies, <sup>32</sup>Pi uptake and translocation to the shoot was significantly greater in the partial submergence treatment [30]. Although the limitation in experimental design in these studies did not allow for valid comparison to bean plants grown in soil, but it was obvious that the bean nodules appeared to be strong competitors for the Pi contained in the nutrient solution.

### **5. Phosphorus Metabolism model in Rhizobia**

The most common model for P metabolism in bacteria has been developed from work on *Escherichia coli* (see [31,32] for reviews) and will be briefly summarized here to serve as a platform to better view the data obtained from studies with the rhizobia. The Pho regulon is controlled mainly by the two component regulatory system

PhoR and PhoB. PhoR is a histidine kinase and PhoB is its equivalent positive regulatory protein. When *E. coli* perceives low levels of Pi in its surroundings (~4  $\mu\text{M}$ ), PhoR phosphorylates PhoB to initiate the Pho stress response. A phosphorylated PhoB (PhoB\*) is essential for transcription of regulated genes [33] and the apparent DNA binding site of PhoB\* consists of 18-base consensus “Pho Box” [31]. PhoU is not involved in Pi transport, but does have a role in regulation as *phoU* mutants constitutively express alkaline phosphatase (AP); AP is the marker enzyme for the Pho stress response [32,34]. PhoR<sup>-</sup> mutants also express AP constitutively (but at lower levels compared to PhoU<sup>-</sup> mutants, reviewed in [32], whereas mutants lacking the positive regulatory protein, PhoB, does not up-regulate the Pho stress response [32].

### 5.1 Growth and phosphorus assimilation in Rhizobia

Rhizobia have different P requirements for growth [35,36], Pi uptake at low Pi concentrations, and ability to store P or to utilize stored P under low Pi conditions [35,37]. Several rhizobia will utilize phosphonate compounds as a P source [38]. Pi limitation leads to significant increased in Pi transport rates and the induction of AP [6,25,39]. The Pi-stress response in *S. meliloti* is observed when Pi concentration in the media depleted to approximately 5  $\mu\text{M}$  [25, 40]. In *R. tropici*, the P-stress response is not apparent until the culture Pi concentration drops below 1  $\mu\text{M}$  [6]. AP activity and Pi transport rates in fully induced *R. tropici* are approximately 10-fold those observed with *S. meliloti* [6,25,41].

### 5.2 Pho regulation

No AP induction and increased Pi transport rates are observed in a *S. meliloti phoB* mutant [25,42], and thus PhoB functions in rhizobia is an analogous fashion to its homologue in other gram-negative bacteria [32]. Another significant similarity is the presence of a Pho box in the promoter region of several Pi-sensitive genes in *S. meliloti* [43,44]. *S. meliloti* PhoU<sup>-</sup> mutants do not have a constitutive Pi stress response phenotype [42] as has been found in *E. coli* [45] and *Pseudomonas aeruginosa* [46]. Nevertheless, this may be due to polar effects of *phoU* mutations on *phoB* expression [42].

Regulation of some *S. meliloti* Pi stress inducible genes looks complex. Under Pi limitation, genes coding for proteins involved in EPSII synthesis (*exp* genes) are under the control of PhoB [43,40], and require ExpG for maximum expression [43]. Further, *exp* gene expression is not entirely dependent on PhoB, as high levels of transcription are apparent in a *phoB-mucR* double mutant [43]; MucR is a transcriptional repressor of *exp* genes [47]. A *S. meliloti lon* protease mutant was isolated while screening for a PhoR mutant [40]. This mutant expresses low levels of AP constitutively, and overproduces both EPSI and EPSII. These observations suggest that Lon protease controls levels of a protein that has a positive regulatory function for *pho* genes (perhaps PhoB, SyrM, and/or ExpG?) [40]. The Lon<sup>-</sup> mutant produces Fix<sup>-</sup> pseudonodules [40].

### 5.3 Phosphate transport

There are two Pi transport systems in *S. meliloti* [48], high-affinity system and low-affinity system. The high-affinity system is encoded by the *phoCDET* operon, while the low-affinity system is encoded by *pit* (part of the *orfA-pit* operon) [49]. Evidence indicates that the expression of both Pi transport systems is controlled by PhoB

[42]. While *phoCDET* operon is positively regulated by PhoB, the latter is negatively controls *orfa-pit*. Under high Pi conditions, the low affinity Pit permease is expressed and primarily controls Pi uptake. When *S. meliloti* experiences P limitation, the Pit system is inhibited whereas the high affinity PhoCDET system is induced and act as the primary mechanism of Pi transport.

Two Pi transport systems have also been identified in *R. tropici*; the high-affinity system has a  $K_m = 0.43 \mu\text{M}$   $P_i$ , and the low-affinity permease has a  $K_m = 34 \mu\text{M}$  [41]. Both systems are expressed constitutively and are inducible by phosphate stress. Both high-affinity and low-affinity systems utilize ATP in energizing the process of Pi transfer across the membrane [41]. *R. tropici* high affinity Pi transport mutant (CAP45) have been isolated [41].

#### 5.4 Acid and alkaline phosphatases

*S. meliloti* strain 104A14 has at least two acid phosphatases [7]. NapD is a nonspecific phosphatase enzyme capable of hydrolyzing a variety of organo-P compounds [7]. It is a homodimer with a subunit MW of 33 kDa and has a reasonably sharp pH optimum located at approximately pH 6.5 [7]. The other acid phosphatase, NapE, is similar in MW, shares some substrates with NadD, and has a pH range that also overlaps fairly with NapD [50]. When *R. tropici* cells grown with adequate Pi, a single acid phosphatase is detected in native gels [6]. However, under Pi-stress conditions, this band weakens while a second distinct band appears and increases in intensity [6]. It has not been determined if these two acid phosphatase activity bands are due to induction and repression of two different genes in response to Pi limitation or if it is a result of post-translationally modification of a single protein such that its electrophoretic mobility is altered [6].

#### 5.5 Pi stress global effects in rhizobia

Global effects of Pi stress in *S. meliloti* have been detected [40]. Mainly interesting were the genes involved with the synthesis of EPSII. Induction of *expC* (complementation group C (Beck and Munns (1984), [47]) occurred in Pi-stressed cells, but not in a PhoB- mutant [40], suggesting that PhoB is a positive regulator of at least some of the *exp* genes [40]. Pi-stress induction of *expC* (as well as three other Pi-stress inducible reporter genes) was found to be considerably (ca. 2 h) delayed relative to induction of AP [40]. Based on the absence of a Pho box preceding *expC* and the delayed induction kinetics, Summers and his colleagues (2000), proposed that an additional regulatory protein might participate as an intermediary between PhoB and Pi-sensitive genes that are not preceded by a Pho box [40]. A similar cascade-like scenario has been established for Pi-sensitive control of alginate synthesis in *P. aeruginosa* [51]. Ruberg and his colleagues (1999) [52] and Salminen and Streeter (1987) [43] have also stated that *S. meliloti expC*, as well as *expA1*, *expD1*, *expE2*, and *expG* are induced by Pi limitation and all appear regulated by PhoB.

The *S. meliloti genes* controlling acetyl phosphate metabolism (*ackA* and *pta*) are under PhoB control [44]. Knowing that acetyl phosphate can act as a non-specific phosphoryl donor for regulatory proteins of two-component regulatory pairs, Pho control of *pta* and *ackA* at least suggests the likelihood that part of the apparent *S. meliloti* global Pi-stress response might be due to Pho regulon-mediated acetyl phosphate synthesis. Specific

blocks in the pathway of acetyl phosphate did not result in a symbiotic defect [44].

## 6. Bacteroid phosphorus acquisition

### 6.1 Potential sources of phosphorus for bacteroids

Irrespective of P nutrition of the host plant, there is now sufficient data that allows us to rationally project that Pi is the primary form of P provided to alfalfa bacteroids, and that high levels of Pi are provided to the bacteroids. Lack of *pho* gene expression in alfalfa bacteroids suggests that alfalfa symbiosome Pi concentrations are at least 5  $\mu$ M and not limiting to bacteroid general metabolism. On the contrary, the evidence obviously implies that Pi is not the primary form of P provided to bean bacteroids and that bean bacteroids are provided very little Pi - even if the host plant is provided with high levels of Pi [6]. Total P levels may be quite high in some plant/nodule organelles or tissues [53], but in fact we know very little about Pi concentrations in determinate legume nodules in general, and there is no information about organo-P (P linked to a carbon skeleton) inventories.

### 6.2 Pi allocation to bacteroids

Assumptions about Pi allocation to bacteroids are unjustified. To illustrate this we can take a comparative example: we now know that although sucrose is the primary photosynthate transported to the nodule, it is not the main carbon/energy source for bacteroids (reviewed in [54]). Knockout mutations in various rhizobia demonstrated that blocks in hexose degradation pathways typically resulted in no symbiotic defect. On the other hand, rhizobia mutants affected in dicarboxylic acid catabolism are always symbiotically defective. Further, the symbiosome membrane is highly selective and at least in some symbioses appeared to transport sugars far less effectively than dicarboxylates and thus is crucial in dictating types and amounts of metabolites shared between symbionts [48].

Regarding the possible P metabolites that may be provided to bacteroids in general, one can roughly categorize them as either Pi or organo-P compounds [55].  $^{31}$ P NMR studies of soybean nodule compartments revealed that the P concentration in soybean nodule vacuoles is higher than in the cytosol [56]. If the plant treats the symbiosome as a vacuole, then it may be pumping Pi into the symbiosome. While stating that nothing is known about Pi transport across the symbiosome membrane, Uvardi and Day (1997) [57] and Voegele and his colleagues (1997) [48], speculated that Pi might share an anion channel that transfers NO<sub>3</sub><sup>-</sup> and/or Cl<sup>-</sup>.

Organo-P compounds might play more than one role in general bean bacteroid and nodule metabolism, including a source of P for bacteroids. Phosphatase reactions with organo-P compounds result in the release of Pi from the carbon skeleton. The released Pi could be taken up for use in normal Pi metabolism, whereas the carbon compound may possibly, in some cases, be important in *R. tropici* bacteroid carbon metabolism. Little information is existing about such presumed phosphatases in terms of nodule physiology, much less their potential role in P allocation to bacteroids. Targeting of host proteins to the symbiosome occurs [58] and could include plant phosphatases. Nodule phosphatases [59,60] and pyrophosphatases [61,62] have been studied but so far there is no evidence that they occur within the symbiosome.

Another possible scenario is that phosphorylated compounds are taken up by bacteroids without phosphatase activity. One example of the latter might be UDP-glucose. Salminen and Streeter (1987) established that soybean bacteroids will readily transport UDP-glucose (rates roughly 6-fold greater than uncharged glucose) [43]. Mellor (1988) assumed that this might serve as a mechanism of transporting hexoses across the soybean symbiosome membrane [33] which is fairly impermeable to glucose [63]. Since *B. japonicum* bacteroids metabolize glucose poorly [43,63], it is likely that some of this putative carbon reappears, minus P, as trehalose, which is a major nodule and bacteroid carbohydrate [24,64]. The P associated with UDP-glucose in this scenario could cycle into general P pools. Other major candidate organo-P compounds earlier found in plant extracts include P-esters such as 3-P-glycerate, glucose-6-P, fructose-6-P, and phytate (and more) (reviewed in Bielecki (1973) [65]).

Drobak (1993) suggested phosphoinositides in the plasma membrane might have an important role in intracellular signaling within plants [66]. Features of this model include interconversion of phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol by means of phosphatases and kinases, and the turnover of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-triphosphate caused by phosphoinositidase activity. A phosphorylated component of the pea plasma and symbiosome membranes has chromatographic properties comparable to polyphosphoinositides [13], although western blot evidence showed its presence on the symbiosome membrane is transient [13]. To the extent that such a model may have relevance to the symbiosome membrane, there are elements that could be linked to P metabolism in addition to that of other ions. Phosphatidylinositol- or inositol-(poly) phosphates may serve as a source of Pi for bacteroids. It is also worth paying attention to the fact that inositol polyphosphates are strongly anionic, and thus are strong iron chelators. If inositol polyphosphates are ultimately shown to occur in the peribacteroid space, this may explain the accumulation of iron in the symbiosome but not in bacteroids [36].

## 7. The *Sinorhizobium meliloti* - *Medicago sativa* symbiosis

A *S. meliloti ndvF* mutant initially described as having a defect in nodulation [67] was later shown to be affected in the *phoCDET* locus, which codes for the high affinity Pi transport system [42]. This mutant forms nodules that are mostly empty of bacteria, inferring that infection is blocked prior to the release of bacteria from the infection thread. This clearly suggested that Pi is important to *S. meliloti* during infection. In addition to that, since under free-living conditions the *phoCDET* operon is only expressed when Pi is limited in the growing environment [42,49], it would be reasonable to conclude that the alfalfa infection thread environment contains low levels of Pi, resulting in the induction of *phoCDET*. In contrast to other studies that suggested alfalfa bacteroids in the effective nodule are not Pi limited: i) regardless of host P nutrition, *S. meliloti* PhoB- mutants form a fully functional symbiosis [25,49]; ii) several Pi-stress inducible genes are not obligatory for symbiosis [68,69]; iii) reporter gene studies obviously imply that Pi-stress inducible genes are not up-regulated during symbiosis in either Pi-sufficient or Pi-limited alfalfa plants [68]; iv) an *S. meliloti* PhoU- mutant has no noticeable symbiotic phenotype [49]. These observations are in line with the conclusion that induction of the *S. meliloti* Pho regulon (includes *phoCDET*) is not obligatory for alfalfa nodule formation or function.

It is likely to explain the symbiotic defect of the *phoCDET* mutant from an analysis of elements controlling the

expression of the two known Pi transport systems in *S. meliloti* and the overall influence of regulatory mutations on Pi transport activity in this bacterium. It is known that the *S. meliloti pit* low-affinity Pi transport system is negatively regulated by PhoB [49,61]. In cultured *S. meliloti* cells, the Pit transporter is expressed under high Pi situations and repressed under low Pi conditions [49,61]. In *E. coli*, most mutations in the high-affinity Pi transport system also ends in constitutive expression of the Pho regulon [70]. Mutations in the *phoCDET* operon have a comparable effect on *S. meliloti* [49], and also result in constitutive suppression of the low-affinity Pit system [48]. Nevertheless, spontaneous second site mutations in *phoB* or in the promoter of the *orfA-pit* operon separate *orfA-pit* from PhoB control (referred to as *sfx* mutations [7]). These *sfx* mutations in the *phoC* mutant result in the restoration of normal symbiotic function [67,71]. Therefore, it has been concluded that the symbiotic phenotype resulting from mutations in the *S. meliloti phoCDET* operon is not caused by loss of Pi uptake through the Pi stress-inducible high affinity Pi transport system *per se*. Rather, it is due to mutations in *phoCDET* that suppress the regular symbiotic expression of Pit and that accidentally eliminate the other main mechanism of Pi uptake, PhoCDET [69,71].

While very likely, it remains to be ultimately shown that Pi is the form of P first presented to alfalfa bacteroids. It is feasibly possible that a P-ester is transported across the symbiosome membrane and then hydrolyzed by a phosphatase to release Pi for uptake by a bacteroid Pi transporter. There was unsuccessful attempts to find AP in alfalfa bacteroids (Al-Niemi and McDermott, unpublished data) but this is consistent with the finding that a PhoB<sup>-</sup> mutant is Fix<sup>+</sup> (a PhoB<sup>-</sup> mutant is phenotypically equivalent to an AP<sup>-</sup> mutant) [25,49]. Both of the two known acid phosphatases in *S. meliloti* strain 104A14 (NapD and NaapE) are present in bacteroids and could play a role in the release of Pi for uptake, however mutants for either acid phosphatase have a normal symbiotic phenotype [7,50]. Given the obvious overlap in their various properties, it is possible that one could compensate for the absence of the other. Therefore, a double acid phosphatase mutant will be necessary to fully assess whether alfalfa bacteroid acid phosphatases are required for bacteroid Pi acquisition. In this context, it is relevant to mention that acid phosphatases had been reported in periplasmic extracts of *B. japonicum* bacteroids and in the surrounding peribacteroid space [54]. If *B. japonicum* bacteroid phosphatase activity is certainly important for any reason, it is probably not under PhoB control as a *B. japonicum* PhoB<sup>-</sup> mutant is Nod<sup>+</sup> Fix<sup>+</sup> [55].

## 8. Conclusions

There is currently little known about P function in the *Rhizobium*-legume symbiosis in legumes that form determinate nodules. There has been a decline in the number of research conducted on this subject in the recent years, possibly due to the lack of financial support from funding agencies.

## 9. Recommendations

Fundamental information regarding how P flows at the "system" level, the kinds of P-metabolites that occur in the nodule and P exchange between symbionts needs to be elucidated. Furthermore, it is essential to determine how P flow may be coordinated with other nutrients such as C (C and P co-metabolism relationships). There are preliminary findings suggest that this may be occurring in the bean nodule. Thus, there is a need for more



research efforts to address these questions by generating key *R. tropici pho* mutants and/or cloning needed genes to facilitate mutant construction and using them in the biochemical analysis of nodule metabolites and in studying symbiosome transport of identified P metabolites.

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