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Rhizobia–*Phaseolus lunatus* Symbiosis: Importance and Diversity in Tropical Soils – A Review

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ABSTRACT

Phaseolus lunatus (Lima bean) is the second most economically important species of genus *Phaseolus* and one of the 12 primary grain legumes. The plant presents great rusticity and has the capacity to resist long dry periods, characteristics that are important for tropical regions. *P. lunatus*, like other legume plants, can establish a symbiosis with soil rhizobia that leads to the development of legume nodules in response to the appropriate nitrogen-fixing bacteria. These symbioses result in biological nitrogen fixation (BNF), the process by which atmospheric nitrogen (N_2) is converted into ammonia (NH_3), making it available for legumes plants. The nitrogen-fixing bacteria of the family *Rhizobiaceae*, including the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* and *Bradyrhizobium*, are collectively referred to as rhizobia. In recent years, however other α- and β-proteobacteria have been shown to produce nodules in legumes. However, the rhizobium–*P. lunatus* symbiosis has scarcely been studied. Recently, some studies have been conducted to evaluate the genetic variability of the rhizobium–*P. lunatus* symbiosis. These studies have evaluated phenotypic and molecular diversity of rhizobia isolated from soils of several countries. This review describes the main research results in this field.

Keywords: Biological Nitrogen Fixation, indigenous strain, legumes, nodulation, rhizobial community diversity
Abbreviations: BNF, Biological Nitrogen Fixation; **PEPC**, phosphoenolpyruvate carboxylase; **GS**, glutamine synthetase; **PCR**, polymerase chain reaction; **RFLP**, restriction fragment length polymorphism

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PHASEOLUS LUNATUS: IMPORTANCE

The genus *Phaseolus* includes five grain legumes of worldwide or regional economic importance, and approximately 50 species, all of neotropical origin (Delgado Salinas 1985). Among these, *P. lunatus* (Lima bean), *P. vulgaris* (common bean), *P. coccineus* (scarlet runner bean) and *P. acutifoliu* (tepary bean) were domesticated by prehispanic civilizations and are widely used for human consumption. Lima bean (*P. lunatus*) is a legume, originally from Peru. Lima bean is the second most economically important species of *Phaseolus* and one of the 12 primary grain legumes (Fofana *et al.* 1999).

The available archaeological evidence supports the hypothesis that the small-seeded Lima beans were domesticated in Mesoamerica while the large-seeded types were domesticated in South America. Based on a review of the available information, Salgado *et al.* (1995) showed that wild forms can be divided into two groups. One is distributed in the lowlands of eastern South America, stretching from the Caribbean coast, through Brazil and eastern Peru, to Salta, Argentina. The other group is distributed in the western Andes, in Ecuador and northern Peru.

The Lima bean is an annual or short-lived perennial species, with a mixed mating system that is predominantly autogamous but without crossing levels up to 48% (Baudoin *et al.* 1998). Wild individuals are characterized by indeterminate climbing growth, a prolonged flowering period, and production of a large number of pods (Zoro Bi *et al.* 2003). Pole type cultivar and wild form of *P. lunatus* are twining, perennial herbs, 2-4 m tall, with an enlarged rootstock (Purseglove 1974). Annual and small bush forms, 30-90 cm high, have been developed in cultivation. Flower size is smaller than that of *P. vulgaris* or *P. coccineus*. Flower color is usually pale green, occasionally violet (Beyra and Artiles 2004). Seeds are very variable in size, shape and color, 1-3 cm long, ranging from flat-seeded types to rounded seeds of potato types with cream, red, purple, brown or black, and various types of mottled color. The hilum is white with translucent lines radiating from it to outer edge of testa. The pod is oblong, generally recurved, 5-12 × 1.5-2.5 cm with 2 to 4 seeds per pod (Vieira 1992).

Lima bean is one of the most important crops associated with other legumes and non-legumes in region Northwest of Brazil. Although this crop shows a higher capacity of adaptation, compared to common bean, the cultivation of Lima

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bean has little relevance. The main reason for the limited cultivation is due the larger tradition in the consumption of common bean. However, the rusticity of Lima bean and its capacity to resist to long dry periods are important characteristics for semi-arid regions, as the Northwest of Brazil, and can increase the economical and social importance of the crop (Azevedo *et al.* 2003). The grain of Lima bean has high protein content and can be used for human alimentation, decreasing the dependence on common bean (Vieira 1992). According to Oliveira *et al.* (2004), the Lima bean is, actually, an alternative food source for human alimentation in the Northeast region of Brazil.

RHIZOBIA–LEGUMES SYMBIOSIS

Legumes can establish an agronomically and ecologically important symbiosis that leads to the development of a new plant organ (the legume nodule) in response to the appropriate nitrogen-fixing bacteria (Schultze and Kondorosi 1998). These bacteria invade root tissues and induce the formation of specialized structures known as nodules where they differentiate and fix atmospheric nitrogen which is supplied to the plant.

Biological Nitrogen Fixation (BNF) is the process by which atmospheric nitrogen (N_2) is converted into ammonia (NH_3) and is subsequently available for plants. In agricultural settings, perhaps 80% of this biologically fixed N_2 comes from symbioses involving leguminous plants and bacteria of the family Rhizobiaceae. The family Rhizobiaceae currently includes six genera: *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium*, and *Bradyrhizobium*, which are collectively referred to as rhizobia (Vance 1998). In recent years, however, other α-proteobacterias have been shown to produce nodules in the legume (Moulin *et al.* 2002) *Methylobacterium* (Sy *et al.* 2001); *Blastobacter* (Van Berkun and Eardly 2002), and *Devosia* (Rivas *et al.* 2002) and β-proteobacterias such as *Burkholderia* strain and *Ralstonia taiwanesis*, isolated from *Mimosa* (Chen *et al.* 2001, 2003, 2007).

It has been estimated that 1 g of soil may contain a community of 10^9 microorganisms, with rhizobia representing around 0.1% of the soil microorganisms or 10^6 rhizobia g^{-1} soil (Thies *et al.* 1991).

Nitrogen fixation by rhizobia is of great importance in agriculture in several ways. Legumes such as peas, beans, lentils, soybeans, alfalfa and clover help to feed the meat-producing animals of the world as well as humans. Crop yields are greatly improved in nodulated plants; legumes can also grow well in poor soils where there is not enough fixed nitrogen to support other types of plants. After harvest legume roots left in the soil decay, releasing organic nitrogen compounds for uptake by the next generation of plants. Farmers take advantage of this natural fertilization by rotating a leguminous crop with a non leguminous one. Symbiotic nitrogen fixation is of great importance not only in the production of leguminous crops but also in the global nitrogen cycle (Araújo *et al.* 2008).

The agronomic implications of this symbiosis have promoted research on biological nitrogen fixation and on the characterization of rhizobia (Fernández-Pascual *et al.* 2007). Rhizobia have the ability to infect the roots of legumes and to produce nodules. Legumes root nodules are highly specialized structures adapted to reduce and fix nitrogen gas for the benefit of the plant system as a whole, thus permitting excellent growth of nodulated legumes plants under nitrogen-deficient conditions. The differentiated forms of rhizobia in the nodule, called bacteroids, fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plant (Long 1989).

Nodule development

Nodulation is a highly host-specific interaction in which, with few exceptions, specific rhizobia strains infect a limited range of plant hosts. Nitrogen-fixing nodules are formed as a consequence of a series of interactions between rhizobia and leguminous host plants. Rhizobia approaching the root of compatible host plants respond to plant derived inducing compounds (usually flavonoids) by expressing their nodulation (nod) genes. Induction of these genes leads to the production and secretion of return signals, the nodulation factors (Nod signals), which are lipochitooligosaccharides of variable structure (Oldroyd *et al.* 2005). Nod factors induce root hair deformations, cortical cell divisions, and on some host plants, fully grown nodule-like structures (Denarie *et al.* 1996).

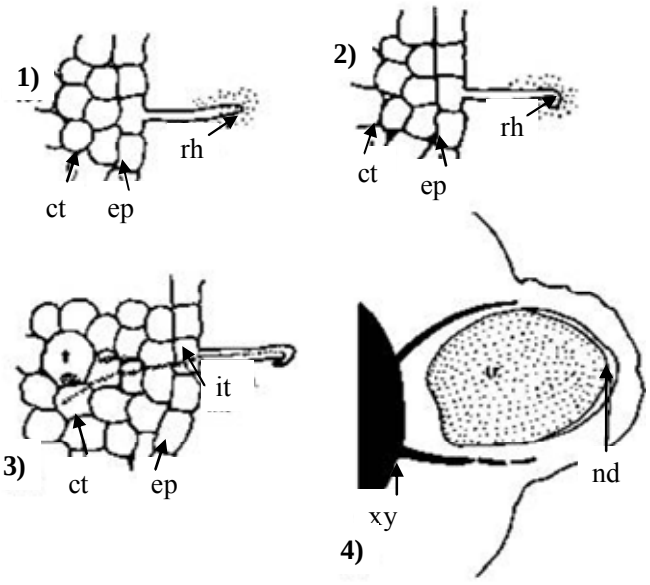

Fig. 1 General steps of nodule formation in legumes. Step 1) Multiplication of rhizobia (rh) in the root hair: cortex (ct) and epidermis (ep). Step 2) Infection of rhizobia (rh) to the root hair. Step 3) Formation of infection thread (it). Step 4) Formation of nodules (nd) connected with plant xylem (xy).

During the infection process, the bacteria enter the plant via the root epidermis and induce the reprogramming of root cortical cell development and the formation of a nodule. First, rhizobia infect the root hairs and form an infection thread that grows in to the inside of the root (**Fig. 1**). The rhizobia multiply inside the infection thread, so that the initial infection of a few bacteria can cause a large colony of bacteria to build up inside the plant. By the time the infection thread has penetrated only 3-6 radial cell layers of the cortex, divisions occur in a small group of inner-cortical cells directly ahead of the thread. The initial divisions in these cells are always anticlinal. These cells are invaded by the bacteria released from the infection thread, and subsequently differentiate into non-dividing bacteroid-containing cells. The infection thread continues to branch in the centre of the expanding meristematic area, and new cells are invaded by the bacteria.

As development proceeds, more cortical layers contribute to the nodule and the thread branches and extend backward towards the epidermis. In this way, the characteristics regions of the young nodule are delimited: a central bacteroid tissue, a peripheral, non-infected nodule cortex with a vascular system, and an apical meristem. The time of process of nodule development varies among different legumes host. For example, in cowpea, common-bean and soybean, both important legumes for tropical regions, the time of infection process and nodule development is approximately seven, nine and 15 days, respectively (Vargas *et al.* 1982; Hungria and Neves 1986; Xavier *et al.* 2007). For Lima bean, there is evidence that the time of infection process and nodule developments is approximately 20-30 days (Jardel O. Santos, pers. comm.). After nodule development and before the occurrence of fixation, nodulins, which are involved in biological nitrogen fixation, form. Nodulins are nodule-specific proteins synthesized in association with the

Table 1 Diversity of rhizobia nodulating some legumes in the world.

Rhizobia genus	Host legume	Location	Reference
Bradyrhizobium	*Glicine max* (soybean crop)	Brazil	Loureiro *et al*. 2006; Giongo *et al.* 2008
		Zimbabwe	Musiyima *et al.* 2005
	Inga derstediana (Inga tree)	Mexico	Grossman *et al.* 2005
Rhizobium	*Phaseolus vulgaris* (bean crop)	Brazil	Kashuk *et al*. 2006
	Pisum sativum (pea crop)	Canada	Vessey and Chemining'wa 2006
	Arachis hypogaea (peanut crop)	Argentina	Ibanez *et al.* 2008
Sinorhizobium Rhizobium	*Retana retam* (forage)	Tunisia	Mahdhi *et al*. 2008
	Sesbania spp. (Sesbania tree)	India	Sharma *et al*. 2005
Bradyrhizobium Mesorhizobium	*Albizia* spp. (Albizia tree)	China	Wang *et al.* 2006

symbiosis process established among leguminous and *Rhizobia*. These proteins play distinct by crucial functions during the biological nitrogen fixation of N_2 (Silveira *et al*. 1999). Nitrogenase is the principal nodulin involving in the BNF and catalyzes both the reduction of N_2 to ammonia and H+ to molecular hydrogen. Additionally, phosphoenolpyruvate carboxylase-PEPC (E.C.4.1.1.31) and glutamine synthetase-GS (E.C.6.3.1.2) are particularly important. PEPC supplies carbon skeletons and as a contributory factor for ATP synthesis, both essential for GS and nitrogenase activity (Rodrigues *et al*. 1998). GS is crucial in the assimilatory process of atmospheric nitrogen, as it catalyzes the first step in the conversion of inorganic nitrogen (ammonium) into its organic form (glutamine) (Gonnet and Diaz 2000). Some evidence indicates that there is synergism played by nitrogenase, PEPC and GS activities on N_2 fixation efficiency by legumes nodules.

The nitrogenase enzyme requires high rates of ATP from oxidative phosphorylation, a process which requires oxygen as the final electron receptor of the respiratory chain. However, nitrogenase (component II) is inactivated by an excess of oxygen, whist the expression of nif and fix genes requires a microaerobic environment (Soupene *et al.* 1995). This implies a very accurate control of oxygen supply to the bacteroids. This control is established in the nodule cortex by an oxygen diffusion barrier and in the infected cells by the oxygen-carrying leghemoglobin and bacteroids' respiration. The leghemoglobin provides a flux of O_2 for rhizobial respiration and maintains O_2 at a concentration that does not render the nitrogenase complex inactive (Appleby 1984).

DIVERSITY OF ISOLATES OF RHIZOBIA NODULATING *PHASEOLUS LUNATUS* IN TROPICAL SOILS

The studies of diversity of rhizobia are related to several legumes of the genera *Phaseolus*, *Medicago*, *Melilotus*, *Trifolium*, *Glycine*, etc. These studies have been conducted with molecular methods that provide the possibility to identify taxonomic groups of rhizobia in soils. Among the methods used, DNA-DNA homology based on quantitative hybridization is considered the standard method for the designation of species (Graham *et al*. 1991). On the other hands, the restriction fragment length polymorphism (RFLP) analysis of 16s rRNA amplified by the polymerase chain reaction (PCR) provides a simplified method for characterization of rhizobial isolates at the molecular level. Laguerre *et al.* (1994) examined 48 strains of *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* by this method, as wel1 as unclassified rhizobia from various host plants, and showed that species assignments were in full agreement with the established taxonomic classification.

A review published by McInnes *et al*. (2004) related the results founded in the world about diversity of rhizobia isolated in several host legumes. The authors observed that some rhizobia species shows low strain richness, for example *Rhizobium leguminosarum* bv. *trifolii* associated with *Trifolium pratense* and *Trifolium subterraneum*. On the other hand, for *R. etli* bv. *phaseoli* populations, there was greater strain richness at Mexican with *Phaseolus coccineus*, while that with *Phaseolus vulgaris*, there was a low strain richness. Additionally, the review shows that the some legume hosts exhibit strain specificity and others is more promiscuous to several rhizobial species, likely it occur for strain related with the legumes host. For example, Shantharam and Wong (1982) shown that *Rhizobium* sp., strain 127E15, which was originally isolated from effective Lima bean nodules was capable of forming effective nodules on the cowpea (*Vigna unguiculata* L., Walp. cv. 'California Blackeye'), lupine (*Lupinus angustifolius* L. cv. 'Frost'), the lima bean (*Phaseolus lunatus* L. cv. 'Henderson'), and partially effective nodules on commom bean (*Phaseolus vulgaris* L. 'cv. Kentucky Wonder').

In the last years, several papers have studied the diversity of rhizobia isolated in several host legumes (**Table 1**). The results show a great diversity of rhizobia in soil in the world, from tropical to temperate regions. For example, Mahdhi *et al*. (2008) investigated the diversity of *Retama raetam* root-nodule bacteria isolated from arid regions of Tunisia. The authors founded twelve isolates and, after characterization by 16S rRNA gene sequencing and phenotypic analysis, these isolates were assigned to *Sinorhizobium*, *Rhizobium* and *Agrobacterium*.

The literature in the world has showed that, among the legumes of genus *Phaseolus*, studies about nodulation and nitrogen fixation refers to symbiosis of the common bean (*P. vulgaris*) which form symbiosis with several species of the *Rhizobium* genus (Martinez-Romero *et al*. 1991; Souza *et al*. 1994; Martinez-Romero 2003). Lima bean plants are nodulated by fast and slow growing rhizobia groups. Studies about *P. lunatus–Rhizobia* symbiosis shows a great genetic diversity among the microsymbionts of *P. lunatus* has been found in a relatively wide collection from some geographic locations (**Table 2**) mainly in the South America. The center of diversity of Lima bean is located in Peru. In this location, some studies were conducted aiming to characterize the diversity of rhizobia symbiont of Lima bean (Ormeño-Orrillo *et al.* 2006, 2007).

The genetic diversity of native rhizobia isolated from Lima bean, in Peru, was conducted by Ormeño-Orrillo *et al*. (2006), who observed that Lima-bean are nodulated mainly by the genus *Bradyrhizobium*. The authors founded 21 alkali-producing isolates from Lima bean nodules. Nine (43%) were slow growers with their colonies reaching a size of 1–3 mm after 5–6 days of incubation on YEM medium, while the remaining 12 isolates (57%) were extra-slow growers with punctiform colonies (<1 mm) visible only after 7–10 days of incubation. The phylogenetic diversity, using *nifH* and *nodB* gene sequences, showed that the Lima bean isolates were dispersed among three distinct

Table 2 Diversity of rhizobia isolated in the world, related to *P. lunatus*.

Rhizobia genus	Location	Reference
Rhizobium, *Sinorhizobium*	Peru	Ormeño *et al.* 2007
Bradyrhizobium	Peru	Ormeño-Orrillo *et al.* 2006
Bradyrhizobium	USA	Thies *et al.* 1991
Rhizobium	USA	Triplett *et al.* 1981
Rhizobium, *Bradyrhizobium*, *Mesorhizobium*	Brazil	Santos 2008

clades. The results evidenced three distinct *nodB* genotypes linked to three *nifH* genotypes within *Bradyrhizobium* and establishing this species as a natural symbiont of Lima bean, in Peru.

However, a recent study conducted in the same location, Ormeño-Orrillo *et al*. (2007) observed growing species nodulating *P. lunatus* commonly belong to the genus *Rhizobium* and more rarely to *Sinorhizobium*. A strain, LMTR32, isolated from *P. lunatus* growing in Peru soils showed a high ability to solubilize bicalcium phosphate. The 16S rRNA sequence of this strain showed a 100% similarity with the type strain of *Sinorhizobium*.

In US soil, Thies *et al*. (1991) examined the degrees of specificity, in terms of both nodulation and effectiveness, exhibited by Lima bean using *Bradyrhizobium* spp. (nodulating cowpea). The results shows that 56-70% of the bradyrhizobia either failed to nodulate or formed ineffective nodules, 12-32% formed symbioses of moderate effectiveness, and only 7-18% were as effective.

On the other hand, the use of morpho-physiological characterization is important to preliminary determination of rhizobia diversity in soils. Using this characterization, Santos (2008) founded a great genetic diversity of rhizobia nodulating Lima bean, in a study conducted in Brazilian soils. In this study, rhizobia found in nodules of Lima bean genotypes were isolated and characterized. Among the isolates, 50 and 60 were fast- and slow-growers, respectively. There was isolates that produced alkali, suggesting a symbiosis with *Bradyrhizobium*. Based on morphological and physiological characterization, five and four groups of isolates formed based in the Tocher and UPGMA methods, respectively. Using the classification of Wang *et al*. (2008), the characteristic shown by isolates indicates species of genus *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* forming nodules in Lima bean in Brazilian soils.

CONCLUSIONS

Phaseolus lunatus is legume species important as a protein source for human nutrition. The plant is very resistant and has the capacity to resist long dry periods, important characteristics in tropical regions. It is important that existing breeding affords incorporate selection to maximize N_2 fixation in the *P. lunatus* legume-rhizobia symbiosis, and both host and microsymbiont must be considered when taking into account the importance of the cultivar × strain interaction in order to increase plant productivity and biological nitrogen fixation. In an agricultural setting, perhaps 80% of this biologically fixed N_2 comes from symbioses involving leguminous plants and bacteria of the family Rhizobiaceae. The studies published have showed, mainly in tropical soils, a great diversity of rhizobia nodulating Lima bean and there are symbionts from the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. In this sense, there is the need to study the isolation and evaluation of the efficiency of strains aiming to increase plant growth and yield.

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