



Diversity of *Rhizobium-Phaseolus vulgaris* symbiosis: overview and perspectives

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Abstract

Common bean (*Phaseolus vulgaris*) has become a cosmopolitan crop, but was originally domesticated in the Americas and has been grown in Latin America for several thousand years. Consequently an enormous diversity of bean nodulating bacteria have developed and in the centers of origin the predominant species in bean nodules is *R. etli*. In some areas of Latin America, inoculation, which normally promotes nodulation and nitrogen fixation is hampered by the prevalence of native strains. Many other species in addition to *R. etli* have been found in bean nodules in regions where bean has been introduced. Some of these species such as *R. leguminosarum* bv. *phaseoli*, *R. gallicum* bv. *phaseoli* and *R. giardinii* bv. *phaseoli* might have arisen by acquiring the phaseoli plasmid from *R. etli*. Others, like *R. tropici*, are well adapted to acid soils and high temperatures and are good inoculants for bean under these conditions. The large number of rhizobia species capable of nodulating bean supports that bean is a promiscuous host and a diversity of bean-rhizobia interactions exists. Large ranges of dinitrogen fixing capabilities have been documented among bean cultivars and commercial beans have the lowest values among legume crops. Knowledge on bean symbiosis is still incipient but could help to improve bean biological nitrogen fixation.

Introduction

About 50 *Phaseolus* species are known (Maréchal et al., 1978) but only a few, such as *P. vulgaris*, *P. coccineus*, *P. acutifolius*, *P. polyanthus*, and *P. lunatus* are domesticated and used as food. Amongst *Phaseolus* the common bean (*P. vulgaris*) is the most extensively cultivated. In developing countries, most beans are grown to feed the local population, where they constitute an important source of dietary protein. The main bean producing countries are Brazil, China, India, Mexico and the USA, where yields vary between 1.65 tonne ha⁻¹ in the USA, to 0.7 tonne ha⁻¹ in Brazil and 0.56 tonne ha⁻¹ in Mexico (<http://apps.fao.org>). Usually, the lower yields can be ascribed to inadequate water supplies, diseases, pests and poor soils. Different sites of origin of *Phaseolus vulgaris* have been located in Mesoamerica (middle America), and in

the Andean region of South America in Northwestern Argentina, in Ecuador and in Northern Peru (Gepts, 1990; Gepts and Debouck, 1991; Tohme et al., 1995). Wild *P. vulgaris* plants exist in Mesoamerica and Argentina (see Singh et al., 1991). Wild bean varieties are tiny climbing plants with very small seeds. Domestication of beans was originally dated over 7000 years ago (Gepts and Debouck, 1991), but more recent analysis indicated that it began around 4000 years ago (Kaplan and Lynch, 1999) by selecting amongst wild *P. vulgaris* ecotypes for larger seeds and plants. Possibly beans were co-domesticated with maize in Mesoamerica, since both are grown in association in traditional agriculture in Mexico and in Peru, where the beans climb over the maize plants. Traditional agricultural practices that include intercropping, seem to promote bean nitrogen fixation and their advantages have been recognized (Andrews and Kassam, 1976; Baudoin et al., 1997; Francis, 1986; Souza et al., 1997; Wooley and Davis, 1991). In the 20th century, agronomists greatly modified traditional agricultural

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practices by selecting for bushy beans in monoculture in the presence of N chemical fertilizer. Most probably, beans with a poor capacity to fix N₂ were selected with the result that the percent nitrogen derived from biological fixation is the lowest among legumes (Isoi and Yoshida, 1991; reviewed in Hardarson, 1994). Graham and Halliday (1976) established good correlations between the growth habit of beans (climbing or bushy) and their nitrogen fixing capabilities, with higher nitrogen-fixing activities found in climbing beans. Levels of N₂ fixation in early maturing lines are lower than those of late-maturing lines (Wolyn et al., 1991), but although nitrogen fixation has been improved by breeding in early maturing lines (St. Clair and Bliss, 1991) these have not been widely used.

To enhance legume nodulation and nitrogen fixation, the introduction of bacterial inoculants to agricultural fields has been a common practice for over 100 years. Whenever the specific rhizobia are absent, inoculation readily enhances plant growth and yield (Singleton and Tavares, 1986; Streeter, 1994; reviews of Triplett and Sadowsky, 1992 and Vlassak and Vanderleyden, 1997). On the other hand, when native bacteria exist in the fields they often out-compete the inoculant strains that only occupy a small proportion of nodules as observed in some areas of Latin America (Aguilar et al., 2001; Burgos et al., 1999; Graham, 1981; Ramos and Boddey, 1987; review in Vlassak and Vanderleyden, 1997). Contrastingly, bean inoculation with *R. tropici* in Brazil has been successful (Hungria et al., 2000; Mostasso et al., 2002) and *Rhizobium* inoculated onto beans enhanced both bean and maize yields when the two were grown together in Peru (Pineda et al., 1994).

Diversity of rhizobia from bean nodules in nature and agricultural fields

The study of the diversity of rhizobia, besides providing valuable ecological information by defining host preferences and predominance of strains, the genetic relationships and structure of bacteria, the dynamics of exchange of genetic material and the basis for the proposal of evolutionary trends, also provides the source of efficient strains to be used in inoculation trials in agricultural fields. Diversity studies which highlight new models such as the novel legume nodulating *Burholderia* (Moulin et al., 2001) contribute to our knowledge of symbiosis.

Diversity of rhizobia isolated from *Phaseolus vulgaris* has been examined almost worldwide both in centers of origin of bean and in introduced areas with a variety of techniques and criteria. DNA hybridization revealed the then designated *R. phaseoli* as an heterogeneous group of bacteria (Crow et al., 1981). Two dimensional protein patterns (Roberts et al., 1980) and intrinsic antibiotic resistance (Beynon and Josey, 1980) also showed heterogeneity among these bacteria. The *nifH* gene hybridization patterns and nodulation host-range allowed the distinction of different types of strains from bean nodules in the Americas (Martínez et al., 1985). These strains exhibited a large genetic diversity in multilocus enzymes electrophoretic studies (Piñero et al., 1988). Representative strains from these studies (CFN42, CIAT899 and FL27) were later designated the type or reference strains of *R. tropici* (Martínez-Romero et al., 1991), *R. etli* bv. *phaseoli* (Segovia et al., 1993), and *R. gallicum* bv. *gallicum* (Sessitsch et al., 1997b).

In the three centers of bean domestication (Mexico, Ecuador-Peru and Argentina), *R. etli* bv. *phaseoli* has been found as the predominant nodule occupant and no *R. tropici* has been isolated from bean nodules. Likewise, *R. etli* bv. *mimosae* has not been isolated from bean nodules although it co-exists in soil with *R. etli* bv. *phaseoli*, at least in Mexico (Wang et al., 1999a). It is notable that neither *R. gallicum* bv. *phaseoli* nor *R. giardinii* bv. *phaseoli* have been reported up to now from bean nodules in the centers of origin of bean in the Americas. Occasionally bacteria other than *R. etli* have been encountered in bean nodules in Mexico and they correspond to *R. gallicum* (Silva et al., 2003). A large genetic diversity has been documented for *R. etli* bv. *phaseoli* from the domestication centers. In Mexico, large diversity indexes were obtained with H>0.6 and 1.3 or 1.1 strains per electropherotype (the latter in a limited geographical area) (Caballero-Mellado and Martínez-Romero, 1999; Segovia et al., 1991; Souza et al., 1994). Similarly, in Argentina no two isolates with identical patterns of REP-PCR were observed and the maximum degree of relatedness among isolates was about 90%. Bean nodule isolates from Ecuador and Peru proved to be very diverse and could be divided by Box A1R-PCR into two clusters distinct from the Mexican isolates (Bernal and Graham, 2001). Furthermore, in Mexico (Souza et al., 1994) and in Argentina (Aguilar et al., 1998) bean wild varieties (small seed beans) have been found to be naturally nodulated by diverse *R. etli* bv. *phaseoli* and in Mexico

R. etli bv. *phaseoli* have been found as maize endophytes in traditional agricultural fields where bean and maize are intercropped (Gutiérrez-Zamora and Martínez-Romero, 2001).

The genetic diversity observed in Colombian and Mexican isolates is so large that it could serve as the basis to propose new species (Caballero-Mellado and Martínez-Romero, 1999; Eardly et al., 1995; Segovia et al., 1991; Souza et al., 1994) but the fact that distantly related isolates share common 16S rRNA genes hampered such a proposal (Eardly et al., 1995). In addition, few isolates defined as *R. etli* by metabolic enzyme electropherotypes had 16S rRNA gene alleles of *R. leguminosarum* (Eardly et al., 1995), raising doubts on species limits or suggesting possible lateral transfer of 16S rRNA genes. Chromosomal recombination among locally related *R. etli* strains has also been considered to account for the large genetic variation observed in *R. etli* bv. *phaseoli* (Souza et al., 1992; Silva et al., 1999).

Sharp differences in symbiotic effectiveness exist within *R. etli* bv. *phaseoli* isolates (Burgos et al., 1999; Epping et al., 1994; Graham and Halliday, 1976; Oliveira and Graham, 1990; Rennie and Kemp, 1983; Vázquez-Arroyo et al., 1998) and these may be related to the large genetic differences observed in these bacteria and also to the co-adaptation of cultivar and bacteria (see later). In *R. etli*, plasmids in addition to the symbiotic plasmid have a role in bean symbiosis (Brom et al., 1992) and they may be transferred among bacteria.

It has been observed that when *R. etli* bv. *phaseoli* populations are low and there are high numbers of other rhizobia, then bean nodules may contain strains other than *R. etli*. This has been the case of bean cultivated in Minnesota fields, where they were nodulated by *Dalea* rhizobia (Bernal and Graham, 2001) or of beans grown in fields where *Leucaena* trees were present and from which *R. gallicum* bv. *gallicum* strain FL27 was obtained (FL stands for Frijol, bean in Spanish, grown in *Leucaena* tree soils) (Martínez et al., 1985), or from sites where beans are introduced that may have scarce or no *R. etli* bv. *phaseoli* populations (see below).

Outside of their sites of origin, where *P. vulgaris* bean has been introduced, nodule isolates have been characterized as well. It seems that in some of introduced sites bean is nodulated by other species in addition to *R. etli* bv. *phaseoli* and the co-occurrence of several species is common (Table 1), although not in the same bean nodule.

In Brazil (Hungria et al., 2000; Martínez-Romero et al., 1991) and in Senegal and Gambia (Diouf et al., 2000), *R. tropici* and *R. etli* bv. *phaseoli* have been found as bean nodule occupants. Additionally *R. giardinii* and *R. leguminosarum* have been detected in bean nodules in Brazil (Mostasso et al., 2002). In Senegal and Gambia, the dominant species in bean nodules was *R. etli* (Diouf et al., 2000) and even in acid soils in some areas of Brazil (Mostasso et al., 2002) the most prominent species was found to be *R. etli* but the strains did not seem to be symbiotically effective.

R. tropici strains are more successful than *R. etli* at nodulating bean in acid conditions (Graham et al., 1994; Vargas and Graham, 1988; 1989) and more tolerant to high temperatures (Hungria et al., 1993; Martínez-Romero et al., 1991; Michiels et al., 1994; Mostasso et al., 2002; Pinto et al., 1998). *R. tropici* strains also seem to be well adapted to sandy soils (Acosta-Durán and Martínez-Romero, 2002; Vargas and Graham, 1989). Originally *R. tropici* were recovered from bean nodules from plants in acid soils in Colombia and Brazil (Martínez-Romero et al., 1991). Two types of *R. tropici* have been clearly distinguished (Table 2) that seem to be diverging lineages that share a common symbiotic plasmid (Geniaux et al., 1995; Martínez-Romero, 1996) but there are also *R. tropici* strains with intermediate characteristics which do not fall into either type A or type B (Hungria et al., 2000; Martínez-Romero, 1996; Mostasso et al., 2002). Type B strains are more diverse than type A strains when a similar number of strains were analyzed from each and a wide spectrum of symbiotic efficiency with bean has been found in *R. tropici* (Mostasso et al., 2002; Oliveira and Graham, 1990; Oliveira et al., 1998) with highly effective strains belonging either to type B (Aguilar et al., 2001; Montealegre et al., 1995), type A (S M Tsai, personal communication) or intermediate types (Mostasso et al., 2002). In Morocco, *R. tropici* type B and other rhizobia were obtained from bean nodules and, some of them were highly tolerant to high salt concentrations (Priefer et al., 2001). *R. tropici* has been catalogued as an opportunistic nodulator of bean having a broad host range (Hernández-Lucas et al., 1995), recently *R. tropici* type A and type B have been isolated from *Gliricidia sepium*, a tropical tree from the Papilionoideae native of the Americas (Acosta-Durán and Martínez-Romero, 2002), that is proposed to be a natural host of *R. tropici*.

The limited genetic diversity encountered in bean isolates (*R. etli* and *R. tropici*) in Senegal and Gambia has been related to the fact that bean is an introduced

Table 1. *Rhizobium* species isolated from *Phaseolus vulgaris* bean nodules

	Site of isolation ^a
<i>R. etli</i> (Segovia et al., 1993)	Mexico , Colombia, Ecuador-Peru , Argentina , Brazil, Senegal, Gambia, Tunisia ^b Spain, Austria, USA
<i>R. tropici</i> (Martínez-Romero et al., 1991)	Brazil (type A, B and others), Colombia (type B), France (type A), Morocco, Kenya, Senegal and Gambia (type B)
<i>R. leguminosarum</i> bv. <i>phaseoli</i> (Jordan, 1984)	England, France, Spain, Colombia, Brazil, Tunisia ^b
<i>R. gallicum</i> (Amarger et al., 1997)	France, Austria, Mexico (bv. <i>gallicum</i> only), Tunisia, Spain
<i>R. giardinii</i> (Amarger et al., 1997)	France, Spain, Brazil

^aCenters of origin are bold.

^bMhamdi et al., 1999

crop in Africa (Diouf et al., 2000). Similarly in England (Young, 1985) and France (Laguerre et al., 1993) *R. leguminosarum* bv. *phaseoli* strains have a narrow genetic diversity that was correlative to beans being an introduced crop in Europe (Laguerre et al., 1993). In France (Laguerre et al., 1993) and Spain (Velázquez et al., 2001) some bean nodule isolates have been found to be very similar to *R. leguminosarum* bv. *viciae* and bv. *trifolii*. *R. leguminosarum* bv. *phaseoli* strains possess a symbiotic plasmid that has characteristics similar to those of the *R. etli* symbiotic plasmid suggesting that the *R. etli* symbiotic plasmid was transferred from *R. etli* bv. *phaseoli* to *R. leguminosarum* when beans were introduced to Europe (Segovia et al., 1993). The presence of *R. etli* bv. *phaseoli* on bean seeds suggests a dispersal mechanism of rhizobia (Pérez-Ramírez et al., 1998) with the bacteria being unadvisedly introduced at the same time as the seed.

The co-occurrence of *R. leguminosarum* bv. *phaseoli* and *R. tropici* type A strains was observed in three sites in France with acidic sandy soils, with *R. leguminosarum* occupying from 2% to 100% of the nodules in four sites sampled. Using plasmid profiles, dominant isolates were identified in each site, but not the same isolate was dominant in all sites (Amarger et al., 1994). In France, temporal variations (from year to year) for the presence of different *Rhizobium* species was also observed with complete absence of *R. gallicum* and *R. giardinii* when some years before *R. giardinii* formed one third of the bean nodules (Laguerre et al., 1996). Genetic relatedness was found among some Austrian, Mexican (Sessitsch et al., 1997b) and French (Amarger et al., 1997) isolates from bean nodules and they were all ascribed to *R. gallicum* in spite of showing some differences

Table 2. Characteristics of the different *R. tropici* types^a

	A	B
Colony morphology in YM medium	wet, translucent	opaque
Colony morphology in PY medium	flat, pearly	white creamy
Motility	-	+
Growth in LB	-	+
Growth at 37 °C	-	+
Acid tolerance (pH 4.5)	+	+
Tolerance to heavy metals and high salt concentration	-	+
Induction of Nod factor by genistein	+	-
Presence of a 200 kb plasmid (pb)	+	-
Presence of <i>teu</i> genes (bean exudate uptake genes) in pSym	+	-
Presence of <i>teu</i> genes in plasmid a	+	+
Presence of <i>exo</i> genes in megaplasmid	+	+
<i>nifH</i> gene hybridization band in EcoR1 digests	8 kb	8 kb
Citrate synthase gene in pSym	+	+
Glutamine synthetase II electromorphs	a	b

^aData taken from Martínez-Romero et al. (1991); Pardo et al. (1994); Geniaux et al. (1995); Rosenblueth et al. (1998); Priefer et al. (2001); Taboada et al. 1996; Martínez-Romero (unpublished).

in total DNA hybridizations (Sessitsch et al., 1997b). In the Austrian soil mentioned *R. etli* strains were encountered as well (Sessitsch et al., 1997a) even though there were no records of bean crops in the soil.

A predominance of *R. etli* bv. *phaseoli* has been found in bean nodules in Spain (Herrera Cervera et al., 1999; Rodríguez Navarro et al., 2000). Remarkably, in a single soil in Spain five rhizobial species (*R. etli*, *R. leguminosarum*, *R. gallicum*, *R. giardinii* and *S. fredii*-like strains) were found to nodulate *P. vulgaris* (Herrera-Cervera et al., 1999). *R. etli* and a majority of all the isolates including the *R. gal-*

licum and *R. giardinii* isolates were found to carry 3 copies of *nifH* genes indicating that they belonged to the biovar phaseoli (Herrera-Cervera et al., 1999). Thus, it is considered that this soil harbors the possible donor and putative receptors in the transfer of the symbiotic plasmid from *R. etli* bv. *phaseoli* to the European resident bacteria *R. gallicum*, *R. giardinii* and *R. leguminosarum* as mentioned above. The *S. fredii*-like strains isolated from bean did not nodulate soybean (Herrera-Cervera et al., 1999) and additional analysis are required to clarify their taxonomic status. Interestingly, the bean *Sinorhizobium* isolates had *nod* genes that resembled those of the phaseoli group suggesting that they were acquired by lateral transfer (Laguerre et al., 2001).

Rhizobium populations in nodules may be largely determined by the environmental conditions or the agricultural practices (Palmer and Young, 2000). The pH of the soil seems to select the type of species that is successful for bean nodulation (Anyango et al., 1995; Giller et al., 1994; Martínez-Romero et al., 1991). Chemical fertilization in doses normally recommended to be used in agricultural fields diminished the genetic diversity encountered in bean nodules (Caballero-Mellado and Martínez-Romero, 1999).

Most diversity studies have addressed the analysis of isolates from bean nodules. Bean rhizosphere isolates in Mexico were mainly non-nodulating *R. etli* most probably lacking the symbiotic plasmid. Rhizosphere isolates exhibited a large genetic diversity that encompassed nodule isolates (Segovia et al., 1991). In contrast to this large diversity, *R. etli* bv. *phaseoli* strains found on bean seeds had a limited genetic diversity suggesting that only some clones survive desiccation stress on seeds (Pérez-Ramírez et al., 1998). To better sample soil diversity, DNA extracted directly from soil was used to amplify *nodD* genes by PCR using primers derived from *R. trifoli nodD* gene sequence and the diversity of *nodD* gene sequences revealed in soil was larger than that found from clover nodules (Zezé et al., 2001). Probably the soils in the bean domestication centers contain even more diverse bean nodulating bacteria than the populations analyzed from bean nodules.

In conclusion, the diversity studies reveal that there is no unique *Rhizobium* strain highly adaptable and efficient for all soils, environmental conditions and bean genotypes. Rhizobia best fitted to nodulate under certain conditions may be recruited every time from the genetic pool of soil rhizobia. Periodic selection

of *R. etli* bv. *phaseoli* in nodules could enrich for better adapted bacteria for a region and cultivar and may constitute the basis for co-evolution. The poor inoculation responses obtained in Latin America in agricultural fields (Aguilar et al., 2001; Burgos et al., 1999; Graham and Halliday, 1976; Graham, 1981; Vieira, 1994; Vlassak and Vanderleyden, 1997; review in Montealegre and Graham, 1996 and in Vlassak et al., 1997) seem to relate to the large and diverse populations of well adapted bean nodulating bacteria encountered therein. The *Rhizobium* species isolated from bean nodules are listed in Table 1.

Diversity of rhizobia nodulating bean in experimental assays

In the laboratory under axenic conditions, *P. vulgaris* is nodulated by many more *Rhizobium* species than in agricultural fields, but they do so with different degrees of efficiency. Host range may depend on the bean cultivar tested and the conditions tested. Taking into account the large number of rhizobia nodulating *P. vulgaris* (Table 3), this species has been recognized as a promiscuous host (Bromfield and Barran, 1990; Martínez et al., 1985; Michiels et al., 1998a) like other species in the Phaseoleae such as *Macropodium* (Bromfield and Barran, 1990) and *Vigna* species (Pueppke and Broughton, 1999). Paradoxically, it has been proposed that in nature bean has some selectivity for *R. etli* bv. *phaseoli* (Aguilar et al., 2001), alternatively we may propose that it was *R. etli* bv. *phaseoli* that developed specificity and competitiveness for bean symbiosis and not the plant, this hypothesis seems consistent with the results obtained by Laguerre et al. (2001) showing a predominance of phaseoli type *nod* genes in different bacterial backgrounds. A corollarium is that the traits that confer nodulation specificity such as those that evolved in the Galeoid phylum of legumes (Yang et al., 1999) did not evolve in the Phaseoleae.

Even though bean is promiscuous, bean plants seem to have some degree of preference for certain rhizobia (Pacovsky et al., 1984). It has been considered that the low effectivity in bean-*Rhizobium* symbiosis frequently observed may be due to the misspairing of host plant and bacteria (Bernal and Graham, 2001). Ecuatorian and Mexican beans, when used as traps, selected different *R. etli* strains both from Ecuatorian and Mexican soils (Bernal and Graham, 2001) and efficiency in nodulation and nitrogen fixation was higher when both partners were from the

Table 3. *Rhizobium* species and strains tested in *Phaseolus vulgaris* plant assays

	Nodulation	N ₂ fixation	Reference
<i>Rhizobium etli</i>	+	+	Segovia et al. (1993)
<i>Rhizobium tropici</i>	+	+	Martínez-Romero et al. (1991)
<i>R. leguminosarum</i> bv. <i>phaseoli</i>	+	+	Jordan (1984)
<i>R. leguminosarum</i> bv. <i>viciae</i>	+/-	-	Michiels et al. (1998a)
<i>R. leguminosarum</i> bv. <i>trifolii</i>	+	+	Michiels et al. (1998a)
<i>R. hainanense</i>	-		Chen et al. (1997)
<i>R. mongolense</i>	+	+	van Berkum et al. (1998)
<i>R. gallicum</i>	+	+	Amarger et al. (1997)
<i>R. galegae</i>	-	-	Michiels et al. (1998a)
<i>R. huautlense</i>	-		Wang et al. (1998)
<i>R. giardinii</i>	+	-/+	Amarger et al. (1997)
<i>Sinorhizobium fredii</i>	+	+ or -	Sadowsky et al. (1988); Pueppke and Broughton (1999)
<i>Sinorhizobium meliloti</i>	+	+ or -	Michiels et al. (1998a); Bromfield and Barran (1990)
<i>Sinorhizobium saheli</i>	-	-	Michiels et al. (1998a; this work)
<i>Sinorhizobium terangae</i> ORS51	+	-	This work ^a
<i>Sinorhizobium arboris</i> HAMB1 1396	+	+	This work ^a
<i>Sinorhizobium kostiense</i> HAMB1 1476	+	-/+	This work ^a
<i>Sinorhizobium</i> sp. BR816	+	+	Laeremans et al. (1997)
<i>Sinorhizobium</i> sp. NGR234	+	+	Pueppke and Broughton (1999)
<i>Mesorhizobium loti</i>	+/-	-	Michiels et al. (1998a)
<i>Mesorhizobium huakuii</i>	+/- ^b	+	Chen et al. (1991)
<i>Mesorhizobium amorphae</i>	-		Wang et al. (1999b)
<i>Mesorhizobium tianshanense</i>	-		Chen et al. (1995)
<i>Bradyrhizobium japonicum</i>	+	-	Michiels et al. (1998a)
<i>Bradyrhizobium</i> sp. (<i>Phaseolus lunatus</i>)	+	+	Ormeño (personal commun.)
<i>Bradyrhizobium elkani</i>	+	-	Ormeño (personal commun.)
<i>Bradyrhizobium</i> sp. (Lespedeza) B038	+	-	This work ^a
<i>Azorhizobium caulinodans</i>	+/-	-	Michiels et al. (1998a)

Bean cultivar used were: Limburgse vroege or Carioca (Michiels et al., 1998a), Negro Xamapa (Segovia et al., 1993; Martínez-Romero et al., 1991; Wang et al., 1998), N-8-116 (Laeremans et al., 1997), Hilds Marona (Pueppke and Broughton, 1999), Vernandon (Amarger et al., 1997), PI 209483 and others (Sadowsky et al., 1988).

^a Bean nodulation was assayed with cultivar Negro Xamapa in 250 ml Erlenmeyer flasks with cotton and Fahraues as described (Pérez-Ramírez et al., 1998). Acetylene reduction activity was measured to determine fixation.

^b Not all plants were nodulated.

same region. While Andean cultivars form large number of nodules with *R. tropici* strains (Nodari et al., 1993), Mesoamerican beans with high capacities to fix nitrogen nodulated poorly with *R. tropici* strains and in these beans *R. tropici* blocked *R. etli* nodulation when both strains were tested together (Martínez-Romero et al., 1998). In other legumes, blocking of nodulation by some strains was observed before (Dowling et al., 1989).

In several cases, *R. etli* bv. *phaseoli* has been found to be more competitive for bean nodule formation than *R. tropici* (Anyango et al., 1998; Borthakur and Gao, 1996; Martínez-Romero and Rosenblueth, 1990). In contrast, in conditions when *R. etli* bv. *phaseoli* is not

adapted, such as in acidity, then *R. etli* bv. *phaseoli* strains are not more competitive than selected *R. tropici* strains (Vargas and Graham, 1989; Wolff et al., 1991). In some cases, even in acid soils the competitiveness of *R. tropici* was enhanced but only formed 35% of bean nodules (Anyango et al., 1998). In bean, *R. tropici* type A isolates from France were moderately effective or ineffective while *R. leguminosarum* bv. *phaseoli* isolates were effective. While the Mesoamerican bean cultivar RAB39 nodulates preferentially with *R. tropici* (Montealegre et al., 1995), wild *P. vulgaris* accessions do not nodulate with some *R. tropici* strains (Kipe-Nolt et al., 1992).

Phaseolus coccineus and *P. vulgaris* share microsymbionts (Piñero et al., 1988; Souza et al., 1994) and in some cases in traditional agriculture both *Phaseolus* species are grown in the same area. *P. coccineus* is also nodulated by a cluster of *Rhizobium* different from *R. etli* bv. *phaseoli* that sometimes nodulates *P. vulgaris* in fields (Silva et al., 1999). *P. lunatus* (lima beans) are nodulated by *Bradyrhizobium* species (Ormeño et al., submitted) that also form nodules on *P. vulgaris* in the laboratory (Table 3). *P. acutifolius* is also naturally nodulated by *Bradyrhizobium* spp. (Somasegaran et al., 1991) or by *R. etli* bv. *phaseoli* (Martínez et al., 1985; Piñero et al., 1988). Some other non-domesticated *Phaseolus* species are nodulated by *Bradyrhizobium* (Parker, 2002).

May it be that domestication and other human selections of beans (for example in the presence of fertilizers, see Caballero-Mellado and Martínez-Romero, 1999) indirectly affected host range? Did domestication provide large densities of hosts so that *R. etli* bv. *phaseoli* evolved mechanisms to be better adapted to bean, sacrificing a larger host range as has been discussed previously (Wang et al., 1999a)? An strategy to select for more specific bean cultivars has been proposed (Rosas et al., 1998) alternatively, engineered rhizospheres of transgenic plants could favour nodulation by desired strains (Rossbach et al., 1995).

Diversity of rhizobia–bean interactions

Due to the diversity of rhizobia nodulating bean one may expect a pleyade of alternative bean-rhizobia interactions. It seems that *R. tropici* nodulation mechanisms or strategies are different in many ways from those used by the bv. *phaseoli* group, namely *R. etli*, *R. gallicum*, *R. giardinii* and *R. leguminosarum*, (Laeremans and Vanderleyden, 1998) and both *R. tropici* and bv. *phaseoli* nodulation strategies seem different from the bv. *gallicum* strategies although for the latter very little information is available. More information exists on *Sinorhizobium* sp. BR816, which was isolated from *Leucaena* and selected as a high temperature tolerant rhizobium strain for bean in Brazil (van Rhijn et al., 1993). BR816 has peculiar characteristics and different Nod factors that grant another strategy for bean interaction (Laeremans et al., 1997; Snoeck et al., 2001).

In *Rhizobium*, NodD is the most important transcriptional regulator of nodulation (Broughton et al., this volume; Perret et al., 2000; reviewed in van

Rhijn and Vanderleyden, 1995). Both *R. etli* (Girard et al., 1991), *R. tropici* (van Rhijn et al., 1994b), *R. leguminosarum* bv. *phaseoli* (Davis and Johnston, 1990) and *Sinorhizobium* sp. BR816 (van Rhijn et al., 1994a) contain multiple copies of *nodD* genes. The *nodD* gene region of *R. etli* bv. *phaseoli* was very similar to that by *R. leguminosarum* bv. *phaseoli* (Laguette et al., 1996). Some of the flavonoids produced by bean roots (Bolaños-Vásquez and Werner, 1997; Hungria et al., 1991a; see also Broughton et al., 2000) have been found to be the inducers of *nod* genes in the different *Rhizobium* species nodulating bean (Bolaños-Vásquez and Werner, 1997; Hungria et al., 1992; Sánchez et al., 1988; van Rhijn et al., 1994a,b) and anthocyanins were also inducers of *R. etli* bv. *phaseoli* *nod* genes (Hungria et al., 1991b; Hungria and Phillips, 1993).

Nod-factors, the key rhizobial molecules that simulate nodule formation (Dénarié et al., 1996; Relic et al., 1994), have been compared from the different species that nodulate bean (reviewed in Laeremans and Vanderleyden, 1998) and found to be heterogeneous, suggesting that bean may not require specific Nod factor structures for nodule formation although a hierarchy of Nod factor chemical modifications is implicated in most efficient nodulation (Laeremans et al., 1999). Other *R. etli* bv. *phaseoli* or *R. tropici* functions that play a role in the early interaction with beans have been identified. Lipo-polysaccharide (LPS) deficient mutants of *R. etli* bv. *phaseoli* do not persist in the infection threads (Cava et al., 1989). In *R. etli* bv. *phaseoli* an extrusion pump that eliminates phytoalexins is produced in the presence of the plant to prevent toxic accumulation of these compounds. *R. etli* bv. *phaseoli* mutants with impaired synthesis of the pump produce less nodules on bean roots (González-Pasayo and Martínez-Romero, 2000). In *R. tropici*, *R. etli* and *R. gallicum* bv. *phaseoli*, ABC transporters of root exuded molecules are required for optimal nodulation. Only bean roots produce these molecules and only rhizobia nodulating bean are capable of taking them up (Rosenblueth et al., 1998). *R. etli* bv. *phaseoli* survival on roots, mediated in part by the resistance to bean phytoalexins but also through the ability to use bean exudates, may determine the success of these bacteria in beans.

Approaches to improve bean nodulation capacity or competitiveness have been undertaken using the different *Rhizobium* species that nodulate bean. Random amplification of parts of the *R. tropici* genome can be used to construct strains more competitive for

nodule formation for bean as were obtained for *Macrorhizium atropurpureum* (Mavingui et al., 1997). Genetically modified *R. etli* bv. *phaseoli* strains with increased competitiveness for nodulation of beans have been constructed (Martínez-Romero and Rosenblueth, 1990). Introduction of a gene for trifolitoxin production into *R. etli* bv. *phaseoli* increased its symbiotic competitiveness against other strains (Robledo et al., 1998), but it remains to be seen if engineered strains can overcome the large diversity of strains existing in fields in Latin America. Quorum sensing mutants were also found to have an enhanced nodulation capacity (Rosemeyer et al., 1998). In *R. etli*, mutants unable to produce poly- β -hydroxybutyrate fixed more nitrogen (Cevallos et al., 1996). A glycogen synthase mutant of *R. tropici* enhanced nodulation and growth of beans (Marroquí et al., 2001).

As with other *Rhizobium* strains, *R. etli* bv. *phaseoli* contains two *nif* gene clusters (*nifHDK*) as well as an additional *nifH* gene (Quinto et al., 1985). Inactivation of the two *nifHDK* operons leads to mutants totally ineffective in nitrogen fixation (Romero et al., 1988). A strong promoter was identified upstream of the third *nifH* copy (Valderrama et al., 1996). Modified strains containing *nif*-genes with inter-changed promoters (e.g. with the strong *nifH* promoter) were constructed; that exhibited enhanced capacities to fix nitrogen (J. Mora, pers. comm.). Furthermore, re-arrangements of the symbiotic plasmid of *R. etli* lead to increased nitrogen fixation in beans (D. Romero, pers. comm.). Various complex modes of *nif* gene regulation seem to exist in different *R. etli* strains (D'hooghe et al., 1995; Girard et al., 2000; Michiels et al., 1994). Different copies of *rpoN* genes have been found in *R. etli* bv. *phaseoli* (Michiels et al., 1998b). An *R. etli* bv. *phaseoli* mutant with an increased capacity to produce cytochrome c terminal oxidase was found to fix more nitrogen (Miranda et al., 1996). Research on bean symbionts is providing a wealth of tools rendering strains that may be used in the field as inoculants to improve bean BNF.

On the other hand, large variations in N_2 fixation amongst bean cultivars has been shown and those with high capacities to fix nitrogen have been identified (Elizondo-Barrón et al., 1999; Peña-Cabriales and Castellanos, 1993; Peña-Cabriales et al., 1993). Some of them are being introduced in agricultural fields in Mexico.

Perspectives

Although a great deal of knowledge has been amassed concerning the diversity and genetics of bean symbionts, the basis of a successful inoculation and efficient nitrogen fixation remain elusive. Some of the problems of bean nodulation and symbiotic nitrogen fixation detected over 20 years ago (Graham, 1981) still exist today. Programmes to enhance bean BNF may benefit from fundamental observations on *Rhizobium* diversity and bean symbiosis genetics that are being made in many laboratories. Phosphate (P) nutrition should be also taken into consideration, since P availability limits N_2 fixation (Pereira and Bliss, 1987). Knowledge of the genetic determinants of bean phosphate transport and more studies on mycorrhiza would be required and this work will be simplified as mycorrhiza and *Rhizobium* apparently share common molecular mechanisms (Catoira et al., 2000).

As the nitrogen fixing capacity of some commercial beans is amongst the lowest of the widely cultivated legumes (see Hardarson, this volume), it is important that it be increased through crop management (Hungria and Vargas, 2000) and plant selection, especially considering those cultivars that have been identified with high capacities to fix nitrogen or by breeding bean lines (Attewell and Bliss, 1985; Bliss, 1985, 1993; Elizondo-Barrón et al., 1999) to obtain less dependence on chemical N fertilization. Beans with high capacity to fix nitrogen may then be used in combination with *Rhizobium* strains with superior capacities to fix nitrogen and compete with native strains. A strategy would be to improve N fixation capacity in the native strains well adapted to different regions highlighted in the bean rhizobia diversity studies. The improvement of bean nitrogen fixation is an important goal, biological nitrogen fixation not only lowers production costs but is also environmentally sound. The global advantages of nitrogen fixation in agriculture have often been emphasised (see Graham and Vance, 2000; Vance, 1998; this volume).

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