Plant and Soil **204:** 147–154, 1998.

Nitrogen fixation and nodule occupancy by native strains of *Rhizobium* on different cultivars of common bean (*Phaseolus vulgaris* L.)

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Key words: common bean, ^{15}N, *Phaseolus vulgaris*, rhizobial diversity

Abstract

A field experiment under rainfed conditions was conducted in Durango, México, to assess N_2-fixation of three cultivars of common bean (*Phaseolus vulgaris* L.) using ^{15}N-methodology. In addition, diversity of rhizobial isolates obtained from nodules of the different plant genotypes was evaluated by intrinsic antibiotic resistance (IAR), PCR using enterobacterial repetitive intergenic consensus (ERIC) primers, PCR-RFLP analysis of the 16S rRNA gene and multilocus enzyme electrophoresis (MLEE). Selected isolates were used to determine acetylene reduction and competitive ability under greenhouse conditions. The three cultivars tested did not show high variation in N_2-fixation, the %Ndfa values ranged from 19 to 26%. Variability in N_2-fixation efficiency among various native rhizobial isolates was very high and our results indicate that differences in competitive abilitiy exist also. PCR-RFLP of the 16S rRNA gene and MLEE revealed that most of the isolates belong to the species *Rhizobium etli*. Intrinsic antibiotic resistance analysis and ERIC-PCR showed high diversity among isolates. In contrast, our results using MLEE show low genetic diversity (H = 0.105).

Introduction

Phaseolus vulgaris L., common bean, represents the main source of protein for the Mexican people (SAGAR, 1995). Enhancement of its nitrogen-fixing capacity is thus a major agronomic goal. The capacity to fix N_2 is variable among genotypes of common bean, ranging from 4 to 59% nitrogen derived from the atmosphere (Hardarson et al., 1993; Peña-Cabriales and Castellanos, 1993; Peña-Cabriales et al., 1993). Breeding programmes with the objective of enhancing nitrogen fixation and yield of common bean are based on this variability. Crop responses to inoculation with selected strains of *Rhizobium* are often low, frequently due to the high competitive ability of native strains. In a survey conducted by Castellanos-Ramos et al. (1993), on field trials during the period 1972–1992, only 12% of the trials showed a statistically significant response to inoculation with elite strains. These observations as well as reports demonstrating the large number and diversity of rhizobial strains nodulating common bean in Mexican soils (Aguilar-Zacarías, 1990; Araujo et al., 1986; Piñero et al., 1988) suggest that the problem is complex. Thus, we decided (i) to assess N_2 fixation capacity of three cultivars of *Phaseolus vulgaris* under rainfed conditions using the ^{15}N methodology; (ii) to isolate and characterize indigenous rhizobial strains nodulating different plant genotypes in a region of México in which common bean production is high, and (iii) to determine N_2-fixation and competitive ability of selected isolates.

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Plant and Soil is the original source of publication of this article. It is recommended that this article is cited as: *Plant and Soil* **204**: 147–154, 1998.

Materials and methods

Field experiment and ^{15}N methodology

A field experiment was conducted at the experimental station of INIFAP in Fco. I. Madero Durango, Mexico. The sandy soil had a pH of 6.8 and the organic matter content was 1.04%. Three common bean (*Phaseolus vulgaris* L.) cultivars were planted: Flor de Mayo-M-38 (FM), Negro Querétaro (Q) and N-3-117 (N). Two non-fixing crops, barley and non-nodulating common bean (Nod-125; Davis et al., 1988), were used as reference crops for the ^{15}N analysis. The experiment was set up using a randomized block design with four replicates. Each genotype was planted in 5 m rows with 10 cm spacing between plants and 76 cm between rows and a ^{15}N-labelled subplot of 1.5 m rows. After planting, 10 kg N ha^{-1} as ammonium sulphate solution (10.075% ^{15}N atom excess) was applied to the labelled plots. The plots were harvested at physiological maturity (96 days after planting) and the plants were separated into straw and seed, which together were taken to represent total dry matter production. Samples were milled and the nitrogen content was determined by the Kjehldahl procedure (FAO/OIEA, 1987). The percentage nitrogen fixed was calculated using the ^{15}N dilution method (Hardarson, 1990) based on the ^{15}N atom excess data obtained by emission spectrometry.

Strain isolation

A total of 53 *Rhizobium* isolates from eight bean plants were obtained as described by Somasegaran and Hoben (1985) from nodules isolated from flowering plants. Nodules were immersed for 30 s in 70% ethanol, transferred to 0.1% $HgCl_2$, soaked for 3 min and rinsed five times with sterile water. Then, the nodules were crushed with a glass rod in a drop of sterile water. Loops of the nodule suspension were streaked out on yeast mannitol agar (YMA) plates containing Congo red or Bromothymol blue.

Intrinsic antibiotic resistance patterns (IAR)

Each isolate was grown in yeast mannitol broth (YMB) for 24 h at 30 °C. 100 μL of each culture were plated on a YMA plate. Multidisks (12 Sensidisks; Sanofi, Diagnostico Pasteur, Mexico) were placed on the surface and incubated at 28 °C for 24–48 h. The antibiotics used were: amikacyn 30 μg, ampicillin 10 μg, cephalotine 30 μg, cephatrioxone 30 μg, chloramphenicol 30 μg, dicloxaciline 1 μg, enoxacine 10 μg, erythromycin 15 μg, gentamycin 10 μg, netilmycin 30 μg, penicillin 10 U, trimethropin-sulphafurzaole 25 μg.

Isolation of template DNA for polymerase chain reactions (PCR)

A loop of cells grown overnight was resuspended in 50 μL TE, held at -70 °C for 4 min, placed on ice for 1 min and put for 2 min in a boiling waterbath. Then, the cells were put on ice for 1 min and boiled for 2 min. Finally, the cell debris was centrifuged at 12000 rpm for 2 min, and the supernatant was used for the PCR reactions (Sessitsch et al., 1997).

PCR using repetitive primers

Enterobacterial repetitive intergenic consensus (ERIC) primers (de Bruijn, 1992) were used to fingerprint native rhizobial strains. PCR amplifications were performed in a total reaction volume of 25 μL containing 1 $\times$ PCR reaction buffer (50 m*m* KCl; 20 m*M* Tris HCl, pH 8.0), 200 μM each of dATP, dCTP, dGTP and dTTP (Pharmacia-LKB), 3 m*M* $MgCl_2$, 4 μM ERIC2 primer, 2 μL of cell extract and 2 U Taq DNA polymerase (Gibco, BRL). All amplifications were performed with a Perkin-Elmer thermocycler (Gene-Amp PCR System 9600) using the following temperature cycle: initial denaturation at 95 °C for 1 min, followed by 30 cycles of 20 s denaturation at 94 °C, 30 s annealing at 40 °C and 2 min extension at 72 °C. The total reaction volumes were examined by electrophoresis on 1.5% agarose gels.

PCR-RFLP analysis of the 16S rRNA gene

PCR amplification of the 16S rRNA gene and the RFLP analysis was performed as described by Laguerre et al. (1994). PCR conditions were as outlined above using a 100 μL reaction volume with 8 μL cell extract and 0.1 μM primers rD1 and fD1 (Weisburg et al., 1991). Initial denaturation for 1 min at 95 °C was followed by 30 cycles of 50 s denaturation at 94 °C, 1 min annealing at 48 °C and 2 min extension at 72 °C and a final extension step of 4 min at 72 °C. Aliquots of 13 μL of the amplified DNA were digested with the restriction enzymes *Hae*III, *Alu*I, *Taq*I and *Msp*I (Gibco, BRL). The resulting DNA fragments were analyzed by horizontal agarose gel electrophoresis in 2.5% agarose gels.

Multilocus enzyme electrophoresis (MLEE)

Starch gel electrophoresis and selective staining of enzymes was done as described by Selander et al. (1986). Seven enzymes were assayed as follows:

Table 1. Nodule number, % N derived from atmosphere (%Ndfa), % N derived from fertilizer (%Ndff) and % N derived from soil (%Ndfs) in three cultivars of common bean (*Phaseolus vulgaris* L.) grown in Francisco I., Madero Durango, Mexico, during the spring and summer 1994

Cultivar	%Ndfa	%Ndff	%Ndfs	Nodule number
Negro Queretaro	26.5[a]	7.6[a]	65.9[a]	82[a]
FM-M-38	18.6[a]	8.6[a]	72.8[a]	52[b]
N-3-117	20.1[a]	8.7[a]	71.2[a]	48[b]

Means (within one column) which are not significantly different from each other at $P = 0.05$ share the same letters as superscripts.

NAD-malate dehydrogenase (1.1.1.38) and xanthine-dehydrogenase (1.1.1.204) in tris-citrate, pH 6.7; isocitrate dehydrogenase (1.1.1.42) and glucose-6-phosphate dehydrogenase (1.1.1.49) in tris-citrate, pH 8.0, phosphoglucomutase (2.7.6.1) and indophenol oxidase (1.11.1.7) in borate, pH 8.2, D-L alanine dehydrogenase (1.4.1.1) in tris-acetate, pH 7.5.

Mobility variants (electromorphs) of each enzyme, numbered in order of decreasing anodal mobility were equated with alleles of the corresponding structural gene. Electromorphic profiles for the seven enzymes (ETs) were equated with multilocus genotypes.

Acetylene reduction activity

Rhizobial isolates representing the nine patterns obtained by ERIC-PCR were grown in YEM broth for 24 h. Seeds of the three cultivars were inoculated with 9×10^8 cells per seed of selected rhizobial isolates (N7, N11, N13, F3, F4, F11, Q14, Q17 and Q21). Plants were grown in Leonard jars using sand as support and a nitrogen-free nutrient solution (Vincent, 1970) and they were removed 47 days after planting. The roots were placed in 650 mL glass containers to assess the acetylene reduction using the technique described by Dart et al. (1972). Acetylene reduction activity was determined using a gas chromatograph with a flame ionization detector and a type N Poropack column.

Competition experiment

A competition experiment was carried out in a growth chamber (Conviron) at 19–22 °C and 14 h photoperiod, where the cultivar Negro Queretaro was grown in pots containing sand. The plants were inoculated seven days after sowing using single inoculations of strains CIAT899::*gusA*10 B (Sessitsch et al., 1997), Q16, Q19 or Q21 or mixed inoculations (1:1) of CIAT899::*gusA*10 B in combination with either Q16, Q19 or Q21. In all treatments, 6×10^8 cells were applied per seed. Plants were harvested 12 days after inoculation and roots were stained as described previously (Sessitsch et al., 1997; Wilson et al., 1995) in order to determine nodule occupancy of the *gusA*-marked strain.

Results

Field experiment. During the experiments the climatic conditions were benign and no stresses were recorded. The mean %Ndfa was 22 and the %Ndfa values ranged from 26 for cv. Negro Queretaro to 19 and 20 for cvs. FM-M-38 and N-3-117, respectively. These data correlated well with the nodule numbers as Negro Queretaro formed on average 82 nodules per plant while FM-M-38 and N-3-117 formed 52 and 48, respectively (Table 1).

IARs of native rhizobial strains. Twenty-eight different intrinsic antibiotic resistance patterns were obtained (Tables 2–4 show the groupings for each cultivar). Two major groups were mainly composed of isolates obtained from the cultivars Q and FM. The identifying codes given to the rhizobial isolates show the plant genotype from which the strain was isolated.

PCR using repetitive primers. ERIC-PCR gave a large number (26) of patterns (Tables 2–4). All isolates obtained from different cultivars formed distinct patterns.

PCR-RFLP of the 16S rRNA gene. This analysis was performed in order to classify the isolated rhizobia from common beans into species. The DNA fragment patterns obtained were compared with those from reference strains of various species of *Rhizobium*. Two

Table 2. Intrinsic antibiotic resistance patterns, ERIC-PCR profiles and MLEE groupings of rhizobial isolates obtained from the *Phaseolus vulgaris* L. cultivar Negro Querétaro

Rhizobial isolate	IAR	ERIC-PCR	MLEE
Q1	IAR-1	PCR-1	MLEE-1
Q2	IAR-2	PCR-1	MLEE-1
Q3	IAR-3	PCR-2	n.a.*
Q4	IAR-2	PCR-3	MLEE-1
Q5	IAR-2	PCR-3	MLEE-1
Q6	IAR-4	PCR-4	MLEE-1
Q7	IAR-5	PCR-5	MLEE-1
Q8	IAR-6	PCR-3	MLEE-1
Q9	IAR-7	PCR-3	MLEE-1
Q10	IAR-8	PCR-6	MLEE-1
Q12	IAR-9	PCR-7	MLEE-1
Q13	IAR-10	PCR-8	MLEE-1
Q14	IAR-11	PCR-7	MLEE-2
Q15	IAR12	PCR-7	MLEE-3
Q16	IAR-7	PCR-9	MLEE-4
Q17	IAR-7	PCR-10	MLEE-1
Q18	IAR-13	PCR-10	MLEE-1
Q19	IAR-14	PCR-7	MLEE-5
Q20	IAR-2	PCR-7	MLEE-1
Q21	IAR-12	PCR-11	n.a.
Q22	IAR-2	PCR-11	MLEE-1
Q23	IAR-7	PCR-12	MLEE-1
Q24	IAR-15	PCR-11	MLEE-1
Q25	IAR-16	PCR-7	MLEE-1
Q26	IAR-17	PCR-13	MLEE-1

*n.a. signifies not analyzed.

different patterns were found. One isolate, Q3, showed the same profile as *R. tropici* type IIA strain CFN299, while the majority of the isolates showed the same pattern as *R. etli*.

Multilocus enzyme electrophoresis. Forty-five isolates were analyzed by MLEE in order to obtain information on the genetic diversity of the isolates. 83% of the isolates and two strains of *R. etli* including type strain CFN42 showed the same electrotype (ET-1), 17% showed seven different patterns. A mean genetic diversity (H) of 0.105 was calculated.

Acetylene reduction assay. Nine isolates obtained from the different cultivars which were different by ERIC-PCR, were used to inoculate three plant genotypes and the acetylene reduction of the resulting nodules was measured. Amounts of ethylene produced (μmol of ethylene plant^{-1} h^{-1}) ranged from 0.35 to 6.5. Isolates from a given plant genotype did not display improved performance with the corresponding cultivar (Figure 1).

Competition experiment. The competitive abilities of three strains isolated from cv. Negro Querétaro against *R. tropici* strain CIAT899::*gusA*10 B were evaluated. The isolates tested had different IAR and ERIC-PCR patterns (Tables 2–4). Isolate Q21 was less competitive than isolates Q16 and Q19 and formed significantly less nodules in the single inoculation treatment (Table 5).

Discussion

Common beans are an important food crop in the Americas and have a high genetic variability for N_2-fixation (Bliss, 1993; Hardarson et al., 1993; Peña-Cabriales and Castellanos, 1993; Peña-Cabriales et al.,

Table 3. Intrinsic antibiotic resistance patterns, ERIC-PCR profiles and MLEE groupings of rhizobial isolates obtained from the *Phaseolus vulgaris* L. cultivar Flor de Mayo-M-38

Rhizobial isolate	IAR	ERIC-PCR	MLEE
FM1	IAR-1	PCR-1	MLEE-1
FM2	IAR-2	PCR-2	MLEE-1
FM3	IAR-3	PCR-2	MLEE-1
FM4	IAR-4	PCR-3	MLEE-1
FM5	IAR-5	PCR-4	MLEE-6
FM6	IAR-6	PCR-2	MLEE-1
FM7	IAR-7	PCR-5	MLEE-1
FM8	IAR-8	PCR-2	MLEE-1
FM9	IAR-9	PCR-2	MLEE-1
FM10	IAR-5	PCR-6	MLEE-1
FM11	IAR-5	PCR-7	MLEE-1
FM12	IAR-10	PCR-7	MLEE-1
FM13	IAR-5	PCR-3	MLEE-1
FM14	IAR-5	PCR-7	MLEE-1
FM15	IAR-11	PCR-8	MLEE-1
FM16	IAR-3	PCR-9	MLEE-1
FM17	IAR-12	PCR-10	MLEE-1
FM18	IAR-12	PCR-11	MLEE-1

1993). The mean %Ndfa (22%) obtained in this study is lower than the values reported previously (Castellanos et al., 1996) for well-watered plants (42% Ndfa) but higher than for plants exposed to drought during the reproductive stage (8.6% Ndfa). The three cultivars tested did not show high variation in N_2-fixation (%Ndfa values ranged from 19 to 26). Higher variation was reported by Hardarson et al. (1993). In that study in two states of México, 37 bean cultivars were tested for their N_2-fixation and %Ndfa ranged from 0 to 58%. The same study revealed that the line Negro Querétaro was one of the best performing lines when grown in Irapuato, México, with a %Ndfa of 50. In our experiments, when the same line was grown in Durango, México, the %Ndfa was only 26%. Nevertheless, the cv. Negro Querétaro performed better than the other cvs. tested. The acetylene reduction assays of selected indigenous strains confirmed these findings. Few of the isolates tested showed high acetylene reduction activity and isolates from a given plant genotype did not necessarily show better performance with the corresponding cultivar. Only one isolate obtained from the cv. Negro Querétaro, Q17, had a high N_2-fixation capacity, while the other two isolates, Q14 and Q21, performed poorly on that cultivar. Furthermore, ERIC-PCR results showed that Q14 and Q21 have the same PCR pattern in common with many other nodule isolates, indicating perhaps, that the nodule occupancy of inefficient strains is high. As variability in N_2-fixation efficiency among various native rhizobial isolates in combination with different plant genotypes was shown to be extremely high, competitive ability has to be considered carefully. Our competition experiment also indicated variability in competitive ability among different native strains. Isolate Q21 showed significantly lower competitive ability than Q16 and Q19 in competing with *R. tropici* strain CIAT899::*gusA*10 B. Nevertheless, competitive abilities against other indigenous, adapted strains might be different. Our results indicate that differences among native strains in competition as well as in N_2-fixation exist. It would be interesting to perform competition experiments with a larger number of isolates under the same conditions as those prevalent in the field from which the isolates were originally obtained.

Intrinsic antibiotic resistance analysis showed high diversity among isolates as was previously reported by Aguilar-Zacarías (1990) with a commercial bean line grown in the state of Zacatecas, México. ERIC-PCR also showed high diversity, but there was no correlation between the antibiotic resistance grouping and ERIC-PCR analysis. Similar results were obtained by

Table 4. Intrinsic antibiotic resistance patterns, ERIC-PCR profiles and MLEE groupings of rhizobial isolates obtained from the *Phaseolus vulgaris* L. cultivar N-3-117

Rhizobial isolate	IAR	ERIC-PCR	MLEE
N1	IAR-1	PCR-1	MLEE-1
N7	IAR-2	PCR-2	MLEE-1
N8	IAR-3	PCR-3	MLEE-1
N9	IAR-4	PCR-4	MLEE-1
N10	IAR-5	PCR-5	MLEE-2
N11	IAR-5	PCR-6	MLEE-1
N12	IAR-6	PCR-7	MLEE-1
N13	IAR-5	PCR-7	MLEE-1
N14	IAR-7	PCR-6	MLEE-3
N15	IAR-7	PCR-8	MLEE-1

Table 5. Number of nodules formed on cv. Negro Querétaro in a competition experiment to determine the competitive ability of three native isolates against strain CIAT899::*gusA*10 B

Strain	Nodule number plant^{-1}		
	Single inoculation	1:1 Mixed inoculation	
		GUS-marked strain	Unmarked strain
Q16	107^a	4^b	96^a
Q19	108^a	7^b	103^a
Q21	67^b	43^a	24^b
CIAT899::*gusA*10 B	90^a		

Means (within one column) that are not significantly different from each other at $P = 0.05$ share the same letters as superscripts.

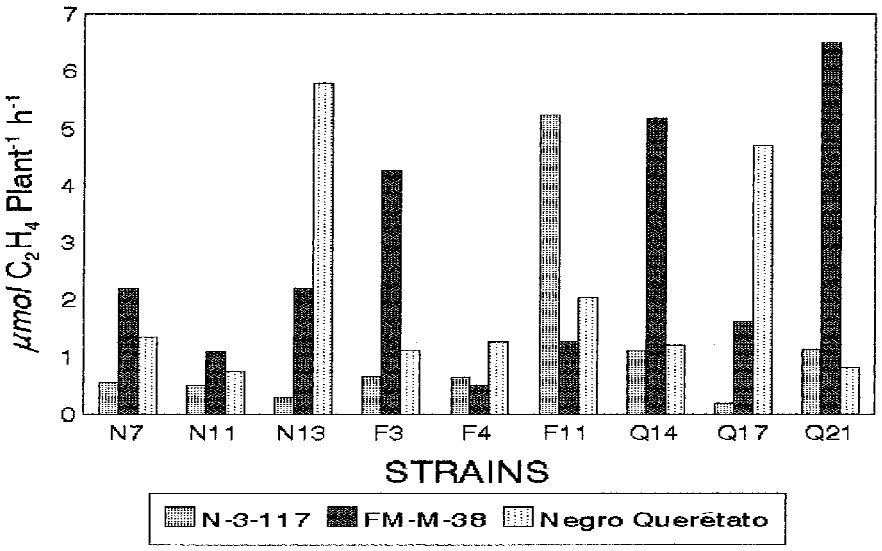

Figure 1. Effect of common bean cultivar and rhizobial strain on acetylene reduction activity at flowering.

van Rossum et al. (1995) on a wide range of *Bradyrhizobium* strains. PCR-RFLP of the 16S rRNA gene and MLEE revealed that most of the isolates are *R. etli*. This is in agreement with previous studies on Mexican rhizobial populations nodulating common bean (Segovia et al., 1993). High genomic instability was reported in *R. etli* (Brom et al., 1991; Flores et al., 1988) perhaps contributing to the diversity of this species. In contrast, using MLEE, our results showed low genetic diversity among the rhizobial isolates (H = 0.105). The majority of these values correspond to a single electrophoretic type. Values of diversity of common bean nodulating rhizobia reported in the literature range from H = 0.4 to 0.6 (Demezas et al., 1991; Eardly et al., 1995; Piñero et al., 1988; Segovia et al., 1991; Souza et al., 1994). Souza et al. (1994) analysed five populations of *R. etli* from Morelos, México, and found a total genetic diversity of H = 0.487. The authors also suggested that environmental constraints for nodulation may reduce the genetic diversity. Similar observations have been made with indigenous soil populations of *R. leguminosarum* bv. trifolii (Leung et. al. 1994a,b,c). The reason why only a limited diversity of the *R. etli* population is encountered in nodules in this region remains to be established and may be related to environmental constraints existing in this area.

Analysis of the genetic diversity of rhizobial strains in combination with symbiotic performance and competitive behaviour may allow the identification of strains useful for agricultural purposes. Our data suggest that among the native populations, we might find successful inoculant strains and further experiments will show whether this approach can improve N_2-fixation in common beans.

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