Soil Fertilization Limits the Genetic Diversity of *Rhizobium* in Bean Nodules

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Abstract

The effect of soil fertilization on the genetic diversity of *Rhizobium* isolates from bean nodules was estimated by multilocus enzyme electrophoresis. In two-year trials, we found that levels of fertilization commonly used in agricultural fields in Mexico (N, P, K, 40-40-20) diminished the genetic diversity in the nodule population in some, but not all *Phaseolus vulgaris* cultivars. The decrease in genetic diversity may be attributed in part to the N fertilization because an additional trial assay using $(NH_4)_2SO_4$, NH_4Cl or NH_4NO_3 in the soil diminished the mean genetic diversity of nodule isolates by 41%, 33% and 26%, respectively. *Rhizobium* strains with genetic distances above 0.8 from the main *R. etli* group were infrequently encountered in bean nodules on plants with added fertilizer.

Abbreviations

MLEE = multilocus enzyme electrophoresis; ET = electrophoretic type.

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1. Introduction

Rhizobium are soil bacteria capable of forming nodules on the roots of legumes. In the nodules, these bacteria contribute to the nitrogen nutrition of the plant. Because of its agronomic importance, the *Rhizobium*-legume symbiosis is the most studied of all symbioses and has become a reference model for others. In recent years, a great deal of effort has been expended to analyze *Rhizobium* population diversity, mainly from isolates obtained from nodules (reviewed in Martínez-Romero and Caballero-Mellado, 1996). Soil physical constraints such as pH, salinity, and temperature may select specific bacterial clones capable of nodulating under certain conditions. Agricultural practices may also influence *Rhizobium* genetic diversity (Souza et al., 1998). The inhibition of nodule formation and N_2 fixation by combined nitrogen has been reviewed by Streeter (1988), Carroll and Mathews (1990) and Schultze et al. (1994). In the presence of 3 mM NO_3NH_4 *Sesbania rostrata* root nodulation was severely reduced with *Sinorhizobium* strains but not with *Azorhizobium* strains (Boivin et al., 1997), while both were equally capable of nodulating without combined nitrogen. Variations in nodulation tolerance to low and medium levels of nitrate exist among strains of *R. meliloti*, *R. leguminosarum* and *Bradyrhizobium japonicum* (Carroll and Mathews, 1990).

Phaseolus vulgaris bean plants were described as the legumes with the lowest nitrogen fixation levels (Hardarson, 1994), and the addition of chemical fertilizer in Mexican agriculture to improve yields has been recommended for the last 35 years. The effect of such additions on the genetic diversity of *Rhizobium* populations in nodules has not been evaluated.

The aim of this work was to analyze the effect of soil fertilization on the genetic diversity of bean-nodule *Rhizobium* by MLEE, which is a standard method for evaluating population diversity based on metabolic enzyme polymorphisms (Selander et al., 1986).

2. Materials and Methods

Experimental design

Phaseolus vulgaris bean cultivars N-8-1-1-6, Negro Xamapa (black seeds); Pinto Villa and L-3-1-1-1 Cm (light seeds), currently used in agricultural fields, were sown with two treatments: with and without chemical fertilization. In the fertilized treatment, the equivalent of 40 Kg N-, 40 Kg P-, and 20 Kg K-per ha were applied at the time of planting. Seeds of the different cultivars were randomly sown at a distance of 40 cm from each other within the row, with 40 cm between rows and a 1 m separation between the plots. Non-sterilized seeds

were placed directly (on 25 March, 1996) into wet brown-sandy loam soil (pH 6.4) containing 0.14% N and 29 ppm P. Plants were watered every other day. Twenty nodules (4 per plant) were collected from each cultivar per treatment 28 days after planting. A total of 80 nodules per treatment were analyzed.

Only Pinto Villa was assayed in a second year trial using the same fertilization scheme described, and bacteria were isolated from 40 nodules with around 4 nodules per plant, 40 nodules were analyzed from non fertilized control plants.

In a different trial, cultivar Pinto Villa was sown (on 25 March, 1997) in the same non-fertilized plot using the same experimental design. The fertilization treatments in this case were only the equivalent of 40 Kg N, using $(NH_4)_2SO_4$, NH_4Cl or NH_4NO_3. Thirty control nodules from the non-fertilized plot and a total of 10 nodules (2 per plant) per treatment were analyzed.

Rhizobium isolation

Nodules were randomly chosen from each plant and surface- sterilized with sodium hypochlorite (1.2% w/v) as described (Martínez-Romero and Rosenblueth, 1990). Nodules were crushed directly on plates of YM (Vincent, 1970) medium containing 20 mg l^{-1} cycloheximide. Individual colonies were picked and washed with 10 mM $MgSO_4$ and 0.01% (vol/vol) Tween 40 to further purify the colonies. Isolates were grown in PY medium (1 g $CaCl_2.H_2O$, 3 g yeast extract, 5 g peptone l^{-1}) and maintained at –70°C in 20% (vol/vol) glycerol.

Multilocus enzyme electrophoresis

Three hundred isolates, reference *Rhizobium etli* strains (CFN42[T], Viking 1, CFN1, F6, and COC-111), and *R. gallicum* strain FL-27 were grown in 75 ml of PY medium inoculated from freshly grown cultures on PY plates and harvested as described (Caballero-Mellado and Martínez-Romero, 1994). Extracts were electrophoresed in starch gels and enzymatic activities revealed following the standard procedures (Selander et al., 1986). The following metabolic enzymes were assayed: malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, hexokinase, phosphoglucomutase, indophenol oxidase (superoxide dismutase), aconitase and phospho-glucoisomerase. The mobility variants of each enzyme were numbered and the distinctive combination of them were designated ETs. The genetic diversity (H) of the rhizobia isolates per each cultivar/treatment was estimated as the arithmetic average of h values for the nine loci, where $h = (1 - X_i^2)\ n/(n-1)$. The dendrogram to illustrate the genetic relatedness of strains was obtained from the programs ETDIV and ETCLUS from T. Whittam kindly provided by B.

Eardly (Department of Biology, Pennsylvania State University, University Park, PA 16802, USA).

Statistical analysis

To determine if the frequencies of isolates from fertilized and non-fertilized plants were significantly different in cluster I and II, a contingency table test and a contrast test for proportions were performed. Expected values (E_{ij}) were calculated according to Cochran's criteria to establish if the conditions required for the contingency table were satisfied (Cochran, 1954).

Plant nodulation assays

Pregerminated plantlets from surface sterilized Negro Xamapa and Pinto Villa seeds were placed in 250 ml flasks with N-free Fahraeus (Fahraeus, 1957) as described (Martínez and Rosenblueth, 1990). Plantlets were inoculated with bacterial suspensions from each *Rhizobium* strain and maintained in a plant-growth chamber for 20 days.

3. Results

Fertilization effects on nodulation

Lower nodule numbers per plant and smaller nodules were obtained upon the addition of chemical fertilizers in all bean cultivars. In L-3-1-1-1 and Pinto Villa nodule numbers were reduced to 26% and 30% and to 50 and 64% in Negro Xamapa and N-8-1-1-6 compared to the non-fertilized controls.

Fertilization effects on genetic diversity

Genetic diversity in nodule isolates was assessed by the estimation of the mean genetic diversity (H) from fertilized and non-fertilized treatments. Fewer ETs were recovered from nodules of fertilized plants of cultivars Pinto Villa and L-3-1-1-1 Cm, although for the latter the difference was small (Table 1). With all cultivars, H was smaller in isolates with the fertilizer treatments, meaning that less diverse populations were recovered from nodules with added fertilizers (Table 1). The genetic diversity of Pinto Villa and L-3-1-1-1 Cm isolates was 50 and 68% of that estimated in non-fertilized controls in the first trial and was 58% of the control's in fertilized Pinto Villa isolates during the second year.

Table 1.   Number of electrophoretic types (ETs) and genetic diversity of *Rhizobium* isolates

Cultivar	Treatment	Number of ETs		H^*	
		1996	1997	1996	1997
Pinto Villa	Control	10	11	0.592	0.311
	Fertilized**	4	7	0.296	0.179
L-3-1-1-1	Control	6		0.590	
	Fertilized**	5		0.401	
Negro Xamapa	Control	8		0.391	
	Fertilized**	11		0.373	
N-8-1-1-6	Control	13		0.549	
	Fertilized**	11		0.408	
Pinto Villa	Control		12		0.456
	NH_4NO_3		4***		0.336
	NH_4Cl		4***		0.304
	$(NH_4)_2SO_4$		4***		0.267

*The genetic diversity (H) is the arithmetic average of h values for the nine loci, where h = $(1 - X_i^2)\, n/(n-1)$. **Fertilization treatment: N, P, K, 40-40-20. ***ETs are shared among all three fertilization treatments. A total of 6 ETs were obtained in the NH_4NO_3, NH_4Cl, and $(NH_4)_2SO_4$ treatments.

In an independent experiment, the mean genetic diversity indexes from Pinto Villa isolates in the presence of NH_4NO_3, NH_4Cl, and $(NH_4)_2SO_4$ were by 26%, 33% and 41% lower than those of the control plants, respectively.

The decrease in genetic diversity is also clearly observed as fertilization virtually eliminated the most divergent ET groups (with a genetic distance >0.8) in the first and in the second year (Figs. 1–3). There are common ETs recovered both from fertilizer and non-fertilizer treatments, but most ETs seem to be confined to a single treatment.

A subsample of strains (40 strains in total), representing different ETs from clusters I and II, from experiment 1, were tested in assays for nodulation with bean plants. All formed red nodules.

Statistical significance

Cluster II is constituted mainly by isolates derived from non fertilized plants in comparison to cluster I (Fig. 1; Table 2). The difference between the proportions of isolates from fertilized and non fertilized plants in both clusters was highly significant with both tests performed (p<0.005).
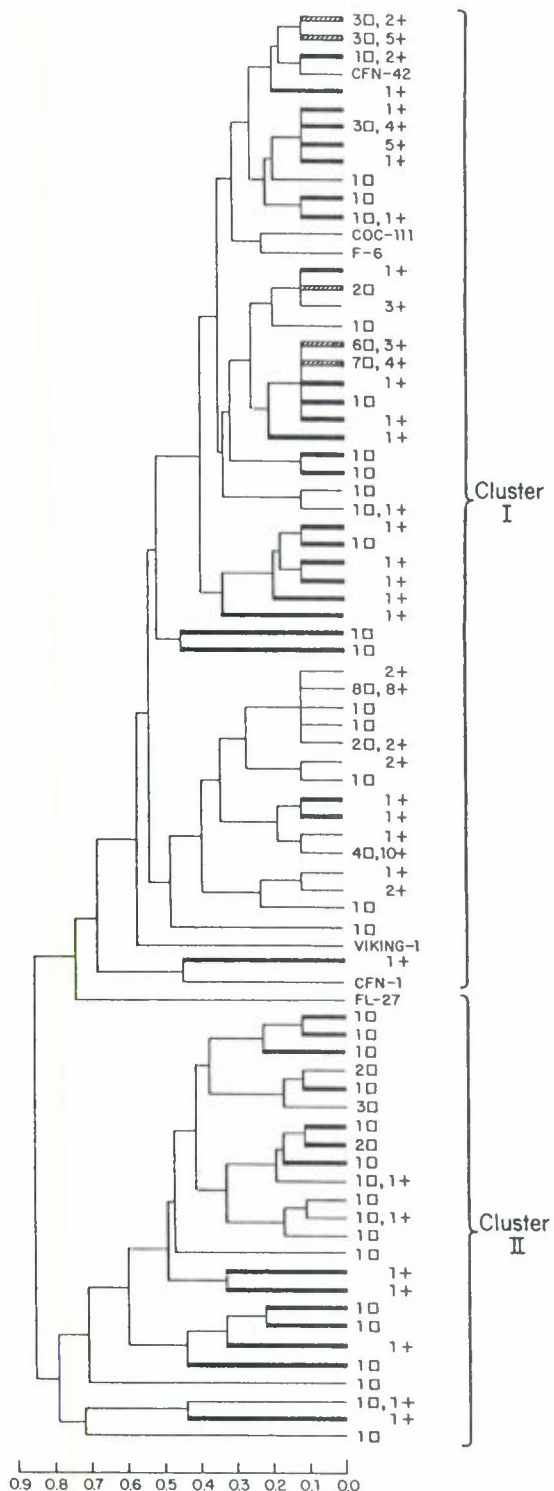
Figure 1.
Dendrogram showing levels of genetic relatedness based on MLEE among 160 nodule isolates obtained in 1996 from cultivars Pinto Villa, L-3-1-1-1 Cm, Negro Xamapa, N8-116, and *Rhizobium* reference strains. The number of isolates for each ET is indicated preceding '+' for those from fertilized plants and square from non-fertilized plants. Fine-lined branches denote ETs represented by isolates from light-seed beans (except for reference strains), bold-lined branches indicate isolates obtained from black-beans and hatched bars show ETs obtained from both types of seeds.
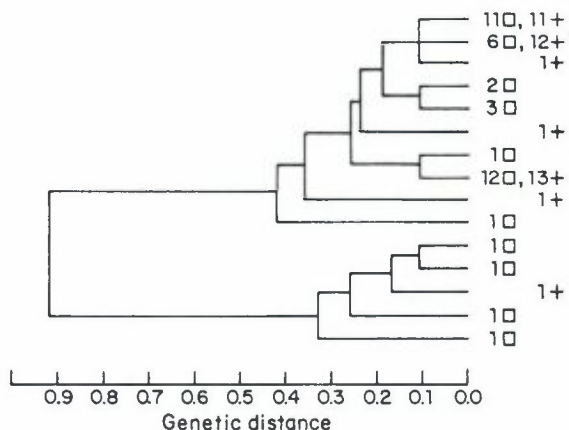
Figure 2. Dendrogram showing levels of genetic relatedness among 80 nodule isolates from Pinto Villa in 1997: 40 from non-fertilized controls and 40 from fertilized treatment. The number of isolates for each ET is indicated preceding '+' for those from fertilized plants and square from non-fertilized plants.
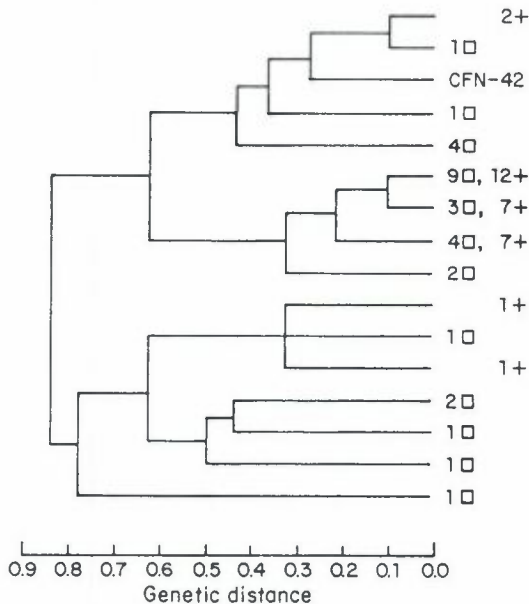

Figure 3. Dendrogram showing levels of genetic relatedness among 60 nodule isolates from Pinto Villa and strain CFN-42: Fifty nodule controls and 10 nodules each from N-treatments [NH_4Cl, NH_4NO_3, or $(NH_4)_2SO_4$]. The number of isolates for each ET is indicated preceding '+' for those from fertilized plants and square from non-fertilized plants.

Cultivar-rhizobia preferences

In general, black-seed beans, Negro Xamapa and N-8-1-1-6 shared strains of similar electrophoretic types, distinct from those isolated from light brown beans Pinto Villa and L-3-1-1-1 Cm. Six out of 42 of the ETs isolated from the black seeds were intermixed with ETs from light seeds. Nine out of 31 of the ETs from light seeds were intermixed with the black-seed derived ETs. *R. etli* strains included as references were intermixed only with cluster I isolates.

4. Discussion

Combined nitrogen limits the establishment of the *Rhizobium*-legume root nodules symbiosis (Streeter, 1988). Plant root exudate composition depends on the presence of nitrate or phosphate (Cho and Harper, 1991; Coronado et al., 1995; Kapulnik et al., 1987). In the presence of nitrate, clover root epidermal cells had lower levels of trifoliin A (a lectin) (Dazzo and Brill, 1978) and alfalfa roots had less apyrase (a special type of lectin that binds *Rhizobium* Nod factors) (M.E. Etzler, personal communication). Similarly, induction of *nod* genes, which participate in the production of Nod factors, is reduced in the presence of combined nitrogen (Dusha and Kondorosi, 1993). Inhibition of nodulation by nitrate may be partially relieved by indoleacetic acid (Munns 1968) and by the ethylene inhibitor aminoethoxyvinylglycine (Ligero et al., 1991).

We describe here a new effect of fertilization, namely a reduced genetic diversity of symbiotic bacteria inside bean nodules. The long term effects of fertilization on soil populations remains to be established and will, in theory, depend on the contribution of bacteria from senescent nodules, as well as on the impact of fertilizers on rhizosphere *Rhizobium* growth and diversity. The observed effects may not be attributed solely to nitrogen fertilizer, since P and K were also added. Since NH_4NO_3, when tested alone, affected diversity but not pH, nitrogen clearly had an effect on nodule *Rhizobium* diversity.

Our results show that different bacterial groups are not equally capable of nodulating in the presence of fertilizer, although all strains tested were symbiotically efficient in the absence of fertilizer.

The decrease of genetic diversity was influenced by the cultivar used. It is worth noting that Pinto Villa was originally selected using no fertilization, while the other cultivars, Negro Xamapa and N-8-1-1-6 were bred and selected with fertilizer. These selection conditions might have also imposed an indirect co-selection of tolerance to nodulation in presence of fertilizer.

In the present report, we confirm the large genetic diversity of bean nodulating bacteria (Eardly et al., 1995; Piñero et al., 1988; Segovia et al., 1991;

Souza et al., 1994). It is mainly the distantly-related cluster that is eliminated upon fertilization. Considering that coefficients of genetic distances at levels higher than 0.5 may be indicative of different species, the taxonomic status of cluster II, not corresponding to *R. tropici* not to *R. gallicum*, is unclear and will be further explored.

Additional results derived from this work show a preference of cultivars for specific bacterial groups. Black seed beans (Negro Xamapa and N-8-1-1-6) share electrophoretic types of rhizobia basically distinct from those of light seed beans (Pinto Villa and L-3-1-1-1). This may be in relation to the anthocyanidin content of the testa. Anthocyanidins have been shown to be *nod*-gene inducers (Hungría et al., 1991). Bean cultivar preferences for peculiar *Rhizobium* strains have been documented previously. *P. vulgaris* cultivar RAB39 preferentially nodulates with *R. tropici* UMR1899 rather than with *R. etli* strains (Montealegre et al., 1995). In contrast, wild bean cultivars have restricted nodulation with all *R. tropici* strains tested but with only a few *R. etli* strains (Kipe-Nolt et al., 1992).

We discussed earlier that little is known of the effects of human activity on bacterial diversity (Martínez-Romero and Caballero-Mellado, 1996). Recently, significant microbial population differences between a mature forest soil and an adjacent pasture were shown to illustrate the impact of deforestation on the soil microbial community (Borneman and Triplett, 1997). We supposed that agricultural practices such as fertilization affected bacterial populations, and have found that this was the case with *Acetobacter diazotrophicus*, an endophytic sugar-cane diazotroph. Few *A. diazotrophicus* strains (Fuentes-Ramírez et al., 1993) and a reduced genetic diversity is encountered in heavily fertilized areas (Caballero-Mellado et al., 1995). Other agricultural practices such as bovine slurry amendments to soil have been shown to affect *Rhizobium* population diversity when evaluated by RAPDS (Labes et al., 1996). All these data warn us about the impact of modern agricultural practices, especially soil fertilization, on microbial diversity.

Acknowledgements

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