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Seeds of *Phaseolus vulgaris* bean carry *Rhizobium etli*

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Abstract

The presence of soil bacteria on seeds could provide an explanation for bacterial geographical spread. We report that *Phaseolus vulgaris* bean seeds naturally carry rhizobia on their testa. One hundred eighteen *Rhizobium* isolates from L-3-1-1-1 seeds were characterized by multilocus enzyme electrophoresis and compared to *Rhizobium* species which form nitrogen fixing nodules on the roots of bean plants. As a result of this and other analyses, seed isolates were classified as *R. etli*. Seed borne rhizobia are probably moisture limited and are acquired from contaminating soil during the harvesting process as seeds from non-opened pods do not carry them. Rhizobia on seeds are in a presumably desiccated or dormant state as they require a rehydration process during which they are antibiotic sensitive. With an improved procedure to detect rhizobia on seeds, we found that 5 out of 13 *Phaseolus vulgaris* bean cultivars tested harbor rhizobia on the seeds.

Keywords: Seeds; *Phaseolus vulgaris*; Rhizobium; Soil bacterium; Nitrogen fixation; Bacterial spread

1. Introduction

Rhizobium spp. form nitrogen fixing nodules on the roots of legumes. Wherever a given legume is native, its specific rhizobia symbionts are also indigenous soil inhabitants [1]. As crops were introduced into new regions, microorganisms harbored by the plants might also have been disseminated. Little is known about the mechanisms by which *Rhizobium* and other bacteria are geographically scattered [2,3]. Most probably man has contributed to their dispersion and seeds may be carriers of both pathogenic [4] and symbiotic bacteria. In 1948, Ash and Allen [5] reported that some alfalfa seeds bear *R. meliloti* and more recent reports showed that the presence of *Azorhizobium caulinodans* on *Sesbania rostrata* seeds explained the nodulation of plants in introduced areas [6,7]. Recently Hagen and Hamrick [3] proposed that the colonization of red clover by several *Rhizobium leguminosarum* bv. *trifolii* strains carried by the seeds contributes to the genetic diversity encountered in nature.

Phaseolus vulgaris (common bean) originated in the Americas [8] but has become an important crop worldwide. Its dissemination pathways have been ex-

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amined [8] and its *Rhizobium* symbionts might have been carried along the same routes. This may help to explain the presence of *R. etli* in areas where the bean was introduced. *Rhizobium* populations nodulating bean have been analyzed from regions where the bean is an introduced crop as well as from its native areas. *R. etli* [9] is the predominant bean nodulating species in Mesoamerica [10,11] but other bean-nodulating species (as *R. gallicum*, [12,13]) and other *Rhizobium* groups have also been described for this region [14,15]. *R. etli* has not been found in England [16] where its legume host is not native. However, *R. etli* strains are found in Spain [17] where beans were introduced from America around 400 years ago [8] and also in Senegal [18] and Austria [19]. Spanish isolates of *R. etli* belong to lineages which are only distantly related to *R. etli* type strain CFN42 [17]. Founder-selection during the bacterial colonization process may cause differences in the populations found on both sides of the Atlantic Ocean. In addition, geographical spread of bacterial clones may also be determined by the types of *Rhizobium* strains that survive on seeds. Different mechanisms of soil transport have also been involved in the dissemination of rhizobia but intercontinental spread may be more easily attributable to seed transport.

The aim of this work was to test for the presence of *Rhizobium* bacteria on *Phaseolus vulgaris* seeds. *Rhizobium* isolated from seeds was then characterized by multilocus enzyme electrophoresis (MLEE). This analysis along with *nif* gene organization and patterns of ribosomal genes of representative strains showed that these rhizobia corresponded to *R. etli*.

2. Materials and methods

2.1. Bean cultivars

The *Phaseolus vulgaris* bean seeds tested were: L-3-1-1-1 harvested in October 1993 from an irrigated field in Celaya, Guanajuato and stored since then in closed paper bags at room temperature; N-8-1-1-6 also from Celaya. All other cultivars (including Peruvian) were harvested in Morelos State in 1996 and obtained in Mexican traditional markets.

2.2. Rhizobium strains and culture conditions

Rhizobium strains, *R. etli*, CFN42^T, F6, F8, F16, Bra5, CNPAF512; *R. tropici* CIAT899^T, and the new isolates were grown in PY medium (5 g peptone, 3 g yeast extract and 0.7 g $CaCl_2$ per liter) or in minimal medium MM [20]. When required, nalidixic acid (20 mg/l), cycloheximide (10 mg/l) or biotin (10 mg/l) were added. All other type strains were grown in PY medium and maintained in YM medium [21].

2.3. Plant nodulation assays

250-ml Erlenmeyer flasks with 7 g cotton or with vermiculite with N-free Fahraeus medium were used under sterile conditions to grow bean plants in sterile conditions (whether inoculated or not) from (i) surface sterilized L-3-1-1-1 seeds, or (ii) pre-germinated 3-day-old plantlets obtained from surface sterilized Negro Xamapa or L-3-1-1-1 bean seeds, or (iii) from non-sterilized seeds from different *P. vulgaris* cultivars.

2.4. Seed washings

Around 20 seeds per bean cultivar tested were placed in tubes with 10 ml sterile water and shaken 30 s by vortexing. The liquid recovered was centrifuged in a microfuge (2040×*g*, 3 min) at room temperature. This was done with the aim of eliminating any compound that may elute from the seeds by this short washing. The pellet was resuspended in 2 ml of sterilized water and 0.1 ml were used as inocula for pregerminated (from surface sterilized seeds) 3-day-old plantlets or for surface sterilized seeds. Otherwise, the bacterial suspensions were incubated for 24 or 72 h at 30°C previous to plant inoculations. In some experiments tetracycline (Tc, 6 μg/ml), kanamycin (Km, 70 μg/ml), or chloramphenicol (Cm, 50 μg/ml) were added to the bacterial suspension for 24 h, during the incubation period, and were then eliminated by centrifugation and washings with water. Controls followed the same procedure except for the addition of the antibiotic. Aliquots from these bacterial pellets resuspended in water were used as inocula for bean plantlets.

2.5. Strain isolation from nodules

Nodules detached from bean plants were surface sterilized by immersion in 1.5% (v/v) sodium hypochlorite for 3–5 min and then rinsed 5 times with an excess of sterile water [22]. Water drops from the surface of the nodule were placed in PY plates to detect any contamination after incubation at 30°C. Nodules were crushed in PY medium and individual *Rhizobium* colonies were selected.

2.6. Microscopy analysis

P. vulgaris L-3-1-1-1 seeds were fixed in 3% glutaraldehyde and 1% osmium tetroxide and after dehydration were gold coated and observed with a JSM-5410LV (Jeol Ltd.) scanning microscope.

2.7. Electrophoretic profiles of metabolic enzymes

Bacterial extracts were prepared from 30-ml liquid PY cultures. Lysates were obtained with lysozyme and analyzed in starch gels after selective staining, as described previously [23], for the following metabolic enzymes: malate dehydrogenase, isocitrate dehydrogenase, phosphoglucomutase, indophenol oxidase (superoxide dismutase), glucose-6-phosphate dehydrogenase, xanthine dehydrogenase, esterases and alanine dehydrogenase.

The distinct electrophoretic patterns were designated ETs. The genetic distance between each pair of ETs is determined from the proportion of loci at which dissimilar alleles occur. Clustering by the UPGMA method of Nei and Lee [24] was determined from a matrix of pairwise genetic distances and a dendrogram was obtained to illustrate the relative positions of isolates to reference strains.

2.8. Analysis of nif gene organization and SSU rRNA PCR-RFLP patterns

DNA was purified as described [25], digested with *Bam*H1 or *Eco*R1 restriction enzymes, and transferred, after electrophoresis in agarose gels to nitrocellulose filters. An internal *nifH* fragment from pEM15 [26] was used as a probe after labelling with ^{32}P by the random primer method (*redi*prime from Amersham Life Science, Amersham Place, Little Chalfont, Buckinghamshire, UK). Hybridization was carried out under high stringency conditions [27].

Primers fD1 and rD1 [28] were used to synthesize SSU rRNA gene fragments with Taq polymerase by PCR. Fragments were digested with *Msp*I, *Sau*3A, *Hin*f1, and *Hha*1 and visualized in agarose gels as described by Laguerre et al. [29]. Patterns were compared to type strains from the majority of described *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* species.

3. Results

3.1. Isolation of seed-borne Rhizobium

Two procedures were followed to recover rhizobia from seeds. The first consisted of placing non-sterilized *Phaseolus vulgaris* seeds from different cultivars directly in sterile flasks containing N-free medium. In the other, we used water from seed washings as inocula for rootlets obtained from sterilized seeds. In four independent assays without rhizobia inoculation, nodules formed on the roots in 70% of the plants obtained from non-sterilized L-3-1-1-1 seeds placed directly in sterilized flasks, but nodulation was largely delayed with nodules appearing after one month. With the first procedure, nodulation was also obtained with N-8-1-1-6 seeds and with one additional cultivar (Peruvian) from Morelos, Mexico. Otherwise, nodulation proceeded faster if bacteria were washed from the seeds and applied as root inocula. With this second procedure, we found that 5 out of 13 bean cultivars tested (including N-8-1-1-6, L-3-1-1-1, and Peruvian) carried *Rhizobium* on their seeds. Control assays using non-inoculated sterile seeds were always devoid of nodules.

In a modification to the seed-washing procedure, we found that if the bacteria were incubated in water for 1–3 days prior to plant root inoculation, nodulation was enhanced (Fig. 1) and nodulation kinetics was similar to that obtained with other reference laboratory strains (not shown) using pregerminated plantlets as described [30]. We compared germinated (Fig. 1B) and non-germinated (Fig. 1A) sterilized seeds to test if some plant metabolites produced during germination were required to stimulate seed rhi-

zobia, but this was not the case. Addition of biotin or PY medium during the rehydration process did not enhance nodulation (not shown). A minimum of 21 h of incubation in water was required to allow *Rhizobium* borne seeds to nodulate in the same manner as normally cultivated rhizobia, forming nodules at 5 days after inoculation (Fig. 2). At 13 days post-incubation, a gradually increasing nodulation response is observed with increasing rehydration time from 2–8 h (Fig. 2).

When bacteria were treated with tetracycline, kanamycin or chloramphenicol for 24 h during the rehydration process, they did not nodulate at all. If the antibiotic was applied for 24 h to the same strains but after seed borne bacteria were grown in PY or in MM plates, then nodulation proceeded normally when these bacteria were used as inocula.

Rhizobium isolates were obtained from 5–10 surface-sterilized nodules per L-3-1-1-1 plant. Alternatively, to directly isolate rhizobia from L-3-1-1-1 seeds, the bacterial suspensions that were washed from seeds and serial dilutions prepared from them were plated on MM with added biotin or on PY medium. This selection allowed *R. etli* to be isolated from the bean rhizosphere [15]. Large numbers of

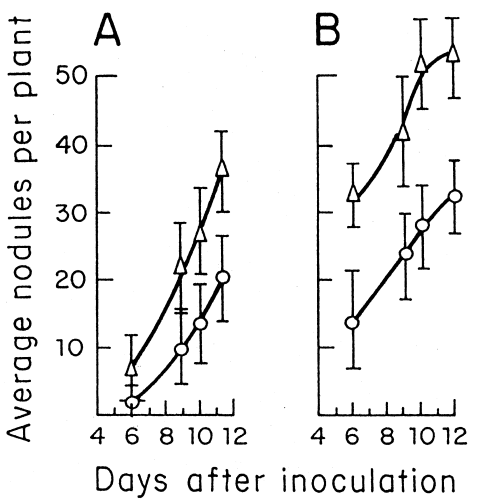

Fig. 1. Nodulation kinetics of *Phaseolus vulgaris* cultivar L-3-1-1-1 showing average nodules per plant from five plants, S.D. indicated. A: Sterilized seeds, or B: 3-day old seedlings were inoculated with bacterial suspensions obtained from L-3-1-1-1 seed washings either applied directly (○); or after incubation for 3 days at 30°C (△). Inc isolates (see text and Fig. 4) were recovered from nodules at day 13 from some of these plants (△).

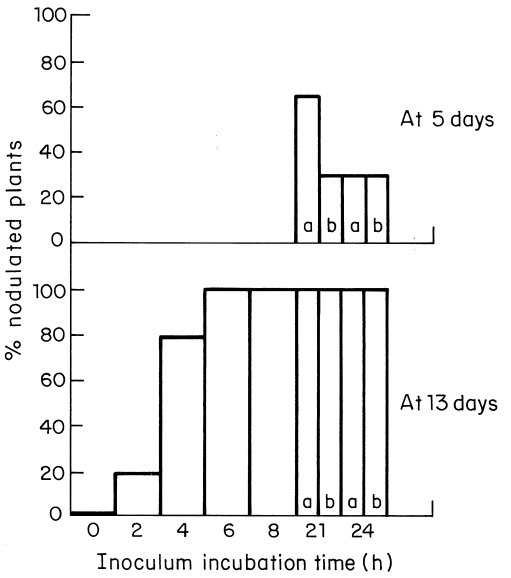

Fig. 2. Nodulated *Phaseolus vulgaris* L-3-1-1-1 plants (percentage) at different times after inoculation with seed-washed suspensions from L-3-1-1-1 incubated at different times in water. The 21- and 24-h inocula were additionally diluted 1:1 with water (a) or applied directly (b) to test if the effects were due to the increase in bacteria number during the incubation period. Ten plants per incubation time were analyzed.

different bacteria grew on the plates and only one *Rhizobium etli* isolate was obtained and recovered from one MM plate out of the more than 50 plates spread with seed wash water or dilutions. This isolate was selected for its resemblance to *R. etli* in its morphology and growth characteristics and further identified using the procedures described (MLEE and PCR-RFLP of ssu rRNA genes). Furthermore, we obtained nodules in bean plants using as inoculate confluent growth of bacteria from PY plates (from which we could not recognize *R. etli*); in this case, bean plants were used to select the rhizobia out of the mass of bacteria (isolates designated PYM).

We tested for the presence of rhizobia on L-3-1-1-1 seeds obtained from non-opened pods, which were not exposed to the air or to soil particles. Dry, non-opened pods were carefully sealed with tape at the agricultural field in Celaya and handled under sterile conditions at the lab. The seeds taken from the pods were used directly in nodulation assays using sterile flasks. Four seeds per bean dry pod were assayed and no nodules were obtained on any of 32 plants derived from these seeds. In addition, water wash-

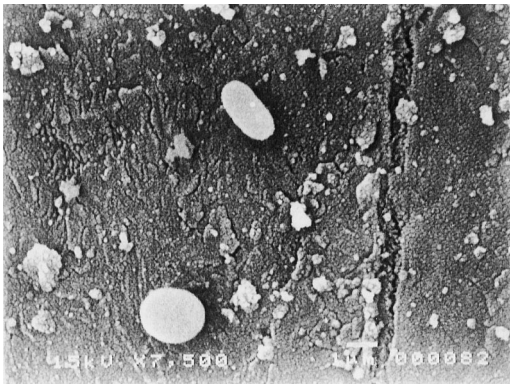

Fig. 3. Scanning-electron-micrograph of a L-3-1-1-1 seed.

ings from 20 additional seeds (as described above) were devoid of rhizobia as well as of many other bacteria as evidenced after growth on PY plates. Simultaneously, L-3-1-1-1 seeds were recovered from the same field by the traditional harvesting process. These seeds contained rhizobia and the 10 isolates obtained, as described before from seed washings, were designated harvest 1996, H96 (see below).

Further evidence to support that rhizobia on seeds arise from contaminating soil was obtained by scanning microscopy. We observed some bacteria on the testa and abundant material resembling soil particles (Fig. 3). When the water suspension from the seed rinses was centrifuged ($2040 \times g \times 3$ min) soil material and rhizobia were recovered in the pellet, but neither the amount of soil from seeds nor their color were in relation to the positive isolation of *Rhizobium* from the seeds (not shown). Rhizobia were eliminated from the seeds by the common seed surface sterilization procedure using 0.3% (v/v) sodium hypochlorite for 15 min.

3.2. *Rhizobium* strain characterization

All 118 *Rhizobium* isolates from L-3-1-1-1 seeds were gummy and most were melanin producers as is characteristic of *R. etli* strains. All were nalidixic acid resistant as are the bean nodulating *R. etli* and *R. tropici* species [31]. None of the isolates grew on LB medium and all were capable of forming nitrogen fixing nodules on both L-3-1-1-1 and Negro Xamapa bean cultivars, with nodules appearing 5–7 days after inoculation.

Bacterial extracts were prepared in PY medium from single colonies of all the isolates and from the *R. etli* CFN42 and *R. tropici* CFN299 reference strains. By MLEE we found that the seed borne rhizobia were related to *R. etli* strains F8 and F16 (Fig. 4) but not to CFN42^T (which is a Guanajuato State isolate). The majority of the strains corresponded to very few electrophoretic types. On average, we got 14 strains per ET which is largely different to what has been observed with bean populations previously analyzed [15,32] with around 1.1 strains per ET and 1.3 strains per ET in a limited geographical area [33].

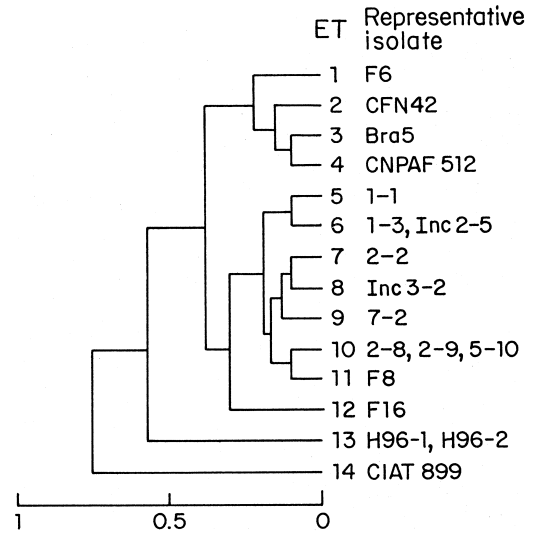

Fig. 4. Dendrogram showing the genetic relatedness of different seed isolates from L-3-1-1-1 and *R. etli* strains CFN42, F6, Bra5, F8, F16, CNPAF 512, and *R. tropici* CIAT899. For designations of seed isolates, the first number indicates the plant, the second the nodule. Letters indicate special procedures (see text): H96 = harvest 96; Inc = incubated (3 days) seed wash; MM = minimal medium isolate; PYM = isolates from PY mass. Additional isolates corresponding to ET5 were: 1-2, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 2-1, 3-1, 3-2, 3-3, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 5-1, 5-3, 5-11, 5-12, PYM1-4; corresponding to ET9: Inc2-3, Inc5-1, Inc5-2, Inc5-5, Inc6-1, Inc6-3, Inc6-5, MM; corresponding to ET7: 2-4, 2-5, 2-7, 4-1, 4-2, 4-3, 4-4, 4-8, 4-9, 4-10, 4-11, 5-2, 6-6, 6-10, 7-1, 7-3, 7-4, 7-5, Inc1-1, Inc 1-2, Inc1-3, Inc1-4, Inc1-5, Inc2-4, Inc3-4, Inc4-2, Inc4-3, Inc5-4, Inc6-2, 4-5, 4-6, 4-7, Inc2-1, Inc2-2, PYM1-1; corresponding to ET8: Inc3-3, Inc3-5, Inc4-1, Inc4-4, Inc4-5, Inc5-3, Inc6-4, PYM2-1, PYM2-4, PYM2-5; corresponding to ET11: 2-3, Inc 3-1, H96-3, H96-4, H96-5, H96-6, H96-7, H96-8, H96-9, H96-10, 2-6, 5-4, 5-5, 5-6, 5-7, 5-8, 5-9, 6-1, 6-2, 6-3, 6-4, 6-5, 6-7, 6-8, 6-9, 6-11, 6-12, PYM1-2, PYM1-3, PYM1-5, PYM2-2, PYM2-3.

Few isolates representing different ETs had three *nifH* gene copies, a typical characteristic of *R. etli* strains [27]. Strains 5-10 (ET9), 6-1 (ET10), H96-1 and H96-2 (ET12) had an identical pattern to CFN42 but strains 4-1 (ET7) and 1-3 (ET5) had a one-band difference (corresponding to CFN42 *nif* region c [15]) both in *Eco*R1 and in *Bam*H1 digests, showing variations to the patterns reported [27].

Almost complete SSU rRNA genes were synthesized by PCR, after digestion, the patterns obtained by *R. etli* CFN42^T and seed isolates (2-4, 4-1, 4-3, 6-6 (ET7), 5-10 (ET9), 2-6, Inc 3-1 (ET10), H96-1 and H96-2 (ET12)) were identical. They were clearly distinct from strains of *R. tropici* CIAT899^T and CFN299, *S. meliloti* USDA1002^T, *R. galegae* HAMBI540^T, *M. loti* NZP2213^T, *R. leguminosarum* USDA2370^T, *M. huakuii* CCBAU2609^T, *M. ciceri* USDA3378^T, *M. mediterraneum* USDA3392^T, *S. fredii* USDA205^T, *R. gallicum* FL27, and *R. giardinii* Ro84 and H152^T.

4. Discussion

We tested two different procedures to demonstrate the natural occurrence of *Rhizobium* in *Phaseolus vulgaris* seeds. In the first procedure with non-sterilized seeds placed directly in sterilized flasks, nodulation was largely delayed probably due to the low accessibility of the rhizobia to susceptible root nodulation sites or to the lack of adequate hydration of the rhizobia on the seed. The hydration process tested in the second procedure may allow the bacteria to recover from a presumably desiccated or dormant stage on seeds or to promote the liberation of rhizobia from soil micropores [34,35] or from other bacteria-soil structures that may protect rhizobia from hydric stress but renders them poorly accessible to roots. The enhanced antibiotic sensitivity of seed borne rhizobia may be due to membrane damage as has been observed during drying and rehydration of nodule bacteria [36].

The results presented indicated that bean seed rhizobia from L-3-1-1-1 are *R. etli* strains and that they are present in a low proportion relative to other bacteria. In addition, the high ratio of different strains/ETs might be explained if there were low numbers of rhizobia on each seed, and if the nodules in one plant were formed by the descendants of a few *Rhizobium* cells located on the seeds. These bacteria, in some cases (as on the L-3-1-1-1 bean seeds), remained viable for nearly four years. There are reports of *Xanthomonas campestris* pv. *phaseoli* and other bacteria isolated from 3-year- [37] and from 15-year-old bean seeds [38]. Differences in longevity of bacteria on seeds seems to be related to moisture, storage temperature and to the specific bacterial species and strains [4].

R. etli strains, like other rhizobia from semiarid regions are normally exposed to long periods of hot and dry soil; for this reason they may be more adapted to desiccation than other *Rhizobium* species from temperate regions and as such, *R. etli* could be a model for the study of the genetic basis of desiccation resistance.

Two of the cultivars that harbor *Rhizobium* on the seeds were L-3-1-1-1 and N-8-1-1-6 that were harvested from fields in Celaya, Guanajuato using traditional methods that consist of pulling out the plant and some roots from the soil once the pods are dry. Plants are then threshed either manually or with a mechanical thresher. In both cases, seeds are separated from the plant residues by means of air ventilation.

For the 8 cultivars of bean seeds tested that did not carry rhizobia we have no information on their harvest process or on possible chemical treatments that could explain the lack of rhizobia. Furthermore, fungal contamination was frequently observed in the plant nodulation assays with non-sterilized seeds or with inocula from non-sterilized seeds of these cultivars, which could have inhibited *Rhizobium* nodulation. Indeed, we have isolated *Fusarium* spp. from these seeds as well (our own unpublished results).

We presume that the harvesting method used in Celaya has been practised for a very long time and it is still commonly used not only in Mexican agriculture but also in other Latin American countries and in the USA. Otherwise this practice is not currently used with other crops such as soybean and pea but is a normal procedure with chick-pea and subterranean clover. It has been observed previously that bacteria on seeds are soil bacteria acquired during the harvesting process through soil contamination [4]. Evidently, during bean harvest, soil and root detritus get mixed with the seeds before they are

separated by screening and ventilation procedures. Recently it was reported that indigenous soil bacteria may be transported by earthworms [39]. Our work gives the first evidence that *Rhizobium etli* is naturally viable on seeds for long periods and it also constitutes a solid basis for researchers to examine their seeds for the presence of indigenous rhizobia. It certainly provides additional elements to address the problem of the origin and distribution of both symbiotic and pathogenic microorganisms.

In addendum *R. etli*, but not other species of *Rhizobium*, like those nodulating *Leucaena leucocephala*, are on bean seeds. *Leucaena leucocephala* is native of Mexico and large populations of rhizobia have been encountered in these soils. With the improved described procedure to reveal seed-borne rhizobia we found no *Leucaena* nodulating rhizobia on *Phaseolus vulgaris* bean seeds, using 13 *Leucaena* plants and a largely increased inoculum (10 times the amount required for bean nodulation). This may indicate that *Phaseolus vulgaris* seed borne rhizobia are either nodule borne or from symbiosis-enriched populations from bean crops.

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References

[1] Martínez-Romero, E. and Caballero-Mellado, J. (1996) *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sciences 15, 113–140.

[2] Strain, S.R., Whittam, T.S. and Bottomley, P.J. (1995) Analysis of genetic structure in soil populations of *Rhizobium leguminosarum* recovered from the USA and the UK. Mol. Ecol. 4, 105–114.

[3] Hagen, M.J. and Hamrick, J.L. (1996) A hierarchal analysis of population genetic structure in *Rhizobium leguminosarum* bv. *trifolii*. Mol. Ecol. 5, 177–186.

[4] Schuster, M.L. and Coyne, D.P. (1974) Survival mechanisms of phytopathogenic bacteria. Annu. Rev. Phytopathol. 12, 199–221.

[5] Ash, C.G. and Allen, O.N. (1948) A comparison of methods recommended for the surface sterilization of leguminous seed. Soil Science Society Proceedings, pp. 279–283.

[6] Ladha, J.K., Watanabe, I. and Saono, S. (1988) Nitrogen fixation by leguminous green manure and practices for its enhancement in tropical lowland rice. In: Sustainable Agriculture: Green Manure in Rice Farming, pp. 165–183. The International Rice Research Institute, Los Baños, Philippines.

[7] Ladha, J.K., Pareek, R.P., So, R. and Becker, M. (1990) Stem-nodule symbiosis and its unusual properties. In: Nitrogen Fixation: Achievements and Objectives (Gresshoff, Roth, Stacey and Newton), pp. 633–639. Chapman and Hall, New York, NY.

[8] Gepts, P. and Bliss, F.A. (1988) Dissemination pathways of common bean (*Phaseolus vulgaris*, *Fabaceae*) deduced from phaseolin electrophoretic variability. II. Europe and Africa. Econ. Bot. 42, 86–104.

[9] Segovia, L., Young, J.P.W. and Martínez-Romero, E. (1993) Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* type I strains as *Rhizobium etli* sp. nov. Int. J. Syst. Bacteriol. 43, 374–377.

[10] Souza, V., Eguiarte, L., Avila, G., Cappello, R., Gallardo, C., Montoya, J. and Piñero, D. (1994) Genetic structure of *Rhizobium etli* biovar *phaseoli* associated with wild and cultivated bean plants (*Phaseolus vulgaris* and *Phaseolus coccineus*) in Morelos, Mexico. Appl. Environ. Microbiol. 60, 1260–1268.

[11] van Berkum, P., Beyene, D. and Eardly, B.D. (1996) Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). Int. J. Syst. Bacteriol. 46, 240–244.

[12] Amarger, N., Macheret, V. and Laguerre, G. (1997) *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int. J. Syst. Bacteriol. 47, 996–1006.

[13] Sessitsch, A., Ramirez-Saad, H., Hardarson, G., Akkermans, A.D. and de Vos, W.M. (1997) Classification of Austrian rhizobia and the Mexican isolate FL27 obtained from *Phaseolus vulgaris* L. as *Rhizobium gallicum*. Int. J. Syst. Bacteriol. 47, 1097–1101.

[14] Eardly, B.D., Wang, F.S., Whittam, T.S. and Selander, R.K. (1995) Species limits in *Rhizobium* populations that nodulate the common bean (*Phaseolus vulgaris*). Appl. Environ. Microbiol. 61, 507–512.

[15] Segovia, L., Piñero, D., Palacios, R. and Martínez-Romero, E. (1991) Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. Appl. Environ. Microbiol. 57, 426–433.

[16] Young, J.P.W. (1985) *Rhizobium* population genetics: enzyme polymorphism in isolates from peas, clover, beans and lucerne grown at the same site. J. Gen. Microbiol. 131, 2399–2408.

[17] Sanjuan, J., Tichy, H.V., Caballero-Mellado, J., Laguerre, G., Herrera-Cervera, J.A., Rodríguez-Alonso, F.I., Simon, R., Martínez-Romero, E., Amarger, N. and Olivares, J. (1996) Complex genetic structure of a Spanish soil rhizobial population isolated from *Phaseolus vulgaris*, E-28. In: Abstracts of

the 8th International Congress on Molecular Plant-Microbe Interactions. International Society for Molecular Plant-Microbe Interactions.
[18] Diouf, A. Personal communication.
[19] Sessitsch, A., Hardarson, G., Akkermans, A.D.L. and de Vos, W.M. (1997) Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Austrian soil. Mol. Ecol. 6, 601–608.
[20] Poupot, R., Martínez-Romero, E., Gautier, N. and Promé, J.C. (1995) Wild-type *Rhizobium etli*, a bean symbiont, produces acetyl-fucosylated, *N*-methylated, and carbamoylated nodulation factors. J. Biol. Chem. 270, 6050–6055.
[21] Vincent, J.M. (1970) A manual for the practical study of root-nodule bacteria. International Biological Programme Handbook, pp. 73–97. Blackwell, Oxford.
[22] Martínez, E., Palacios, R. and Sánchez, F. (1987) Nitrogen-fixing nodules induced by *Agrobacterium tumefaciens* harboring *Rhizobium phaseoli* plasmids. J. Bacteriol. 169, 2828–2834.
[23] Selander, R.K., Caugant, D.A., Ochman, H., Musser, J.M., Gilmour, M.N. and Whittam, T.S. (1986) Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51, 873–884.
[24] Nei, M. and Li, W.H, (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269–5273.
[25] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[26] Morett, E., Moreno, S. and Espín, G. (1988) Transcription analysis of the three *nifH* genes of *Rhizobium phaseoli* with gene fusions. Mol. Gen. Genet. 213, 499–504.
[27] Martínez, E., Pardo, M.A., Palacios, R. and Cevallos, M.A. (1985) Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. J. Gen. Microbiol. 131, 1779–1786.
[28] Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.
[29] Laguerre, G., Allard, M.R., Revoy, F. and Amarger, N. (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. 60, 56–63.
[30] Martínez-Romero, E. and Rosenblueth, M. (1990) Increased bean (*Phaseolus vulgaris* L.) nodulation competitiveness of genetically modified *Rhizobium* strains. Appl. Environ. Microbiol. 56, 2384–2388.
[31] Martínez-Romero, E., Segovia, L., Martins Mercante, F., Franco, A.A., Graham, P. and Pardo, M.A. (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41, 417–426.
[32] Piñero, D., Martínez, E. and Selander, R.K, (1988) Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar *phaseoli*. Appl. Environ. Microbiol. 54, 2825–2832.
[33] Bouchet, V. and Souza, V., personal communication.
[34] Bottomley, P.J. (1992) Ecology of *Bradyrhizobium* and *Rhizobium*. In: Biological Nitrogen Fixation (Stacey, G., Burris, R.H. and Evan, H.J., Eds.), pp. 293–348. Chapman and Hall, New York, NY.
[35] Ozawa, T. and Yamaguchi, M. (1986) Fractionation and estimation of particle-attached and unattached *Bradyrhizobium japonicum* strains in soils. Appl. Environ. Microbiol. 52, 911–914.
[36] Salema, M.P., Parker, C.A., Kidby, D.K., Chatel, D.L. and Armitage, T.M. (1982) Rupture of nodule bacteria on drying and rehydration. Soil Biol. Biochem. 14, 15–22.
[37] Basu, P.K. and Wallen, V.R. (1966) Influence of temperature on the viability, virulence, and physiologic characteristics of *Xanthomonas phaseoli*. Can. J. Bot. 44, 1239–1245.
[38] Schuster, M.L. and Sayre, R.M. (1967) A coryneform bacterium induces purple-colored seed and leaf hypertrophy of *Phaseolus vulgaris* and other *Leguminosae*. Phytopathology 57, 1064–1066.
[39] Daane, L.L., Molina, J.A.E., Berry, E.C. and Sadowsky, M.J. (1996) Influence of earthworm activity on gene transfer from *Pseudomonas fluorescens* to indigenous soil bacteria. Appl. Environ. Microbiol. 62, 515–521.