

Inoculation with indigenous *rhizobium* strains increases yields of common bean (*Phaseolus vulgaris* L.) in northern Spain, although its efficiency is affected by the tillage system

Daniel Mulas¹ · Victoria Seco² · Pedro A. Casquero³ · Encarna Velázquez⁴ · Fernando González-Andrés¹

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Abstract Common bean (*Phaseolus vulgaris* L.) crops hold the potential to obtain higher yields by enhancing their biological nitrogen fixation (BNF) with *Rhizobium*. However in contrast to other legumes, common bean has shown a lack of positive response to inoculation with *Rhizobium* in many cases. This has led to a limited use of rhizobial inoculants in this crop, especially in Europe. The adaptation of bacterial strains to the rhizosphere is a key factor in the success of any inoculant, especially in a promiscuous legume such as common bean. This research aimed at increasing common bean yields via inoculation with effective indigenous *Rhizobium leguminosarum* strains. Three highly effective strains (LCS0306, LBM1123 and ZBM1008) which were selected according to their effectiveness at BNF in hydroponic conditions were separately inoculated onto common bean in a field experiment. The experiment was carried out under three environments and three tillage systems: conventional-tillage (CONVT), no-tillage (NT) and a cover-crop (CC). The grain yield observed with seed inoculation was significantly higher than the yield obtained with uninoculated seed under CONVT and CC. However, under NT inoculation had no effect.

Furthermore, under CONVT and CC, inoculation with *R. leguminosarum* LCS0306 produced even higher yields than those obtained in nitrogen-fertilised or control plots. This is the first attempt to explain the inoculation performance of common bean under different tillage systems in Europe. A gene-based hypothesis has been used to explain the effectiveness of indigenous common bean rhizobia as nitrogen fixers in this crop.

Keywords *Rhizobium leguminosarum* · No-tillage · Cover-crop · Conventional-tillage · Yield · Field experiment

1 Introduction

Common bean (*Phaseolus vulgaris* L.) is the second most cropped legume worldwide after soybean (*Glycine max* L. Merr), with a combined production of 44.2 million tons for green and dry beans in the year 2015 (FAOSTAT 2015). *Phaseolus vulgaris* L. was domesticated in America, with two main domestication centers in the Mesoamerican and the Andean regions (Gepts and Debouck 1991). This legume has co-evolved with rhizobia carrying the symbiotic plasmids typical of symbiovar phaseoli (Aguilar et al. 2004), which seems to be exclusive for bean nodulation (Amarger et al. 1997). This symbiovar was dispersed worldwide probably attached to bean seeds and the symbiotic plasmid was transferred to native rhizobial strains, for example *R. leguminosarum* in Spain (García-Fraile et al. 2010), which is considered to be a secondary diversification center of common bean (Santalla et al. 2002). Although common bean is a promiscuous host nodulating with rhizobial species native to soils from different continents, erratic and poor nitrogen fixation has been frequently reported (Buttery et al. 1987; Graham 1981; Rodríguez-Navarro et al. 2000). Therefore, the

✉ Fernando González-Andrés
fgona@unileon.es

¹ Instituto de Medio Ambiente, Recursos Naturales y Biodiversidad, Universidad de León, Av Portugal 41, E24071 León, Spain

² Departamento de Ingeniería y Ciencias Agrarias, Universidad de León, Av Portugal 41, E24071 León, Spain

³ Escuela Superior y Técnica de Ingeniería Agraria, Universidad de León, Av Portugal 41, E24071 León, Spain

⁴ Departamento de Microbiología y Genética, Universidad de Salamanca, Edificio Departamental de Biología, Lab. 209. Av Doctores de la Reina sn., E37007 Salamanca, Spain

inoculation of this legume with effective rhizobial strains may increase its production. In Spain, a lack of response to inoculation in field conditions when non-native strains were used as inoculant has been reported (Rodríguez-Navarro et al. 2000; Daza et al. 2000), but we have shown that selected local strains can increase bean production up to the levels obtained with 166 kg ha⁻¹ nitrogen (N) fertiliser application (Mulas et al. 2011). This is in agreement with results obtained with soybean, which usually shows high yields after a correct seed inoculation, mainly with elite *Bradyrhizobium* strains (Hungria and Vargas 2000; Albareda et al. 2008).

Although common bean has not been widely bred in terms of its potential for a yield increase, as has happened with soybean, there is an increasing interest in developing sustainable agronomic practices that also enhance productivity. Tillage and fertilisation are key agronomical practices, and thus the selection of effective rhizobial strains to be used as inoculants for N supply to the plant, instead of N mineral fertilisation, is the optimum strategy to improve plant nutrition in the best economic and environmental conditions. On the other hand, the development of a rhizobial inoculant must take into consideration the conditions in which the common bean is established in terms of local agricultural practices. Important differences in the soils reported under different tillage systems have a direct impact on the soil microbial community and, therefore, in the case of legumes, on the rate of BNF by the nodules.

Conservation tillage systems are gaining popularity and replacing conventional tillage (CONVT) in the agricultural areas of several continents. In Europe, conservation tillage is performed on approximately 16 million hectares (10–12 % of the arable land), of which 2 million hectares are in Spain (Abdalla et al. 2013). Conservation tillage includes soil management with a minimal impact on its composition, structure and biodiversity, by restricting any land preparation activities to a shallow depth and eliminating soil inversion, while conserving and managing crop residues. This leads to a higher organic matter content and a reduction in nutrient losses through leaching and erosion (Cunningham et al. 2004). The effect of different tillage systems on rhizobia has been mainly studied in tropical or subtropical areas of America and Oceania, where no tillage (NT) is widely used in soybean crops (Wheatley et al. 1995; Loureiro et al. 2007; Bizarro et al. 2011) and common bean crops (Kaschuk et al. 2006a, b; Alguacil et al. 2011). Most of the aforementioned studies analyzed the effect of tillage on rhizobia or bradyrhizobia diversity, which in general is increased by NT, and they focused on BNF, which usually is increased under NT (Hughes and Herridge 1989; Wheatley et al. 1995; Alguacil et al. 2011).

Under CONVT, the mouldboard plow is used for primary tillage, and one or several cultivations are practiced before the next crop is established, leaving soils exposed in the inter-crop periods. The use of a cover crop (CC) for these intervals is

increasing in temperate regions worldwide, with growing evidence for the sustainability of this agricultural practice. It prevents leaching and erosion, buffers soil temperature and prevents predominant weeds because of competition and by allelopathic effects of the CC and its residues on weeds (Urbano et al. 2006; Inderjit et al. 2005). Although some authors have reported a negative effect of cover crops on nodulation (Batish et al. 2007), other studies have reported an improvement in BNF (Sturz and Christie 2003). Ruffo et al. (2004) reported that the introduction of a CC showed no reduction in soybean yield, but did increase soil fertility by trapping N and reducing nitrate leaching to the subsoil.

Although several studies have analyzed the effects of tillage on soil properties in the Mediterranean Basin and, specifically, in northern Spain (Sombrero and De Benito 2010), there are no studies in this region about the interaction between tillage and BNF by legumes. The objective of the present work was to evaluate under field conditions and in different environments the effect of the combination of inoculation with effective indigenous *Rhizobium* strains and different tillage practices in order to increase the profitability and sustainability of common beans by achieving yields at the same level as a N-fertilised crop. The work was conducted in the region designated as the Protected Geographical Indication (PGI) ‘Alubia de La Bañeza-León’ (León, Spain) with a landrace of common bean ‘Riñón’. This area was the first region in Spain to come into common bean production and it has produced an average of over 6000 t per annum during the last decade. Three strains isolated from the local common bean landrace ‘Riñón’ were used as inoculants; two of these were tested under CONVT in a previous study, and yields higher than those obtained with conventional N fertilization were obtained (Mulas et al., 2011).

2 Materials and methods

2.1 Rhizobial strains

Three rhizobial strains, LCS0306, LBM1123 and ZBM1008 were used as inoculants. Strain LCS0306 was isolated in a loamy soil with a pH of 5.1 in Sueros de Cepeda and strain LBM1123 in a silty loamy soil with pH 6.8 in Miñambres de la Valduerna (Mulas et al. 2011). Strain ZBM1008 was isolated in a silty loamy soil from the locality of Maire de Castroponce with a pH of 7.0 (Mulas et al. 2008).

2.2 Symbiotic effectiveness analyses

The three strains used in this study were selected on the basis of the IR BNF effectiveness (Mulas et al. 2011), which was assessed following the procedure described by Howieson et al. (2000) which consists of growing common bean plants

Table 1 Edapho-climatic conditions of soils from the sites in which the field experiment was established

A. Edaphic characteristics										
Texture (%)		pH 1:2 (soil:water)	Organic matter (%)	Total nitrogen* (%)	Ratio C/N	Phosphorus (Olsen) (mg kg ⁻¹)	Potassium (cmol ⁽⁺⁾ kg ⁻¹)	Calcium (cmol ⁽⁺⁾ kg ⁻¹)	Magnesium (cmol ⁽⁺⁾ kg ⁻¹)	Sodium (cmol ⁽⁺⁾ kg ⁻¹)
Sand	Silt clay									
León	30	40	6.66	0.33	9.31	18.60	0.28	18.70	2.80	0.15
	26	42	7.80	0.14	8.33	77.20	1.39	3.90	0.72	0.06
B. Climatic characteristics [‡]										
Year 2008	Month	Temperatures (°C)		Tavg	Lavg	Lmin	R (mm)	PET (mm)	I (%)	D < 10 °C
		Hmax	Havg							
León	May	23.6	17.0	12.2	7.3	0.0	144.6	150.2	39.5	27
	June	30.4	23.3	16.9	10.4	5.6	9.9	161.6	69.5	15
	July	34.0	26.2	18.5	10.8	5.4	0.7	181.6	81.1	10
	August	33.0	26.6	19.0	11.4	5.8	3.6	177.9	82.3	5
	September	25.8	21.5	15.1	8.6	3.4	10.3	134.3	68.6	18
Year 2009	León	May	21.4	14.2	6.9	1.2	25.0	154.2	69.3	24
		June	31.6	18.1	11.2	6.0	25.2	172.2	61.9	10
		July	31.0	18.8	11.1	5.4	20.4	189.8	80.9	10
		August	32.6	20.9	13.3	7.8	2.0	184.6	82.3	3
		September	31.6	16.9	9.7	3.4	0.7	147.3	78.2	16
	Cogorderos	May	22.9	14.6	6.2	0.0	46.4	-	-	28
		June	33.4	18.5	10.6	4.9	24.8	-	-	14
		July	33.6	19.3	10.4	5.0	4.2	-	-	14
		August	34.3	21.1	11.9	7.3	11.2	-	-	4
		September	33.6	17.6	8.9	2.4	0.2	-	-	20

* Total N: organic + nitric + ammonia nitrogen. Hmax: maximum highest temperature (°C); Havg: average highest temperature (°C); Tavg: average mean temperature (°C); Lavg: average lowest temperature (°C); Lmin: minimum lowest temperature (°C); R: monthly precipitation (mm); PET: potential evapotranspiration (mm); I: solar radiation (%); D < 10 °C: number of days with temperature under 10 °C. [‡] The climatic data were recorded at the León and the Astorga (for Cogorderos) meteorological stations, which are the closest ones to the León and Cogorderos sites, respectively

under axenic conditions in modified Leonard jars with hydroponic N-free medium (Rigaud and Puppo 1975) and vermiculite as a substrate. Sixteen plants were inoculated with each individual strain, and the dry biomass (aerial parts and roots), the N content in the biomass, and the number and weight of nodules were compared with two uninoculated controls, one of which was fed with a N-supplemented medium.

2.3 Phylogenetic analyses

The sequences of the *recA*, *atpD* and *nodC* genes were previously obtained for strains LCS0306, and LBM1123 (Mulas et al. 2011), and in this study we compared these genes with those of the strain ZBM1008 obtained in this study according to the methodology described in Mulas et al. (2011). The sequences were aligned using the Clustal W software (Thompson et al. 1997), and the distances were calculated according to Kimura's two-parameter model (Kimura 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou and Nei 1987), and the bootstrap analysis was based on 1000 resamplings. The MEGA 4 package (Tamura et al. 2007) was used for all analyses.

2.4 Edaphic and climatic conditions at the experimental sites

The soils of the experimental sites for the field trial (Table 1) were located in the municipalities of Cogorderos (42°32'50" N, 6°01'39"W, 903 m asl) and León (42°34'59"N, 5°35'29"W, 818 m asl). The two experimental sites are included in the PGI 'Alubia de La Bañeza-León'. The soil in León had no previous tradition of common bean cultivation, whereas that located in Cogorderos had a long tradition of cultivation of this crop. During the years before the experiment, common bean had been cultivated every three or four years, with the previous cultivation four years before the start of the experiment. The combination of locations and years provided three different environments: León-2008, León-2009 and Cogorderos-2009. The soils showed differences in their pH, organic matter content and cation exchange capacity (CEC). The soil from Cogorderos was slightly basic, with low organic matter content and low CEC of 5.67 cmol(+) kg⁻¹. On the other hand, the soil from León was slightly acidic, with a higher content of organic matter and, therefore, the CEC was several times higher, up to 23.77 cmol(+)kg⁻¹.

For each soil, the number of infective rhizobia for common bean was calculated using the most probable number methodology (MPN), using a 10-fold dilutions series up to 10⁻⁶, and inoculating four common bean replicate plants cv. 'Riñón' with 1 ml of each corresponding dilution. According to the MPN results, in the soil from León there were less than 10 rhizobia capable of nodulating common bean per gram of soil, whereas in the soil from Cogorderos, with a long tradition of

common bean cultivation, the MPN was 3.1×10^4 bean-nodulating rhizobia per gram of soil.

The field experiment was performed in the years 2008 and 2009. The most remarkable climatic conditions during 2008 were a very wet spring and a cool summer, particularly during August and September. The amount of precipitation registered during May 2008 was 144.6 mm whereas the average for this month is 58.0 mm. On the other hand, summer conditions (June to September) were dry in 2008 (24.5 mm, whereas the average rainfall for that period is 130.0 mm), with low temperatures towards the end of the summer and some minimum temperatures around 5 °C in August and September.

During 2009, both meteorological stations recorded a dry spring, which reduced the residue coverage in the NT and CC tillage systems. This summer was also drier than average in León (48.8 mm between June and September) and Cogorderos (40.4 mm). Regarding the temperature readings, this summer was warmer than the average, nearly 2 °C higher each month on average in León, particularly towards the end of the summer (see in Table 1 Tavg in August and September 2009 vs. 2008).

2.5 Description of the field experiment

In each environment, the experimental design was a split plot in a randomized complete block that included two experimental factors, the tillage system and the inoculation treatment. The tillage system was assigned to the main plot, and the inoculation treatment to the subplot. The experimental subplot was 18 m², with six plant rows leaving 0.50 m distance between them and 0.15 m distance between plants, for a total of 240 plants per plot. A buffer area was established surrounding each plot, with a width of 2.25 m. This buffer area remained non-cropped to avoid inoculant and fertiliser transfer.

The experiment included the three different tillage systems (CONVT, NT and CC) in León whereas only CONVT and CC were used in Cogorderos. The reason for this was that there was only one plot with more than 5 years under NT system in the demarcation of the PGI, and it was located in León. The tillage operations for CONVT included mouldboard plowing in November. In February (February 2nd in 2008 and February 13th in 2009) a disk harrow was used to remove the weeds that appeared during the winter. In the CC plots, the soil was prepared with a disk harrow to sow the rye (*Secale cereale* L.), on the same dates in February already indicated. Rye was sown one week after harrowing with a conventional seed drill, at a seed density of 200 kg ha⁻¹, which is a high density in order to enhance the competition of the rye against the weeds. The rye cover was partially buried by means of two passes with a disk harrow. Burying and incorporation of the rye cover was planned for the phenological stage corresponding to the first node, stage I according to Keller and Baggiolini (1954), but the excessive rainfall during 2008 delayed the

incorporation until the phenological stage of flag leave appearance (stage L). This operation led to a partial incorporation of the rye biomass, leaving some crop residues on the surface for at least 50 % of the soil cover. Seedbed preparation with a light cultivator was the final operation in both CONVT and CC, the day before sowing. Sowing was on May 29th 2008 and May 20th 2009. On the area cropped under NT the only practice that was carried out prior to sowing was a weed control with the broad-spectrum systemic herbicide glyphosate at a rate of 2.5 l per ha of a commercial product containing 41.5 % (m/v) of the active ingredient isopropylamine salt of V-(phosphonomethyl)-glycine.

There were three inoculation treatments, the three *R. leguminosarum* strains (LCS0306, LBM1123 and ZBM1008) as well as two uninoculated controls, one non-fertilised with N and the other fertilised with 166 kg N ha⁻¹, which is the amount of N theoretically extracted by the crop for an expected yield of 4000 kg ha⁻¹. Half of this amount was provided five days before sowing and half at the beginning of flowering, in both cases as ammonium nitrate (33.5 % N). The inoculants were peat-based, with a final density of 3 × 10⁸ cells g⁻¹ of peat. The peat had previously been ground and packaged in low density polyethylene bags and sterilized with 50 KGy of gamma irradiation (Albareda et al. 2008). The saturated bacterial culture that contained the inocula was aseptically injected into the bag at an initial content of 3 × 10⁹ cells ml⁻¹ of broth, obtained after six days of incubation of each strain at 28 °C in YMB medium (Vincent 1970). The seeds were mixed with the inoculant just prior to sowing, at a rate of 1 × 10⁶ rhizobia seed⁻¹ allowing it to attach to the seed for a few hours, while avoiding direct sunlight.

According to the soil analyses (Table 1), Cogorderos showed a high P and K content in the soil, whereas León had a medium P and a low K content. In Cogorderos, due to high soil P and K levels, fertilisation was unnecessary. In León the soil was amended with 25 kg P₂O₅ ha⁻¹ in the form of Ca superphosphate (18 % P₂O₅) and 100 kg K₂O ha⁻¹ as KCl (60 % K₂O) five days prior to sowing. These fertiliser applications were calculated on the basis of the soil content and the expected yield of 4000 kg ha⁻¹. All the non-cropped areas between plots were kept free of weeds using glyphosate at the rate indicated previously. Within the plot, manual weeding was used. The crop was drip irrigated in order to maintain the soil moisture between 80 % and 100 % of the available soil water. The appearance of silverleaf whitefly (*Bemisia tabaci* Gennadius) in some plants led to a single treatment with the systemic insecticide imidacloprid, at a dose equivalent to 75 mg ha⁻¹ of the active ingredient.

2.6 Data collection and statistical analysis

The common bean plants were harvested at grain maturity after approximately 4 months of growth, on October 6th

2008 and September 25th 2009. Forty random plants from the central lines of each experimental unit were individually hand harvested. Each plant was kept separate. From each plant, the following traits were recorded: (i) grain yield (oven-dried at 60 °C to a constant weight), (ii) the yield components, (iii) total dry aerial biomass (oven-dried at 60 °C to a constant weight), (iv) harvest index (calculated by the ratio of grain yield/total dry aerial biomass), (v) nitrogen accumulated in the seeds determined from the dry weight of seeds per plant and the total N content determined by Kjeldahl method. Combined analysis of variance appropriate to split plots in a randomized complete block design was performed using the general linear models (GLM) procedure of the SPSS statistics v. 17.0. The tillage system, the inoculation treatment and the environment were considered to be fixed effects.

3 Results

3.1 Phylogeny of inoculated strains

The strains LCS0306 and LBM1123 have identical 16S *rrs* gene sequences (Mulas et al. 2011), but different *recA* and *atpD* genes that allows them to be placed in groups I and II, respectively, within the species *R. leguminosarum* (Mulas et al. 2011). The analysis of these two genes in strain ZBM1008 showed that it belongs to the group I of the species *R. leguminosarum* and has *recA* and *atpD* genes 100 % identical with respect to those from strain LCS0306 (Fig. 1).

Strains LCS0306 and LBM1123 were previously placed into the symbiovar phaseoli and it was shown that they carry the γ allele of the *nodC* gene (Mulas et al. 2011). The analysis of the *nodC* gene in strain ZBM1008 showed that it also belongs to the symbiovar phaseoli, carrying the same *nodC* allele as the other two strains (Fig. 2).

3.2 Symbiotic characteristics of strains

Studies of symbiotic effectiveness performed previously for strains LCS0306 and LBM1123 in hydroponic conditions showed that the plants inoculated with these strains had 45.4 and 42.9 mg N per plant, respectively, and a dry shoot weight of 1341.8 and 1187.0 mg per plant, respectively, being the most effective out of a collection of 33 strains (Mulas et al. 2011). In the present study we analysed the BNF of strain ZBM1008 under the same hydroponic conditions and using the same controls, and observed that the plants inoculated with this strain had an average total N content of 37.4 mg per plant, and a total dry aerial biomass of 1033.8 mg per plant. Although these values are lower than those obtained for several strains in the study of Mulas et al (2011), we decided to include the strain ZBM1008 in the field experiments in the

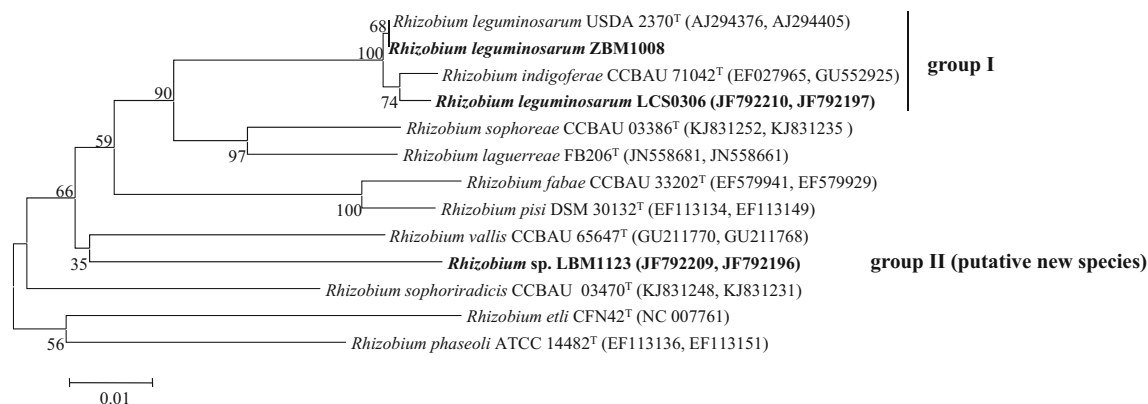


Fig. 1 Neighbour-joining phylogenetic tree based on *recA* and *atpD* concatenated gene sequences (520 and 500 nt, respectively) showing the position of representative strains from each RAPD group. Bootstrap

values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Accession numbers from GenBank are given in brackets

present study because it was isolated from a different province to strains LCS0306 and LBM1123.

3.3 Field experiment results

The results were subjected to an analysis of variance (ANOVA) of several dependent variables, the sources of variation being the environment, the tillage system, and the inoculation treatment. The results of the combined ANOVA using all data from the environments and treatments are shown in Table 2. Table 3 shows the comparison of the mean values observed for the following dependent variables: total dry aerial biomass, harvest index, grain yield, yield components, and total nitrogen in the seeds, for each of the three sources of variation. There

were highly significant differences in all the dependent variables analyzed for the environment, the tillage system and the inoculation treatment (Table 2).

3.3.1 Grain yield and total n in seeds

Grain yield showed highly significant differences among all the environments, the highest being in Cogorderos-2009, intermediate in León-2009 and minimum in León-2008. Regarding tillage, in the NT and in the CC, the yield was significantly lower than in the CONVT. With respect to the inoculation treatments, they produced the same yield as the control with N fertiliser, regardless of the strain used, in all the cases being significantly higher than the yield of the control without N fertiliser (Tables 2 and 3).

Fig. 2 Neighbour-joining phylogenetic tree based on *nodC* gene sequences (840 nt) showing the position of representative strains from each RAPD group. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Accession numbers from GenBank are given in brackets

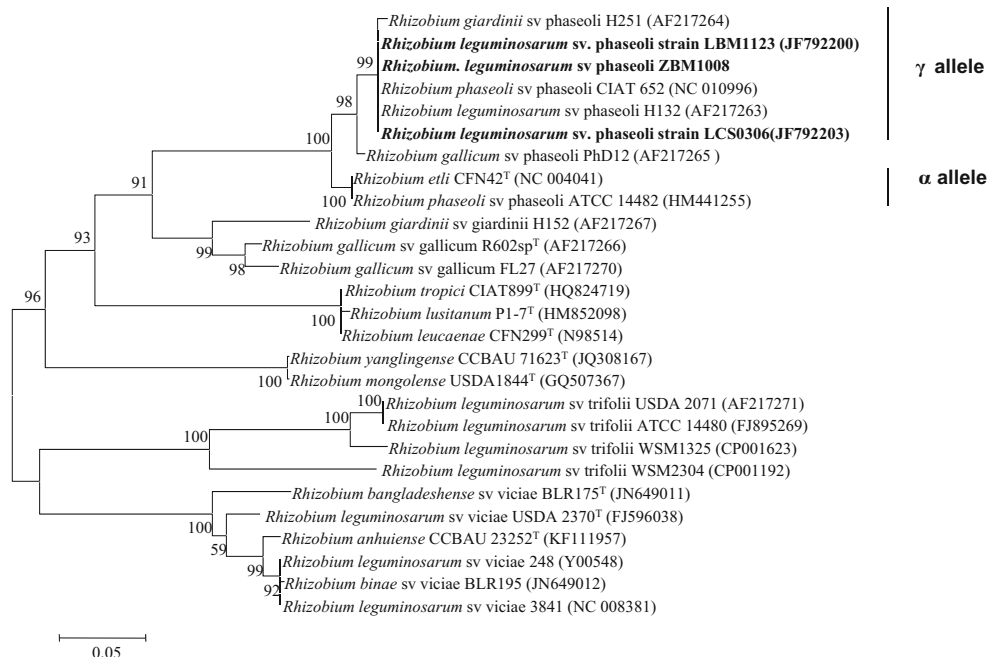


Table 2 ANOVA of all the dependent variables analyzed in the field experiment with environment, tillage system and inoculation treatment as factors

Source of variation	Total dry aerial biomass (g plant ⁻¹)	Harvest Index		Yield (g plant ⁻¹)		Yield components				N in seeds (mg plant ⁻¹)				
						Pods per plant		Seeds per pod		1000 seeds weight (g)				
		Mean square	F	Mean square	F	Mean square	F	Mean square	F	Mean square	F			
Environment (E)	1.2 10 ⁶	1782.2***	95.505	4940.8***	3.3 10 ⁵	1257.5***	1.0 10 ⁵	1833.6***	6993.1	1838.0***	8.3 10 ⁷	1167.4***	4.0 10 ⁸	1149.8***
Main plot Analysis														
Tillage (T)	1.2 10 ⁶	1869.5***	94.789	4464.4***	3.3 10 ⁵	1241.0***	1.0 10 ⁵	1850.5***	6879.6	1675.5***	8.2 10 ⁷	1067.8***	4.0 10 ⁸	1185.3***
E * T	5340.6	8.457***	0.052	2.713	4533.7	3.706***	240.2	4.496**	22.17	5.905**	3.1 10 ⁵	4.362**	1.9 10 ⁶	5.611**
Subplot Analysis														
Inoculation (I)	7.2 10 ⁵	1068.1***	56.88	2678.1***	2.0 10 ⁵	723.2***	6.0 10 ⁴	1074.4***	4126.4	1001.7***	4.9 10 ⁷	640.8***	2.4 10 ⁸	688.0***
E * I	1620.3	2.460	0.026	1.369	381.9	1.485	79.40	1.04510 ⁴	1.203	0.314	2.4 10 ⁴	0.333	5.6 10 ⁵	1.638
T * I	2396.4	3.822***	0.036	1.724	501.9	1.937	130.3	2.419	5.174	1.260	2.1 10 ⁴	0.276	6.1 10 ⁵	1.819
E * T * I	1269.3	2.124	0.041	2.199	149.3	0.369	159.2	3.118***	4.084	1.085	2.7 10 ⁴	0.369	7.6 10 ⁵	2.363**

** Significant at $P < 0.01$ *** Significant at $P < 0.001$

The N accumulated in the seeds was higher in Cogorderos-2009 and León-2009 and lower in León-2008. When analyzed under different tillage systems, the CONVT produced a higher N content in the seeds than the NT and CC, and when analyzed under different inoculation treatments, the bacteria were able to provide the same amount of N to the seeds as the N fertiliser, and a higher amount than the control without N fertiliser (Tables 2 and 3).

3.3.2 Yield components

The yield component most affected by the tillage system was the number of pods per plant. Therefore, the higher yield of the plots under CONVT was a consequence of a higher number of pods per plant, although this resulted in significantly smaller seeds (Table 3). The inoculation and the fertilisation induced a higher number of pods per plant, but it did not affect either the number of seeds per pod, or the size of the seed. Conversely, the environment affected the number of pods per plant, the number of seeds per pod, and the 1000 seed weight in different ways (Table 3); for instance the lowest yield in León-2008, was a consequence of the lowest seeds weight.

3.3.3 Total dry aerial biomass and harvest index

The total dry aerial biomass was higher in Cogorderos-2009 and León-2009 than in León-2008, and the harvest index showed the same tendency (Table 3). Regarding the tillage system, the plants grown under CONVT produced the highest total aerial biomass, although the harvest index was intermediate between the other two treatments. Regarding inoculation, the plants that had been inoculated and those which received N fertiliser developed higher amounts of total aerial biomass than the control without N fertiliser; two of the inoculation treatments (ZBM1008 and LBM1123) produced a significant increase in the harvest index compared with the N fertilised control.

3.3.4 Interaction between factors

For all the dependent variables analyzed there were interactions between the environment and the tillage (Table 2), as the environment in León-2008 was unfavorable for NT and CC, but it did not affect the CONVT. The total dry aerial biomass, the grain yield and the N in seeds were significantly lower in León-2008 compared with the environment in 2009 for the NT and the CC, but not for the CONVT (data not shown).

There was also an interaction between the tillage system and the inoculation treatment for the total dry aerial biomass, but not for the grain yield or the total N in seeds (Table 2). The interaction was due to the fact that inoculation had a higher positive impact on the total aerial biomass under CONVT and CC than under NT (Table 4). In the CONVT the biomass of

Table 3 Comparison of means of the common bean field experiment variables and comparison of their values to the N-fertilized control (*% values in italics) (values followed by the same letter do not show significant difference according to the LSD test, $p < 0.05$)

Source of variation	Total dry aerial biomass (g plant ⁻¹)		Harvest Index	Yield (g plant ⁻¹)		Yield components			N in seeds (mg plant ⁻¹)		
						Pods per plant	Seeds per pod	1000 seeds weight (g)			
Environment											
Cogorderos - 2009	60.86 b		0.54 b	33.19 c		18.66 b	3.81 a	471.84 b	1090.47 b		
León - 2009	57.57 b		0.53 b	30.78 b		15.43 a	4.96 b	537.83 c	1054.34 b		
León - 2008	51.56 a		0.44 a	24.51 a		15.66 a	3.82 a	375.18 a	936.44 a		
Tillage system											
No tillage (NT)	50.49 a		0.49 a	26.02 a		14.82 a	4.32 a	486.39 b	951.26 a		
Conventional tillage (CONVT)	64.65 b		0.50 ab	33.36 b		18.34 b	4.13 a	438.02 a	1167.25 b		
Cover crop (CC)	52.45 a		0.51 b	27.60 a		15.50 a	4.36 a	477.55 b	933.57 a		
Inoculation											
Non-fertilized control	49.33 a	85*	0.50 ab	104	25.43 a	87	14.78 a	4.17 a	460.69 a	906.22 a	84
N fertilized control	58.25 b	100	0.48 a	100	29.20 b	100	16.13 ab	4.31 a	467.64 a	1073.72 b	100
Inoculated with LCS0306	60.00 b	103	0.50 ab	104	31.22 b	107	17.52 b	4.28 a	458.52 a	1071.62 b	100
Inoculated with ZBM1008	56.31 b	97	0.51 b	106	29.66 b	102	16.28 b	4.22 a	466.04 a	1021.93 b	95
Inoculated with LBM1123	58.23 b	100	0.51 b	106	31.02 b	106	17.10 b	4.36 a	474.89 a	1048.77 b	98

Table 4 Comparison of mean yield for the inoculation treatments by tillage system and comparison of their values to the N fertilized control (*%, in italics) (values followed by the same letter do not show significant difference according to the LSD test, $p < 0.05$)

Inoculation treatments	Tillage * Inoculation				Environment * Inoculation		
	Total dry aerial biomass (g plant ⁻¹)		Yield (g plant ⁻¹)		Yield (g plant ⁻¹)		
	Conventional tillage (CONVT)				Cogorderos 2009		
Non-fertilized control	54.37a	89*	27.21a	86	26,61a	a	86
N fertilized control	61.27ab	100	31.65a	100	30,94ab	ab	100
Inoculated with LCS0306	76.12c	124	38.75b	122	36,79bc	bc	119
Inoculated with ZBM1008	63.44ab	104	32.50ab	103	34,16bc	bc	110
Inoculated with LBM1123	67.29bc	110	36.51b	115	37,48c	c	121
	No tillage (NT)				León 2009		
Non-fertilized control	50.59a	92	25.49a	95	28,11a		96
N fertilized control	55.24a	100	26.94a	100	29,25a		100
Inoculated with LCS0306	45.29a	82	24.11a	89	32,38a		111
Inoculated with ZBM1008	50.99a	92	27.33a	101	31,65a		108
Inoculated with LBM1123	50.31a	91	26.21a	97	32,55a		111
	Cover crop (CC)				León 2008		
Non-fertilized control	43.71a	76	23.71a	84	21,50a		78
N fertilized control	57.24b	100	28.21b	100	27,68a		100
Inoculated with LCS0306	53.17b	93	28.20b	100	25,97a		94
Inoculated with ZBM1008	53.09b	93	28.51b	101	24,12a		87
Inoculated with LBM1123	55.80b	97	29.54b	105	23,42a		85
F value of the interaction	3.822***		1.937 ns		1.485 ns		

** Significant at $P < 0.01$ *** Significant at $P < 0.001$

ns: not significant

inoculated plants even exceeded the N fertilised control, although the difference was only significant for the strain LCS0306. Conversely, neither the inoculation nor the N fertilisation in the NT system had an effect on the aerial biomass. The grain yield showed a similar behaviour to that of the total aerial biomass, but the observed differences were not statistically significant (Table 4). Notwithstanding the lack of statistical significance, the inoculation was apparently highly effective in the CONVT, effective in the CC and ineffective in the NT.

Although there was no statistical interaction between the environment and the inoculation treatment for any dependent variable, the effect of the inoculation on the common bean yield was higher in Cogorderos-2009 and León-2009 than in León-2008 (Table 4).

4 Discussion

European agriculture is now undergoing considerable changes that are focused on increasing the productivity of the land and reducing the farmers' dependence on Common Agricultural Policy (CAP) subsidies, introducing a 'greening' policy and also focusing on quality products.

Under these circumstances, the recognition of European-wide labeling like PGI, PDO or TSG to some food-stuffs can revalue them, thereby increasing their competitiveness for local farmers. This is the case for common bean in Northern Spain which has been recognized by the award of the PGI 'Alubia de La Bañeza-León'. The designated area covers nearly 5000 km² in the provinces of León and Zamora where common bean is cropped under irrigation and grown during spring and summer. This area produced roughly 40 % of the Spanish *P. vulgaris* production in 2014. There are records of common bean cultivation in this area since the 18th century (Rubio Pérez 1987), but it was neglected during the last three decades of the 20th century (Valeciano et al. 2006). However, a significant reduction in the application of fertilisers in the whole crop rotation as a consequence of the introduction of a legume crop, and a rise in competitiveness of the common bean after being awarded a quality label at European level could make this crop more attractive for farmers and consumers.

Core genes such as *recA* and *atpD* are commonly used to define *Rhizobium* species and they have been used for the analysis of strains isolated in León from *P. vulgaris* (García-Fraile et al. 2010; Mulas et al. 2011). The strains LCS0306 and LBM1123 were assigned to the species *R. leguminosarum* on the basis of an analysis of these two house-keeping genes (Mulas et al. 2011). However, when clustering the strains studied by Mulas et al. (2011), two groups were established (I and II). *R. leguminosarum* LCS0306 belonged to group I, whereas *R. leguminosarum* LBM1123 was

included in group II. Interestingly, the new strain *R. leguminosarum* ZBM1008 (isolated in a different province, Zamora) also belonged in group I, the least abundant group in León soils (Mulas et al. 2011). Despite the scarcity of group I of *R. leguminosarum* in León province, it is widely distributed in Spanish soils, and also Worldwide. Indeed, *R. leguminosarum* USDA 2370^T isolated in the USA is also included in group I (Mulas et al. 2011). Concerning group II, it probably represents a different species from *R. leguminosarum* considering its phylogenetic position after the description of several new species of genus *Rhizobium* such as *R. vallis* (Wang et al. 2011), *R. laguerreae* (Saïdi et al. 2014) and *R. sophorae* (Jiao et al. 2015) which has changed the topology of the phylogenetic tree of concatenated *recA* and *atpD* genes with respect to that found by Mulas et al. (2011).

The ability to induce nodulation by a given strain depends on the nodulation genes, the *nodC* gene being essential for the nodulation and specificity of rhizobia, as well as for symbiovar delineation (Rogel et al., 2011; Peix et al. 2015). In the case of the species *R. leguminosarum* the analysis of this gene allowed the delineation of three symbiovars, namely *viciae*, *trifolii* and *phaseoli* (García-Fraile et al. 2010). According to the *nodC* gene analysis, the three strains LCS0306, LBM1123 and ZBM1008 belonged to the symbiovar *phaseoli* (García-Fraile et al. 2010; Mulas et al., 2011). Several alleles of this gene have been found in the American distribution centers of common bean, with the allele denominated γ being the most widely distributed in Spain (Aguilar et al. 2004; García-Fraile et al. 2010; Mulas et al. 2011). This *nodC* allele has been also found in *Rhizobium etli* Viking 1, of an American origin (Josephson et al. 1991). Therefore, it has been postulated that the plasmid containing the *nodC* gene typical of biovar *phaseoli* has persisted over time within the strains of *R. leguminosarum* native to Northern Spain, and are thus well-adapted to such areas (García-Fraile et al. 2010). This can firstly help explain the optimal agronomic results in terms of effective BNF in common bean obtained in the present study using inoculants based on indigenous strains. This contrasts with the poor effectiveness that other authors observed in field conditions using foreign strains with proven BNF effectiveness (Rodríguez-Navarro et al. 2000; Daza et al. 2000). Although at least two different chromosomal groups of strains with high BNF effectiveness (Mulas et al. 2011 and this study) are distributed in the area belonging to the PGI analysed, both showed an optimal agronomic performance regardless the specific environment within the PGI-designated area, indicating that strains have an adaptation range.

In Cogorderos-2009 there was a significant increase in yield after inoculation with the three strains compared to the N fertilised and uninoculated control (Table 4), even though the MPN of rhizobia in Cogorderos (3.1×10^4 rhizobia g⁻¹ soil) indicated the presence of common bean-nodulating rhizobia in that soil. Although there was no analysis of the

rhizobia nodulating the common bean plants in the experiment, the positive response to inoculation in this soil indicates that the selection of indigenous rhizobial strains is important to overcome the lack of effectiveness of rhizobia present in the soil, which has been considered as responsible for the poor N fixation in common bean (Hungria et al. 2005). Further studies analyzing the *nodC* gene abundance in the rhizobia found in Cogorderos would confirm the relevance of the γ allele in the nodulation and BNF effectiveness of the rhizobial inoculants in Northern Spain.

The global yield and the response to the inoculation in terms of total aerial biomass and grain yield were higher in the CONVT, intermediate in the CC and lowest in the NT. In the literature, there is some controversy about the effects of tillage on BNF because the soil parameters affected by the tillage may have independent effects on the BNF (Liu et al. 2010). Interestingly, the yield and the response to inoculation in terms of yield in our field experiment, was inversely proportional to the soil area covered by residues (0 % in CONVT, roughly 50 % in CC and over 90 % in NT). Moreover, in León-2008 both in the CC and in the NT systems, the residue cover was higher than normal because of the wet spring, which delayed the burial of the rye in the CC system and the elimination of the weeds in the NT system. In such situations, the total dry matter and grain yield in the NT and the CC was significantly lower than in the CONVT. The buffering effect on the soil temperatures when the soil is covered with plant residues (CC and NT) has been widely reported. This effect is particularly beneficial in tropical and temperate regions, as it also helps to maintain the soil moisture, with a consequent beneficial effect on common bean crops (Urchei et al. 2000). But for cooler areas, such as the European regions above a latitude of 42 °N, it might pose a threat to the performance of crops such as common bean, especially at flowering and grain filling stages.

To our knowledge, this is the first practical and productive field experiment in Europe aimed at developing a commercial inoculant for common beans based on elite native strains of *R. leguminosarum*, which is the only species found nodulating common bean in Northern Spain. The success of inoculation was consistent in terms of yield with the study of Mostasso et al. (2002), which used common bean in Brazil together with native strains phylogenetically close to *Rhizobium tropici*, which are different from *R. leguminosarum*. In both studies the yield increase in common bean when compared with the non-fertilised control was similar to the N-fertilised treatment.

We conclude that the optimal common bean inoculant must consist of a well-adapted indigenous rhizobial species (in Northern Spain it was *R. leguminosarum*) holding highly efficient nodulation genes. Concerning this, the results also show that the plasmid containing the *nodC* gene determines the effectiveness and the performance of inoculants of the rhizobial strains regardless of their chromosomal

background. As Oliveira et al. (2011) postulated, and as we have demonstrated in this work, it is possible to identify individuals with a high N-fixing capacity within the high diversity of indigenous rhizobia. Furthermore, they can be selected for use in commercial inoculants, producing a positive response in terms of crop yield even in soils with a significant presence of native rhizobia. Moreover, as strains belonging to different chromosomal groups of *R. leguminosarum* have shown high BNF effectiveness in field conditions regardless of the specific environment within the PGI-designated area, this indicates that the strains have an adaptation range.

The results obtained in this study have shown not only the importance and the potential of local elite rhizobial strains as inoculants, but also their performance under different tillage systems. The designed inoculant has been effective in CONVT and CC, but unlike results obtained in other parts of the World, for NT inoculation was ineffective for this tillage system in the studied area. The results obtained in this work are of particular interest in terms of microorganism-based agricultural inputs when their role in the soil is buffered by the native microbial community. Currently, most of these inputs are based on culture collection strains, whose origin is very distinct from their application region.

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References

- Abdalla M, Osborne B, Lanigan G, Forristal D, Williams M, Smith P, Jones MB (2013) Conservation tillage systems: a review of its consequences for greenhouse gas emissions. *Soil Use Manag* 29:199–209. doi:10.1111/sum.12030
- Aguilar OM, Riva O, Peltzer E (2004) Analysis of *Rhizobium etli* and of its symbiosis with wild *Phaseolus vulgaris* supports coevolution in centers of host diversification. *Proc Natl Acad Sci U S A* 101: 13548–13553. doi:10.1073/pnas.0405321101
- Albareda M, Rodríguez-Navarro DN, Camacho M, Temprano FJ (2008) Alternatives to peat as a carrier for rhizobia inoculants: solid and liquid formulations. *Soil Biol Biochem* 40:2771–2779. doi:10.1016/j.soilbio.2008.07.021
- Alguacil MM, Roldan A, Salinas-Garcia JR, Querejeta JI (2011) No tillage affects the phosphorus status, isotopic composition and crop yield of *Phaseolus vulgaris* in a rain-fed farming system. *J Sci Food Agric* 91:28–272. doi:10.1002/jsfa.4180
- Amarger N, Macheret V, Laguerre G (1997) *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* 47:996–1006. doi:10.1099/00207713-47-4-996
- Batish DR, Lavanya K, Singh HP, Kohli RK (2007) Phenolic allelochemicals released by *chenopodium murale* affect the growth, nodulation and macromolecule content in chickpea and pea. *Plant Growth Regul* 51:119–128. doi:10.1007/s10725-006-9153-z
- Bizarro MJ, Giongo A, Kayser L, et al. (2011) Genetic variability of soybean bradyrhizobia populations under different soil managements. *Biol Fertil Soils* 47:357–362. doi:10.1007/s00374-010-0512-6

- Buttery BR, Park SJ, Findlay WJ (1987) Growth and yield of white bean (*Phaseolus vulgaris* L.) in response to nitrogen, phosphorus and potassium fertilizer and to inoculation with *rhizobium*. Can J Plant Sci 67:425–432. doi:10.4141/cjps87-061
- Cunningham MH, Chaney K, Bradbury RB, Wilcox A (2004) Non-inversion tillage and farmland birds: a review with special reference to UK and Europe. Ibis 146:192–202. doi:10.1111/j.1474-919X.2004.00354.x
- Daza A, Santamaría C, Rodríguez-Navarro DN, Camacho M, Orive R, Temprano FJ (2000) Perlite as carrier for bacterial inoculants. Soil Biol Biochem 32:567–572. doi:10.1016/S0038-0717(99)00185-6
- FAOSTAT (2015) FAOSTAT Agriculture Data. Food and Agricultural Organisation, Rome. <http://faostat.fao.org>. Accessed 23 April 2015
- García-Fraile P, Mulas-García D, Peix A, Rivas R, González-Andrés F, Velázquez E (2010) *Phaseolus vulgaris* is nodulated in northern Spain by *rhizobium leguminosarum* strains harboring two *nodC* alleles present in American *Rhizobium etli* strains: biogeographical and evolutionary implications. Can J Microbiol 56:657–666. doi:10.1139/W10-048
- Gepts P, Deboucq D (1991) Origin, domestication, and evolution of the common bean (*Phaseolus vulgaris* L.). In: van Schoonhoven A, Voysest O (eds) Common beans: research for crop improvement. CAB, Wallingford, pp. 7–53
- Graham PH (1981) Some problems of nodulation and symbiotic nitrogen fixation in *Phaseolus vulgaris* L.: a review. Field Crop Res 4:93–112. doi:10.1016/0378-4290(81)90060-5
- Howieson J, Malden J, Yates RJ, O'Hara GW (2000) Techniques for the selection and development of elite inoculant strains of *rhizobium leguminosarum* in southern Australia. Symbiosis 28:33–48
- Hughes RM, Herridge DF (1989) Effect of tillage on yield, nodulation and nitrogen fixation of soybean in far north-coastal New South Wales. Aus J Exp Agric 29:671–677. doi:10.1071/EA9890671
- Hungria M, Loureiro MF, Mendes LC, Campo RJ, Graham PH (2005) Inoculant preparation, production and application. In: Werner D, Newton WE (eds) Nitrogen fixation in agriculture, forestry, ecology, and the environment. Springer, Dordrecht, pp. 223–253
- Hungria M, Vargas MAT (2000) Environmental factors affecting N₂ fixation in grain legumes in the tropics, with an emphasis on Brazil. Field Crop Res 65:151–164. doi:10.1016/S0378-4290(99)00084-2
- Inderjit LA, Weston B, Duke SO (2005) Challenges, achievements and opportunities in allelopathy. J Plant Interact 1:69–81. doi:10.1080/17429140600622535
- Jiao YS, Yan H, Ji ZJ, Liu YH, Sui XH, Wang ET, Guo BL, Chen WX, Chen WF (2015) *Rhizobium sophorae* sp. nov. and *rhizobium sophoriradicis* sp. nov., nitrogen-fixing rhizobial symbionts of the medicinal legume *sophora flavescens*. Int J Syst Evol Microbiol 65:497–503. doi:10.1099/ijs.0.068916-0
- Josephson KL, Bourque DP, Bliss FA, Pepper IL (1991) Competitiveness of KIM-5 and VIKING-1 bean rhizobia - strain by cultivar interactions. Soil Biol Biochem 23:249–253. doi:10.1016/0038-0717(91)90060-W
- Kaschuk G, Hungria M, Andrade DS, Campo RJ (2006a) Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in southern Brazil. Appl Soil Ecol 32:210–220. doi:10.1016/j.apsoil.2005.06.008
- Kaschuk G, Hungria M, Santos JCP, et al. (2006b) Differences in common bean rhizobial populations associated with soil tillage management in southern Brazil. Soil Tillage Res 87:205–217. doi:10.1016/j.still.2005.03.008
- Keller B, Baggiolini A (1954) Les stades repères dans la végétation du blé, d'après l'échelle élaborée par freekes. Revue Romande Agriculture et Arboriculture 10:17–20
- Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. doi:10.1007/BF01731581
- Liu Y, Wu L, Baddeley JA, Watson CA (2010) Models of biological nitrogen fixation of legumes. A review. Agron Sustain Dev 31:155–172. doi:10.1051/agro/2010008
- Loureiro MF, Kaschuk G, Alberton O, et al. (2007) Soybean [Glycine max (L.) Merrill] rhizobial diversity in Brazilian oxisols under various soil, cropping, and inoculation managements. Biol Fertil Soils 43:665–674. doi:10.1007/s00374-006-0146-x
- Mostasso L, Mostasso FL, Dias BG, Vargas MAT, Hungria M (2002) Selection of bean (*Phaseolus vulgaris* L.) rhizobial strains for the Brazilian cerrados. Field Crop Res 73:121–132. doi:10.1016/S0378-4290(01)00186-1
- Mulas D, García-Fraile P, Carro L, Ramírez-Bahena MH, Casquero P, Velázquez E, González-Andrés F (2011) Distribution and efficiency of *rhizobium leguminosarum* strains nodulating *Phaseolus vulgaris* in northern Spanish soils: selection of native strains that replace conventional N fertilization. Soil Biol Biochem 43:2283–2293. doi:10.1016/j.soilbio.2011.07.018
- Mulas D, Ramírez-Bahena MH, García-Fraile P, Velázquez E, González-Andrés F (2008) *Rhizobium leguminosarum* is the predominant species found among rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in León (Spain). 8th European Nitrogen Fixation Conference Gent (Belgium), pp 106
- Oliveira JP, Galli-Terasawa LV, Enke CG, Cordeiro VK, Tavares Armstrong LC, Hungria M (2011) Genetic diversity of rhizobia in a Brazilian oxisol nodulating Mesoamerican and Andean genotypes of common bean (*Phaseolus vulgaris* L.). World J Microbiol Biotechnol 27:643–650. doi:10.1007/s11274-010-0501-9
- Peix A, Ramírez-Bahena MH, Velázquez E, Bedmar EJ (2015) Bacterial associations with legumes. Crit Rev Plant Sci 34:17–42
- Rigaud J, Puppo A (1975) Indole-3-acetic acid catabolism by soybean bacterioids. J Gen Microbiol 88:223–228. doi:10.1099/00221287-88-2-223
- Rodríguez-Navarro DN, Buendía AM, Camacho M, Lucas MM, Santamaría C (2000) Characterization of *rhizobium* spp. bean isolates from south-west Spain. Soil Biol Biochem 32:1601–1613. doi:10.1016/S0038-0717(00)00074-2
- Rogel MA, Ormeño-Orrillo E, Martínez-Romero E (2011) Symbiovars in rhizobia reflect bacterial adaptation to legumes. Syst Appl Microbiol 34:96–104. doi:10.1016/j.syapm.2010.11.015
- Rubio Pérez LM (1987) La bañeza y su Tierra, 1650–1850. Un modelo de sociedad rural leonesa. Universidad de León, León, Spain
- Ruffo ML, Bullock DG, Bollero GA (2004) Soybean yield as affected by biomass and N uptake of cereal rye in winter cover crop rotations. Agron J 96:800–805. doi:10.2134/agronj2004.0800
- Saïdi S, Ramírez-Bahena MH, Santillana N, Zúñiga D, Álvarez-Martínez E, Peix A, Mhamdi R, Velázquez E (2014) *Rhizobium laguerreae* sp. nov. nodulates *vicia faba* on several continents. Int J Syst Evol Microbiol 64:242–247. doi:10.1099/ijs.0.052191-0
- Saitou N, Nei M (1987) A neighbour-joining method: a new method for reconstructing phylogenetics trees. Mol Biol Evol 4:406–425
- Santalla M, Rodiño AP, De Ron AM (2002) Allozyme evidence supporting southwestern Europe as a secondary center of genetic diversity for common bean. Theor Appl Genet 104:934–944. doi:10.1007/s00122-001-0844-6
- Sombrero A, De Benito A (2010) Carbon accumulation in soil. Ten-year study of conservation tillage and crop rotation in a semi-arid area of castile-Leon, Spain. Soil Tillage Res 107:64–70. doi:10.1016/j.still.2010.02.009
- Sturz AV, Christie BR (2003) Beneficial microbial allelopathies in the root zone: the management of soil quality and plant disease with rhizobacteria. Soil Tillage Res 72:107–123. doi:10.1016/S0167-1987(03)00082-5
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599. doi:10.1093/molbev/msm092

- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The clustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24:4876–4882
- Urbano B, González-Andrés F, Ballesteros A (2006) Allelopathic potential of cover crops to control weeds in barley. *Allelopath J* 17:53–64
- Urchei MA, Rodrigues JD, Stone LF (2000) Growth analysis of two bean cultivars under irrigation in no tillage and the conventional tillage. *Pes Agrop Bras* 35:497–506. doi:[10.1590/S0100-204X2000000300004](https://doi.org/10.1590/S0100-204X2000000300004)
- Vincent JM (1970) The cultivation, isolation and maintenance of rhizobia. In: Vincent JM (ed) *A manual for the practical study of root-nodule*. Blackwell, Oxford, pp. 1–13
- Wang F, Wang ET, Wu LJ, Sui XH, Li Jr Y, Chen WX (2011) *Rhizobium vallis* sp. nov., isolated from nodules of three leguminous species. *Int J Syst Evol Microbiol* 61:2582–2588. doi:[10.1099/ijs.0.026484-0](https://doi.org/10.1099/ijs.0.026484-0)
- Wheatley DM, Macleod DA, Jessop RS (1995) Influence of tillage treatments on N₂ fixation of soybean. *Soil Biol Biochem* 27:571–574. doi:[10.1016/0038-0717\(95\)98633-Y](https://doi.org/10.1016/0038-0717(95)98633-Y)