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ORIGINAL PAPER

Evaluation of bradyrhizobia strains isolated from field-grown soybean plants in Argentina as improved inoculants

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Abstract Bradyrhizobium strains were isolated from nodules obtained from field-grown soybean plants sampled in 12 soybean production locations in Argentina. These fields had been annually cropped with soybean and did not show decreases in yields even though they had been neither N-fertilized nor inoculated for at least the last 5 years. We hypothesized that the isolated strains maintained high competitiveness and efficiency in fixing adequate N_2 levels. A set of strains that showed the highest nodular occupancy in each sampling location were assayed for symbiotic performance under greenhouse and field conditions and comparatively evaluated with *Bradyrhizobium japonicum* E109, the strain officially recommended for inoculant formulation in Argentina. An inoculant pool, formed by four strains obtained from nodules collected from Cañada Rica, developed higher nodular biomass than *B. japonicum* E 109 in assays carried out in greenhouses under well irrigated conditions. Additionally, neither nodule production nor specific nitrogenase activity decreased with respect to *B. japonicum* E 109 when plants were drought stressed during 7 days from sowing. The mean yields obtained under field conditions and plotted against the principal component one (CP1) obtained with an additive main effect and multiplicative interaction (AMMI) model showed that the inoculant pool from Cañada Rica had higher contribution to yield than strain E 109, although with lower environmental stability. The inoculant pool from Cañada Rica could be considered an improved inoculant and be used for preliminary assays, to formulate inoculants in Argentina.

Keywords *Bradyrhizobium japonicum* · Biological nitrogen fixation · Inoculant · Soybean

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Introduction

Soybean (*Glycine max* L. Merrill) is the most important legume produced in Argentina, representing more than 70% of Argentina's agricultural land (SAGPyA 2007). The wide use of legumes as food crops and forages is mainly associated with their ability to establish symbiotic associations with stem and root nodulating N_2-fixing bacteria, collectively called rhizobia (Allen and Allen 1981). These bacteria are among the most intensively studied groups of microorganisms mainly because of their potential to replace N-fertilizers, with emphasis on their key role in environmental sustainability.

Inoculation in Argentina has been adopted as a common practice. Recent data from a survey commissioned by the main inoculant companies in Argentina showed that inoculation is performed in more than 90% of soybean fields (icaSA, unpublished data) and that it favors biological nitrogen fixation (BNF). Success in nodulation process after inoculation is determined by several factors, such as

soil fertility and the competitiveness of inoculant strains, which have to be able to survive and colonize plant roots in an environment with abundant native microorganisms, producing fixing nodules. The strains selected for use as inoculants need to possess two important characteristics: they should show high nitrogen-fixing ability in their target host legume (Howieson et al. 2000; Rodriguez Blanco et al. 2010) and they should also be able to compete with indigenous rhizobia in soils (Martensson 1989). The low nodule occupancy by strains from inoculants with high ability to fix atmospheric nitrogen could limit the contribution of inoculants to crop yield (Berg et al. 1988; Ham 1980). Additional efforts to understand the mechanisms involved in competitiveness with indigenous bradyrhizobia in soybean seem to be critical to increase nodular occupancy by the selected strains (Bhagwat and Keister 1992). Thies et al. (1991) reported that significant increases in soybean yield were observed only when no less than a doubling of nodule mass and 66% nodule occupancy by inoculated rhizobia were reached, showing the close inverse relationship between the presence of indigenous rhizobia and of those introduced by inoculation. Searching for more effective rhizobia, in terms of nitrogen-fixing capabilities and competitiveness, is a time-consuming process that requires several greenhouse experiments and field trials.

In Argentina, a variant of *Bradyrhizobium japonicum* USDA 138, also named *B. japonicum* E109, is the strain recommended by *Instituto Nacional de Tecnología Agropecuaria*—Argentina (INTA) for use in inoculant formulation because of its high BNF capacity and high survival in inoculated soybean seeds. However, as for many other strains, *B. japonicum* E109 showed high variability in its nodular occupancy when used in soybean fields for many years. In these fields with a long soybean production history, it has been observed that nodule occupancy by strains from commercial inoculants did not exceed 18% (Date 2000) due to the presence of strains initially introduced and subsequently naturalized. Despite the considerable efforts invested in selecting more effective strains than *B. japonicum* E109, the results obtained were not always successful.

It has been stated that strain competitiveness and persistence in soils might not be closely related characteristics (Brockwell et al. 1982); however, in non-inoculated and non-N_2-fertilized fields, soybean yields higher than the mean in the region are usually observed, suggesting that native strains have both high ability to nodulate and to fix atmospheric nitrogen.

The aims of this work were to isolate, from nodules of field-growing soybean plants, native strains whose competitiveness and nitrogen fixing capacity were maintained after several crop years without inoculation and that showed higher symbiotic performance than *B. japonicum* E109. In addition, the symbiotic performance of the selected strains under drought conditions was evaluated. Nodules for strain isolation were collected from plants at phenological stage R5 (Fehr and Caviness 1977), sampled in 12 soybean production locations in Argentina. These fields had been annually cropped with soybean and did not show decreases in yields even though they had been neither N_2-fertilized nor inoculated for at least the last 5 years. From each location, the strains with high nodular occupancy were selected to compare them with *B. japonicum* E109 in terms of nodulating ability and nitrogen fixing capacity. Additionally, the behavior of the selected strains under drought conditions was determined in a greenhouse assay, and the strains that showed better responses were then assayed under field conditions.

We have hypothesized that native strains isolated from nodules in non-inoculated and non-N_2-fertilized fields maintain high competitiveness and efficiency in providing adequate N_2 levels, contributing to high yields; this strategy might allow us to find strains with better performance.

Material and methods

Reference strains and soybean rhizobial isolates

Bradyrhizobium japonicum E109, a variant of *B. japonicum* USDA 138, provided by IMyZA-INTA strain bank, is the strain used in all commercial inoculants manufactured in Argentina.

Nodules used for strain isolation and genetic characterization were obtained from field-grown commercial soybean cultivars sown in 12 locations in the main soybean production region of Argentina, the provinces of Santa Fe (Cañada Rica, Chazón, Firmat, Reconquista, Sancti Spiritu, Venado Tuerto, and Uranga), Buenos Aires (Chacabuco and Alberti), and Córdoba (La Carlota, Oncativo and Laguna Larga). Nodules located in the main root of five plants per location were collected and pooled. Ten nodules from each (between 27% and 43% of total nodules per plant) were surface-sterilized with 70% ethanol 1 min, and with 5% (v/v) commercial hypochlorite (55 mg active chloride) 10 min, and then rinsed with water six times using a water jet pump. The nodules were individually crushed under sterile conditions and bacteria were isolated using standard procedures (Vincent 1970). Purity of cultures was confirmed by repeatedly streaking the bacteria on yeast extract-mannitol-agar (YMA) medium and verifying a single type of colony morphology (color, mucosity, diameter, transparency, border elevation), and absorption of Congo red and Gram-stain reaction after they were grown in the dark at 28°C for 6 days.

DNA extraction

Ten single colonies from each nodule were individually transferred to 3 ml YM liquid broth (YMB), grown (6 days)

on rotary shaker at 100 cycles min^{-1}, mixed with glycerol (50%), and stored at −80°C. Working cultures were maintained on YMA slants at 4°C. Freeze–thaw lysis protocol was used for DNA extraction (Yu-Li and Olson 1992); briefly, 3 ml bacterial cultures were centrifuged at 12,000×*g* for 5 min; the pellet was re-suspended in 300 μl of sterile water and subjected to three cycles of freezing in liquid air and thaw in water bath at 60°C; with final centrifugation at 12,000×*g* for 5 min. DNA of at least 50 strains isolated from each location was amplified by PCR.

Rep-PCR fingerprinting with BOX primer

Five microliters of supernatant containing DNA was used for rep-PCR fingerprinting (interspersed repetitive sequence, Versalovic et al. 1994), with BOX-A1R primer (5′ CTACGGCAAGGCGACGCTGACG-3′). The final volume of the PCR reaction was 25 μl and contained: dNTPs (1.5 mM of each), 5.0 μl; buffer 10× (500 mM KCl; 100 mM Tris–HCl, pH 8.3), 2.5 μl; $MgCl_2$ 50 mM, 1.5 μl; primer (50 pmol μl^{-1}), 1 μl; Taq DNA polymerase (5 U μl^{-1}), 0.2 μl; DNA template, 5 μl; sterile milli-Q water to complete the volume. The following cycles were used: one cycle of denaturation at 95°C for 7 min; 35 cycles of 1 min denaturation at 94°C, annealing at 53°C 1 min, and extension at 65°C 8 min; one cycle of final extension at 65°C 16 min; and a final soak at 4°C. The reactions were carried out in an Eppendorff® thermocycler and amplified fragments were separated by electrophoresis on 1.5% agarose gel (20×25 cm), at 120 V, for 4 h in 0.5× TBE buffer; 1 kb plus (Life Technology) was included as ladder. Gels were stained with ethidium bromide, visualized under UV light and photographed.

Analysis of electrophoretic pattern

Electrophoretic band patterns were analyzed using the Gel Compar II software version 4.1 (Applied Mathematics, Kortrijk, Belgium). For each location, similarity of band patterns was evaluated with cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA) clustering method and the coefficient of Dice as similarity coefficient. The similarity matrix generated by UPGMA and Dice coefficient was used to perform the principal coordinate analysis (PCoA) to evaluate the relationship among strains. The analysis was performed with Infostat software (Di Rienzo et al. 2009).

Inoculant pool

The strain clustering for each location was obtained using the UPGMA algorithm and Dice coefficient. The cluster formed by strains that produced the highest number of nodules and that showed 70% or higher of similarity coefficient was selected. From this cluster, a "strain pool" formed by four strains that had shown highest within-cluster distance was subsequently used to evaluate their symbiotic behavior in a greenhouse assay. Each isolate was individually grown in 50 ml YMB for 6 days. Cultures were collected by centrifugation at 5,000×*g* for 10 min and resuspended in 0.9% g L^{-1} NaCl physiological saline solution. Each "inoculant pool" contained equal proportion of each strain at a final concentration of 1×10^9 cell mL^{-1}. A total of 11 inoculant pools were assayed.

Greenhouse assay

Four seeds of Don Mario 4,800 commercial soybean were sown in 10-L pots filled with a mix of sterile sand/soil (1:1 v/v). Seeds were inoculated at sowing with 500 μL culture of each inoculant pool. Soil moisture was maintained at 100% field capacity from sowing to R2 (70 days after sowing) and drought treatment was applied maintaining 50% field capacity (determined by gravimetric measurement) for 7 days. Then, plants were watered to field capacity up to R2. Plants were grown in greenhouse under 14 h photoperiod (900 μmol m^{-2} s^{-1}) and day/night temperatures of 32/20°C.

Twenty replicates (five pots with four plants each), per treatment and per inoculant pool were carried out. Nodule dry weight (g $plant^{-1}$) and Nitrogenase activity were determined (Hardy et al. 1968). An ANOVA was adjusted for completely randomized design. Comparisons were made using DGC test model with Infostat software (Di Rienzo et al. 2009).

Nitrogenase activity

Nitrogenase activity was measured as acetylene reduction activity (ARA) by GC analysis using a 5,890 HP chromatograph (Hewlett Packard, Palo Alto, CA, USA) with an ionization flame detector at 135°C and porapak N 1/8 inch column. It was measured after incubation of nodulated roots in 500 ml glass flasks containing 10% (v/v) C_2H_2, as described previously Hardy et al. (1968). Total reductive acetylene activity (ARAt) (nmol ethylene $plant^{-1}$) and specific reductive acetylene activity (ARAs) (nmol ethylene g $nodule^{-1}$) were determined.

Field assays

The inoculant pools that showed the best responses in nodular production and/or ARAs in the greenhouse assays were evaluated with soybean under field conditions in Córdoba (Cañada de Machado and Monte Cristo), Buenos Aires (Carhué, Ferré, Pergamino, Trenque Lauquen, Bolivar, Junín,

and Gonzalez Chavez), and Santa Fe (Laguna Paiva) in 2007, 2008, and 2009. Five replicates per treatment were planted using no-tillage following the common agronomical practices in each location. Seeds were inoculated with inoculant pools or *B. japonicum* E109 and non-inoculated (NI) and NI fertilized with 400 kg ha^{-1} urea, equivalent to 46% N (NI+N_2), were included as controls.

At the phenological stage R8 (crop maturity), grain yield was determined from a 2-m^2 harvested area comprising the two central rows in each four-row plot. Number and weight of seeds were determined.

The performance of inoculant pools in their respective environment (location*year) was evaluated with the additive main effect and multiplicative interaction (AMMI) model (Gauch 1988), adjusted for matrix yield residuals obtained from ANOVA (DGC test model for comparisons). AMMI had the effects of the environment and inoculant pool interaction. Comparisons were made with Infostat software (Di Rienzo et al. 2009).

Results

All strains isolated from nodules were slow growers in YMA medium, and they were *Bradyrhizobium* spp. according to their Gram-negative staining and colony morphological characteristics. Genetic diversity was analyzed within isolates obtained from each sampling location (Table 1), except isolates from Oncativo and Laguna Larga, and Sancti Spiritu and Venado Tuerto, which were analyzed together due to their agro-climatic similarities.

A high genetic diversity was observed among sampling locations. The strains that colonized the highest number of nodules and showed 70% or higher similarity coefficient level in BOX clustering were evaluated. In agreement with the clustering results obtained, the PCoA showed similar strain clustering, indicating that each of the main clusters obtained with both procedures included the strains that had occupied the highest number of nodules. A dendrogram, based on UPGMA algorithm and Dice coefficient using isolates collected from field-grown soybean nodules in Alberti and the reference strain *B. japonicum* E109, is shown as an example (see box in Fig. 1, where strains with 70% similarity are highlighted).

The PCoA was performed for each sampling location using the similarity matrix for the isolates and *B. japonicum* E109. An example of strains isolated from Alberti showed that PCoA grouped the same strains that were included in the main cluster in Figs. 1 and 2). Similar responses were observed when data of the other sampling locations were analyzed using both methods (data not shown). An analysis based on UPGMA and Dice coefficient showed that all sampling locations had one main cluster with 70% similarity or higher, except in Firmat, where two main clusters were observed.

Table 1 shows the total number of strains obtained in each sampling location, the number of strains included in the main/s cluster/s, and the number of nodules from which each strain came from; the branching (similarity) to *B. japoni*cum E 109 and the strains selected to be included in each inoculant pool (see below) are also shown. The number of strains that formed clusters in each sampling location ranged from to six to 19; these strains came from two to seven nodules, and had the highest nodular occupancy. The main cluster in Chazón, La Carlota and Reconquista showed the lowest similarity to *B. japoni*cum E 109 (30%); by contrast, *B. japoni*cum E 109 was included in the main cluster in Uranga, whereas branching to E109 in the other sampling locations ranged between 40% and 60% (Table 1).

From each cluster, an inoculant pool (Table 1) formed by four strains that had shown the highest within-cluster distance was subsequently used to evaluate BNF capacity of the strains in a greenhouse assay carried out under control and drought stress for a period spanning 7 days from sowing. The relative performance of plants inoculated with inoculant pool with respect to those inoculated with *B. japonicum* E 109 was compared by nodular biomass production and total (ARAt, nmol ethylene $plant^{-1}$) and specific reductive acetylene activity (ARAs, nmol ethylene g $nodule^{-1}$).

Inoculant pools from Cañada Rica, Chazón, Chacabuco, Oncativo-L. Larga and Uranga induced higher nodular biomass than *B. japoni*cum E 109 under optimal irrigation conditions ($p \leq 0.05$), whereas, under drought conditions, higher differential values were only maintained using inoculant pools from Oncativo-L. Larga and Uranga ($p \leq 0.05$) (Table 2). Plants inoculated with pools from Reconquista, Chacabuco, and Chazón had between one and five times higher specific (ARAs) and total (ARAt) ARA, respectively, than those inoculated with *B. japoni*cum E 109 ($p \leq 0.05$) under control condition, whereas, under drought, only plants inoculated with pools from Chazón maintained their ARAt and ARAs (9.85 and 2.43 times, respectively) higher than those inoculated with *B. japoni*cum E 109 ($p \leq 0.05$) (Table 2).

Values of relative nodule production, ARAt, and ARAs obtained with the inoculant pools under drought with respect to control conditions are shown in Table 3. Nodule dry weight, ARAt, and ARAs did not decrease under drought conditions in nodules induced by *B. japonicum* E 109 compared with control conditions ($p \leq 0.05$), whereas nodular dry weight decreased significantly ($p \leq 0.05$) by 71%, 58%, and 61% with respect to control values in plants treated with inoculant pools from Cañada Rica, Chacabuco, and Chazón, respectively. In plants treated with inoculant pool from Firmat II and Cañada Rica, ARAt did not vary

Table 1 Bradyrhizobia strains isolated from soybean field-grown nodules and Cluster analysis (UPGMA with the Dice coefficient) of the rep-PCR (primer BOX-A1R)

Sampling location	Number of strains	Main cluster	No. strains in main cluster	No. nodules plant^{-1}	Branching to E109 (similitude)	Strains selected inoculant pool
Uranga	44	1	6	2	72	H555–H543–H551–H541
Cañada Rica	54	1	17	6	56	A351–A363–A513–A523
V Tuerto-S Spiritu	104	1	19	5	56	F133–F531–F532–C155
Firmat	98	2-I	17	6	50	B571–G121–G541–B353
		2-II	18	7	62	G181–B523–B561–G591
Chacabuco	46	1	9	4	46	D542–D112–D552–D511
Oncativo-L Larga	79	1	6	3	44	I565–J311–J314–J131
Alberti	46	1	15	5	43	E531–E552–E142–E131
Chazon	46	1	8	2	30	K535–K531–K562–K561
La Carlota	41	1	10	3	30	L141–L131–L154–L135
Reconquista	25	1	7	2	30	M141–M133–M142–M134

Number of main clusters formed by strains that had the highest nodular occupancy and number of strains included. Similarity to *B. japonicum* E 109 (branching); strains selected to prepare an "inoculant pool" (which have 70% similarity and highest within-cluster distance)

under drought conditions, whereas ARAs increased by 60% and 66% with respect to control, showing their BFN capacity under restrictive water conditions. Nodules developed with inoculant pools from Chacabuco, Chazón, and Reconquista showed a 77%, 39%, and 57% decrease in ARAt, respectively, under drought conditions with respect to control. Likewise, ARAs under drought conditions decreased by 35% and 64% in nodules developed with inoculant from Chacabuco and Reconquista, respectively, whereas it increased by 22% in nodules formed with pools from Chazón (Table 3). Based on responses observed in greenhouse assays, inoculant pools from Cañada Rica, Chacabuco, Chazón, Oncativo-L. Larga, and Uranga were then assayed under field conditions in several locations during 2007, 2008, and 2009.

The adjusted AMMI model showed that CP1 and CP2 explained 39.85% and 22.4%, respectively, of variability in yields obtained in the environments tested. The higher inertia on CP1 could be attributed to inoculant pools from Cañada Rica and *B. japonicum* E 109, whereas the lower incidence is related to NI+N_2 treatment and pools from Oncativo-L. Larga and Uranga (Fig. 3).

The greatest contribution to explain yields in the environment Ferre-2007, Pergamino-2007, Cañada de Machado-2007, and Monte Cristo-2009 could be attributed to inoculant pool from Cañada Rica and *B. japonicum* E 109. On the other hand, NI+N_2 and pools from Oncativo-L. Larga and Uranga mainly contributed to explain yields obtained in Ferre-2008, Bolivar-2009, Saenz Peña-2009, Junin-2009, and Gonzalez Chavez-2009.

Mean yields were plotted against the CP1, obtained with AMMI model, to show inoculant stability and its contribution to yield in all the environments where they were assayed (Fig. 4). NI treatment showed the smallest contribution to yield; by contrast, inoculant pool from Cañada Rica had the highest contribution but smaller stability than *B. japonicum* E 109. Stability of a strain pool shows its capacity to contribute to yields and means that its contribution does not change across the environments tested. Likewise, NI+N_2 and inoculant pools from Chacabuco and Chazón made a smaller contribution to yield than Cañada Rica and *B. japonicum* E 109, but showed the highest stability in all environments assayed (Fig. 4).

Discussion

Efficient nitrogen fixation and high competitiveness are both desirable characteristics in selection of strains to be used as improved inoculants. In this work, a collection of bradyrhizobia isolates from field-grown soybean plants was obtained. Fields were selected since they had not been inoculated or N_2 fertilized for at least 5 years, and that they did not show yield decreases. The strains that developed the highest number of nodules from each location were chosen to compare their fixation capabilities with those of *B. japonicum* E 109. Even though several molecular procedures allow bacteria identification and classification with high level of taxonomic resolution, the use of rep-BOX-PCR genomic fingerprinting allows the assessment of intra-species genetic diversity (van Belkum 1999) in the sampling locations and with respect to *B. japonicum* E109. High variability in branching to *B. japonicum* E 109 was observed among the strains that developed the highest number of nodules in the sampling locations analyzed. The cluster branching to *B. japonicum* E 109, a

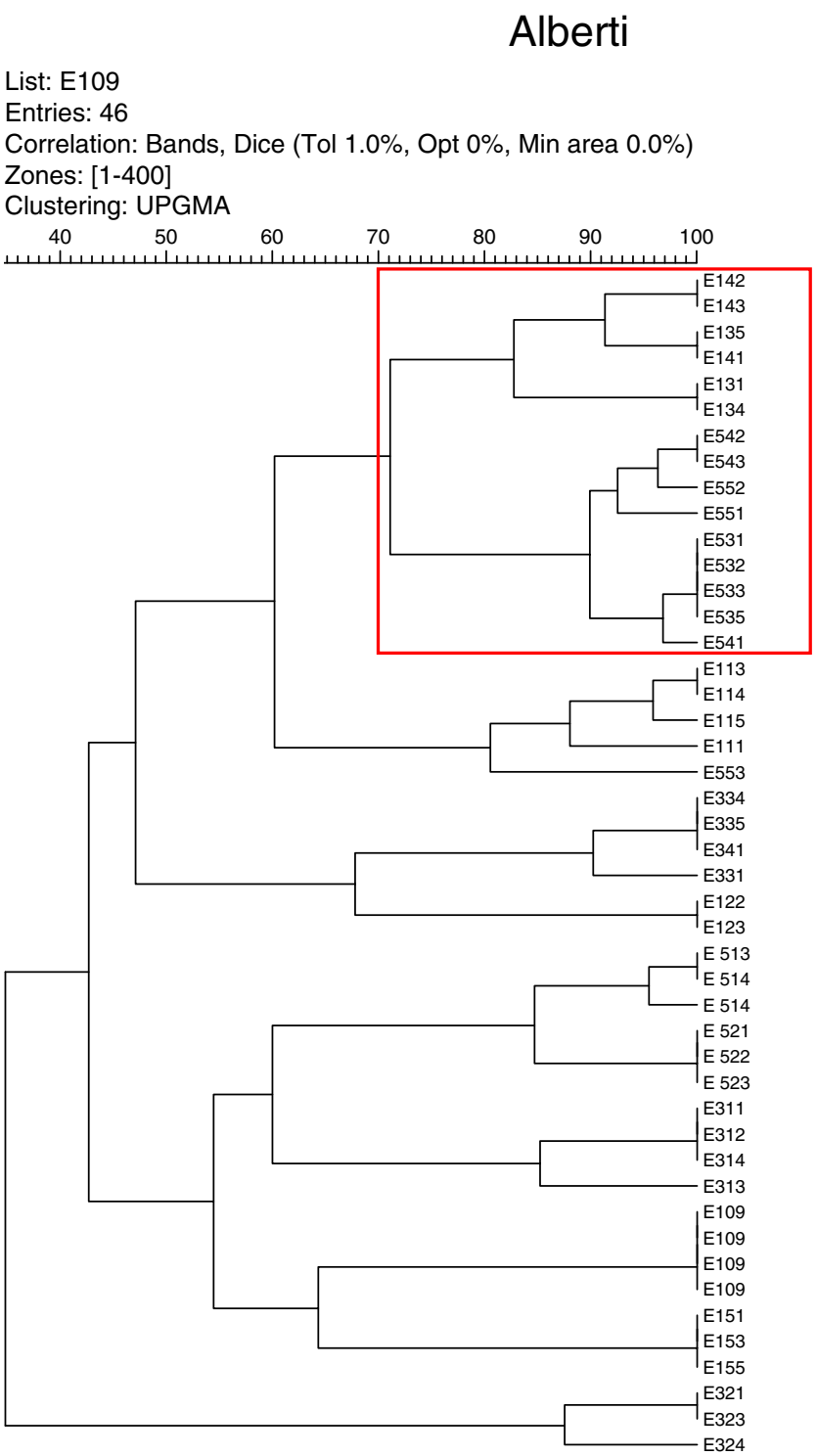

Fig. 1 Cluster analysis (UPGMA with the Dice coefficient) of the rep-PCR (primer BOX-A1R) products of rhizobial isolates obtained from field-grown soybean nodules in Alberti. The *box* highlights the strains that formed the highest number of nodules, grouped with 70% similarity

similarity measurement made in each location, allowed us to estimate possible differences of the isolated strains from the reference strain; this information is important because strains present in inoculants are known to establish and persist in soils, even when commercial inoculants were not used for several years (Straliotto and Gouvea Rumjanek 1999; Montecchia et al. 2004; Batista et al. 2007).

The criterion for the initial selection of strains was competitiveness, and the approach based on clustering by BOX-PCR analysis allowed the initial discrimination that

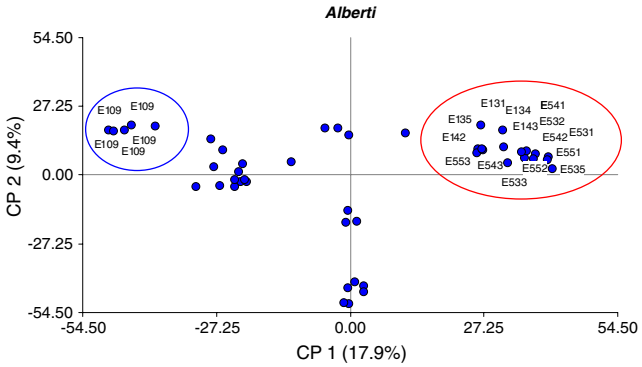

Fig. 2 PCoA based on similarity matrix generated by UPGMA and Dice coefficient of the rep-PCR (primer BOX-A1R) products of rhizobial isolates obtained from field-grown soybean nodules in Alberti. The *circle* highlights the strains that formed the highest number of nodules grouped by PCoA and *B. japonicum* E109 as reference strains

was reinforced by the PCoA, with similar levels in strain grouping. The strains selected as inoculant pools were assayed in pots in terms of their nodulation and BNF capacity compared with *B. japoni*cum E 109 and of their response variation under drought stress in a period spanning 7 days from sowing. Nodulation and BNF under drought conditions have been extensively reported in a legume–microsymbiont system using several approaches (Zahran 1999; Marino et al 2006), applying drought stress at different times during symbiosis development. Responses are mainly managed by plant metabolism if drought stress is applied when nodules are formed (Arrese-Igor et al 1999). However, the symbiotic process is markedly conditioned by bacterial survival and infection ability under drought stress at sowing. Focusing on this aspect, we evaluated the plant–inoculant pool relationship at early stages in terms of their capacity to develop nodules and BNF. In dehydrated soils, bacterial movement could be lower, reducing bacterial capacity to reach root hair to initiate infection process and develop infection threads (Zahran et al. 1994; Graham 1992). It has been observed that microsymbionts are quite more tolerant than their plant host in soils because they could survive in water films, the slow growers being more tolerant than the fast ones (Serraj et al. 1999). Drought treatment in soybean applied immediately after sowing causes great bacterial death, mainly to those bacteria that had not started the infection process, whereas prolonged drought stress induces plant host responses that inhibit nodulation (Racca 2009). In our work, the timing of drought exposure might have influenced mainly the initial steps in plant–bacterial relationship, thus limiting nodular development; hence, drought incidence on nodular development could be closely related to bacterial survival as free-living forms or with abortion of infection thread. Even though new bacterial infection could have happened during re-watering after the drought period, inoculant pools from Cañada Rica and Chazón, which drastically decreased their nodule dry weight under drought with respect to control conditions, compensated the decreases with higher ARAs.

The highest number of nodules formed in field-grown soybean plants in the sampling locations has been colonized by native strains that had not suffered inoculation pressure during several culture cycles, which suggests that these strains could have acquired competitiveness and ecological advantages. This hypothesis was recently discussed in an analysis of strains from Brazil Cerrados soils. Gomes Barcillos et al.

Table 2 "Inoculant pools" performance in soybean plants cultivated under control conditions and drought during 7 days since sowing

Inoculant	Nodule DW (mg plant^{-1})		Total ARA (nmol ethyl plant^{-1})		Specific ARA (nmol ethyl g nod^{-1})	
	Control	Drought	Control	Drought	Control	Drought
E 109	90 (10)a	40 (4.1)a	1,420.8 (196)a	450.4 (117)a	6.63 (1)b	3.93 (1.1)b
Uranga	*350* (30)b	*270* (40)b	1,727.9 (202)a	1,992 (280)a	1.35 (0.3)a	2.44 (0.6)a
Cañada Rica	*280* (30)b	80 (20)a	2,289.9 (240)a	1,750 (288)a	2.89 (0.6)a	4.79 (0.9)b
V Tuerto-S Spiritu	150 (30)a	60 (10)a	2,256.7 (416)a	363.2 (42)a	4.26 (1)b	2.63 (0.5)a
Firmat I	40 (10)a	110 (20)a	396.48 (65)a	1,155 (118)a	2 (0.4)a	2.25 (0.3)a
Firmat II	110 (22)a	100 (20)a	1,517.5 (178)a	1,919 (316)a	2.69 (0.5)a	4.31 (1)b
Chacabuco	*310* (40)b	130 (10)a	*7,238.5* (637)c	1,650 (192)a	*7.14* (0.9)c	4.64 (0.9)b
Oncativo-L Larga	*330* (30)b	*230* (30)b	1,788.2 (186)a	1,191 (111)a	1.94 (0.4)a	1.77 (0.3)a
Alberti	130 (40)a	100 (20)a	1,271.3 (396)a	231.7 (61)a	2.02 (0.6)a	0.85 (0.4)a
Chazon	*280* (20)b	110 (30)a	*7,296.3* (443)c	*4,434* (668)b	*7.82* (0.9)c	*9.55* (1)c
La Carlota	60 (10)a	70 (10)a	205.61 (43.8)a	882.8 (156)a	0.88 (0.3)a	3.1 (0.2)a
Reconquista	140 (30)a	130 (30)a	*4,529.4* (755)b	1,958 (522)a	*6.38* (1)c	2.29 (0.4)a

Nodules dry weight and total and specific nitrogenase activity. Letters indicate significant differences with DGC test ($p \leq 0.05$) in inoculant*treatment interaction, whereas italic numbers indicate significant differences respect to *B. japonicum* E109 under each condition

Table 3 Relative variations in nodule dry weight and nitrogenase activity (total and specific) in soybean plants treated with inoculant pools under drought stress with respect to control conditions during a period spanning 7 days from sowing

Inoculant	Nodule DW (g plant^{-1}) relative to control (% variation)	Total ARA (nmol ethyl plant^{-1}) relative to control (% variation)	Specific ARA (nmol ethyl g nod^{-1}) relative to control (% variation)
E 109	–	–	–
Uranga	–	–	–
Cañada Rica	−71.40	–	+66.00
V Tuerto-S Spiritu	–	–	−38.30
Firmat 2-I	–	–	–
Firmat 2-II	–	–	+60.00
Chacabuco	−58.00	−77.2	−35.00
Oncativo-L Larga	–	–	–
Alberti	–	–	–
Chazon	−61.00	−39.20	+22.00
La Carlota	–	–	–
Reconquista	–	−56.70	−65.00

(2007) and Batista et al. (2007) showed that the great diversification in morphological, physiological, genetic, and symbiotic properties in relation to the originally introduced parental strains was strongly related to horizontal gene transfer, strain dispersion, and genomic recombination, which did not result in symbiotic disadvantages. However, to test this hypothesis, deeper molecular analyses are needed using the inoculant pools obtained in this work from different locations. It is noteworthy that the inoculant pool from Cañada Rica made the highest contribution to yield in the field environments assayed, but with lowest stability. Stability might be improved by assaying each inoculant pool in their places of origin. Likewise, *B. japonicum* E 109 showed lower contribution to yield than Cañada Rica, but it proved to be more stable.

In conclusion, we were able to recover a set of strains and made a first attempt to discriminate their similarity to *B. japoni*cum E 109 and analyze their performance, nodular development, and BNF under environmental controlled conditions; we also correlated this behavior with yield in field assays, showing that the inoculant pool of strains obtained from Cañada Rica could be selected to be preliminarily assayed as an improved inoculant. Further molecular work will be needed to define their characteristics accurately and compare them with *B. japonicum* E 109, the strain currently used to formulate inoculants in Argentina.

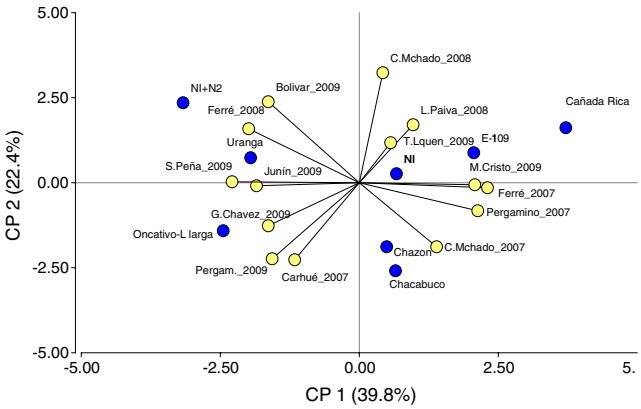

Fig. 3 PCA of inoculant pools (as shown in Table 1), obtained in each sampling location and evaluated in soybean production locations during 2007, 2008, and 2009. Inoculant pools were evaluated in the environment (location*year), where they were assayed with AMMI model, adjusted for matrix yield residuals obtained from ANOVA that had the interaction effects between environment and inoculant pool

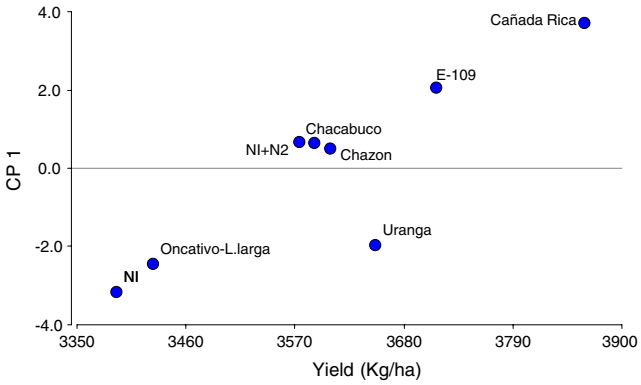

Fig. 4 Stability of inoculant pools. Principal component one (*CP1*) obtained with AMMI model (see Fig. 3 legend) plotted against mean yields

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