

## **Selection of *Rhizobium leguminosarum* strains via symbiotic and production variables in *Pisum sativum* L.**

### **Selección de cepas de *Rhizobium leguminosarum* por variables simbióticas y productivas en *Pisum sativum* L.**

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#### **ABSTRACT**

Field pea (*Pisum sativum* L.) is a winter symbiotic legume that associates with *Rhizobium leguminosarum* sv *viciae*. This work aimed to evaluate strains of *R. leguminosarum* for their infective ability and early-plant growth, BNF contribution, biomass and grain yield. Seventy-eight specific strains and four pea cultivars were evaluated in a growth chamber, five strains and three cultivars were evaluated in a greenhouse, and three strains and two cultivars were evaluated in a field experiment. Only 44-55% of all evaluated strains were infective in the four cultivars. In the greenhouse, D70 and D156 strains showed the best nodulation variables as well as higher N content and yield. The field experiment showed D156 and D70 yielded a similar behavior for N content in canopy biomass and individual nodule biomass, whereas D191 had a higher nodule number per plant, aerial biomass and grain yields. D70 provided good nodulation, N content in biomass, and yield in the growth chamber, greenhouse, and field experiments, whereas D156 had a like or superior behavior in the greenhouse and field experiments. Therefore, D156 could constitute a good candidate for bacterial single-strain inoculants, as well as for formulating microbial consortia.

#### **Keywords**

Field pea • *Rhizobium leguminosarum* • strain selection • symbiotic efficiency

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## RESUMEN

La arveja (*Pisum sativum L.*) es una leguminosa simbiótica del invierno que establece asociación con *Rhizobium leguminosarum sv viciae*. Los objetivos de trabajo fueron evaluar cepas de *R. leguminosarum* por infectividad y crecimiento temprano, aporte de BNF, producción de biomasa y rendimiento. Setenta y ocho cepas y cuatro cultivares se evaluaron en cámara de crecimiento, cinco cepas y tres cultivares en invernadero, mientras que tres cepas y dos cultivares en experimento de campo. Solo el 44-55% de las cepas fueron infectivas en los cultivares. En invernadero, las cepas D70 y D156 mostraron los mejores valores de nodulación y los mayores contenidos de N en biomasa y rendimiento. En campo, D156 y D70 mostraron comportamientos similares que en invernadero, mientras que D191 tuvo más nódulos por planta, mayor biomasa aérea y mayor rendimiento. La cepa D70 mantuvo su comportamiento de nodulación, contenido de N en biomasa y rendimiento en cámara de crecimiento, invernadero y campo; mientras que D156 tuvo igual o mejor comportamiento que D70 en invernadero y campo. Por lo tanto, D156 podría ser una buena candidata para formular inoculantes bacterianos con esta cepa o para formular consorcios microbianos.

### **Palabras clave**

Arveja • *Rhizobium leguminosarum* • selección de cepas • eficiencia simbiótica

## **INTRODUCTION**

Field pea (*Pisum sativum L.*) is a winter symbiotic legume able to establish a specific mutualistic association with *Rhizobium leguminosarum* symbiotic variant (sv) *viciae*. This association contributes to satisfying part of the crop nitrogen (N) demand via biological nitrogen fixation (BNF). From an agronomic perspective, field peas may be included as an alternative winter crop in agricultural rotations, with a shorter cycle duration and lower water consumption than other winter crops like wheat or barley, and the N-fixing ability (19). The N-fixing efficiency of the legume-rhizobium relationship depends mainly on crop genotype (1, 2), rhizobium strain (23), and soil-water interactions associated with management practices (10, 13, 14). Contributions of BNF in field pea range between 45 and 286 kg N ha<sup>-1</sup> (1, 13, 16, 22, 31, 36), representing 33-91% of total N uptake (1, 13, 16, 21, 22, 26, 31, 36).

*Rhizobium leguminosarum* strains have three distinct symbionts: (i) *R. leguminosarum* sv *phaseoli*, which nodulates beans (*Phaseolus vulgaris*); (ii) *R. leguminosarum* sv *trifolii*, which nodulates clovers (*Trifolium* sp); and (iii) *R. leguminosarum* sv *viciae*, which nodulates field pea, vetch (*Vicia* sp), lentil (*Lens* sp), faba beans (*Vicia faba*) and *Lathyrus* (*Lathyrus* sp) (7, 8, 27). In field peas, statistical interactions between strains and crop genotypes have been observed for traits associated with BNF, plant growth and photosynthesis (11). Rhizobia strains have greater relevance on BNF and N content, biomass and grains, than plant genotype (23). This strain-dependent behavior is associated with the expression of genes related to *R. leguminosarum* sv *viciae* nodulation, ensuring nodule establishment (7).

In Argentina, although field pea is not a native plant, native soil N-fixing rhizobia can nodulate this crop. Currently, the *R. leguminosarum* native strain D70 is the elite strain inoculating field peas, vetches, and lentils. This strain can nodulate all commercial cultivars and has shown greater BNF contribution in all agro-ecological environments evaluated in Argentina (13). However, even showing good behavior regarding grain yield and biomass production, it does not necessarily respond equally in all agricultural environments. Newly incorporated commercial cultivars still require evaluation regarding symbiotic behavior. In this regard, strains of the collection of the Instituto de Microbiología y Zoología Agrícola (IMyZA) of the Instituto Nacional de Tecnología Agropecuaria (INTA) could potentially perform better than D70 and be used in new inoculant formulations. Thus, this work aimed to i) evaluate *Rhizobium* sp. soil isolates and strains from the IMyZA-INTA collection for infective ability and early growth promotion under controlled conditions (growth chamber), ii) evaluate symbiotic efficiency of strains selected from the growth chamber, under greenhouse conditions, and iii) evaluate inoculation effects of the greenhouse selected strains on BNF contribution, nodulation, and production variables under field conditions.

## MATERIALS AND METHODS

### Growth chamber experiments

Seventy-one to seventy-eight specific strains and/or soil isolates from the IMyZA-INTA collection (Additional material 1) were combined with four field pea cultivars (Facón, Bicentenario, Manantiales, and Pampa). *Rhizobium* sp. inocula were grown in glass tubes (20 mm x 195 mm) with 10 ml of yeast extract mannitol (YEM) broth (35) on an orbital shaker (180 rpm, 48 h at 28°C). Each inoculum contained at least  $3 \times 10^8$  colony-forming units per milliliter (CFU ml<sup>-1</sup>). Field pea seeds were disinfected superficially with 80% ethanol for 1 min, 4% sodium hypochlorite for 2 min, at 80% ethanol for 0.5 min, and rinsed 5 times with sterile distilled water. Then, these seeds were placed in plastic trays with moistened absorbent paper for 72 h in an incubation stove at 28°C for germination. Two germinated seeds were inoculated with 1 ml of inoculant and planted in a 220-ml pot with sterile vermiculite substrate and daily irrigated with N-free nutrient solution (17). Each pot was kept at a constant temperature of 25°C and 16 h of light. Each strain and cultivar combination was considered a treatment, and a non-inoculated control was added for each field pea cultivar. The experimental design was set as completely randomized with three replications. Finally, the number of nodules per plant ( $N_{Nod}$ ) and biomass per plant ( $Biom_{Total}$ ) were evaluated 25 days after planting (DAP).

### Greenhouse experiment

Five strains selected from the growth chamber experiment (D70, D156, D191, D192, and D193) were evaluated for nodulation and early growth promotion abilities in the greenhouse. The experiment was planted in 2 L pots on August 21<sup>st</sup>, 2013, using four seeds per pot and sterile vermiculite as substrate. Seeds were disinfected as described above. Treatments consisted of a factorial combination of the five selected strains plus a non-inoculated control, with three commercial field pea cultivars (Viper, Facón, and Bicentenario) arranged in a complete randomized block design totaling 17 replicates. The inoculant was peat-based with a count of at least  $1 \times 10^8$  CFU g<sup>-1</sup> of product. Sugared water (20% w/v) was used as adherent at a rate of 10 ml kg seed<sup>-1</sup>. The inoculant was applied to seeds, mixed to homogenize, and allowed to stand for 30 min before planting. Pots were irrigated three days a week with an N-free nutrient solution (17). Variables per plant were evaluated at 56 DAP and 78, 92, and 103 DAP per pot. Root biomass ( $Biom_{Root}$ ), aerial biomass ( $Biom_{Aerial}$ ), plant biomass ( $Biom_{Plant}$ ), number of nodules ( $N_{Nod}$ ), dry nodule biomass ( $Biom_{Nod}$ ) and dry biomass per nodule ( $Biom_{Nod-J}$ ) were evaluated at two sampling moments (at 56 and 78 DAP), with additional measurements such as root and aerial length ( $Leng_{Root}$  and  $Leng_{Stem}$ , respectively) at 56 DAP. Then, the following variables were also evaluated: vegetative, grain, and aerial biomass ( $Biom_{Veg}$ ,  $Biom_{Grain}$ , and  $Biom_{Aerial}$ , respectively), and plant yield components as pod numbers, number of grains, number of grains per pod, and individual grain biomass ( $N_{Pod}$ ,  $N_{Grain}$ ,  $N_{Grain-pod}$ , and  $Biom_{Grain-p}$ , respectively) at harvest (ca. 103 DAP). Additionally, N content (% $N_{upt}$ ) and isotopic composition ( $\delta^{15}N_{upt}$ ) on whole plant biomass were determined at 92 DAP to estimate the β factor for each strain and field pea cultivar combination according to Boddey *et al.* (2000).

### Field experiment

Strains D70, D156, and D191, first evaluated in the greenhouse, were then compared in a field experiment. All field treatments (*i.e.* the combinations between these three strains and two pea cultivars, Facón and Viper) were carried out in triplicate. The field experiment was performed in the locality of Rueda (33°21'48.48" S and 60°27'39.12" W), in Santa Fe province (Argentina), on a Typic Argiudoll soil. The experimental unit (plot) had six furrows (at 0.175 m interrow) and 5 m in length. The experiment was sown on July 29<sup>th</sup>, 2014, with 119 plants m<sup>-2</sup> (110-127 plants m<sup>-2</sup>) and harvested on November 14<sup>th</sup> (108 DAP). Plants were fertilized with 100 kg ha<sup>-1</sup> of monoammonium phosphate (*i.e.* 20 kg P ha<sup>-1</sup>). Seeds were inoculated as described in the greenhouse experiment. Weeds and pests were controlled. During the cycle, total rainfall was 284 mm and mean temperature was 17.3°C.

At 60 DAP (September 14<sup>th</sup>), 0.5 m of two rows (0.18 m<sup>-2</sup>) was sampled and the number of nodules in each plant ( $N_{Nod}$ ) was counted. Then, plants were oven-dried at 65°C for 72 h

to determine nodule biomass per plant. At 95 DAP, 1 m<sup>2</sup> of the total aboveground biomass of field pea of each plot (N-fixing crop) plus one linear meter of wheat (non-fixing crop) were sampled. These samples were dried in an air circulation stove at 65°C for 72 h. A subsample of each mentioned sample was ground with a Wiley mill to determine N content in biomass (%N<sub>upt</sub>) according to the micro-Kjeldahl method. The N uptake (N<sub>upt</sub>) was obtained by multiplying %N<sub>upt</sub> by Biom<sub>Aerial</sub>. BNF was determined by the <sup>15</sup>N natural abundance method, using an elemental analyzer Carlo Erba EA 1108 coupled to a ThermoScientific Delta V Advantage isotopic mass spectrometer of continuous flow through a ConFLo IV interface. Then, %N<sub>FBN</sub> was estimated according to Collino *et al.* (2015) and N<sub>BNF</sub> was calculated by N<sub>BNF</sub> = %N<sub>FBN</sub> \* N<sub>upt</sub>.

At harvest (108 DAP), two samples of aboveground biomass (Biom<sub>Aerial</sub>) were taken from each plot of 1 m<sup>2</sup>. The, grained (Biom<sub>Grain</sub>) and vegetative structures (Biom<sub>Veg</sub>) were separated, dried in an air circulation stove at 65°C for 72 h and weighed. The harvest index was calculated. Grain yield (Yield<sub>Grain</sub>) was adjusted to grain moisture of 0.135 kg water kg grain<sup>-1</sup>.

### Molecular characterization

Three of the strains evaluated (D70, D156 and D191) were characterized by partial sequencing of the 16S rRNA gene by amplification reactions. Pure colonies were grown on plates in YEM culture medium, then suspended in 50 µl of ultrapure water and boiled in a water bath for 10 min to obtain DNA extracts. The universal primers fd1 and rP2 proposed by Weisburg *et al.* (1991) were used and amplification was carried out in a volume of 25 µl of the reaction containing 2.5 ml 1X Buffer, 0.75 µl of 50 mM MgCl<sub>2</sub>, 0.5 µl of 10 mM dNTP, 0.25 µl Taq DNA Polymerase, 0.5 µl of each primer (fd1 and rP2), 1 µl of tempered genomic DNA and 19 µl of ultrapure water. Amplification conditions consisted of an initial denaturation of 2 min at 94°C, followed by 35 cycles of denaturation (94°C, 40 seconds), annealing (52°C, 40 seconds) and extension (72°C, 1.5 min) and a final extension at 72°C for 10 min. A negative control without template DNA was included. Electrophoresis was performed at 90 V. The amplified products were analyzed by 0.8% agarose electrophoresis stained with SYBR Safe DNA gel stain (Invitrogen™) for 30 min and then purified with the commercial Gel Extraction kit QIAEX II (Qiagen). Partial sequences of 16S rRNA were compared with those deposited in the NCBI BLAST database (<http://blast.ncbi.nlm.nih.gov/>).

### Data analysis

Statistical analysis of the growth chamber, greenhouse, and field experiments was carried out using a two-way ANOVA. Means were compared with the DGC test (p≤0.05). All analysis was performed using Infostat software version 2018p (Di Rienzo *et al.*, 2018).

## RESULTS

### Growth chamber experiment

Only 44-55% of all the strains tested (71-78) presented nodules in the four cultivars (*i.e.* they were infective), varying according to the cultivar. Facón and Pampa cultivars had the highest number of infectious strains (41), whereas Bicentenario and Manantiales had the lowest (32 and 31, respectively).

Twenty-three strains were able to infect the four cultivars evaluated. The isolates obtained from nodules of the genus *Lathyrus* had a higher proportion of infective and effective nodules in the four cultivars evaluated (60%), followed by those from *Lens*, with 50%, and those from *Vicia*, with 29%, whereas the isolates from *Pisum*, with 8%. Soil isolates had 38% nodulation ability in the four cultivars.

Results from the growth chamber showed highly significant (p<0.001) variability in the N°<sub>Nod</sub> per plant (*i.e.* strain x cultivar), whereas the effects of strains on Biom<sub>Total</sub> were significant at p=0.025 (Additional material 2). N°<sub>Nod</sub> ranged from 76 to 1. D70 presented the highest N°<sub>Nod</sub> values, with an average of 43 nod pl<sup>-1</sup>, followed by D191, with 35 nod pl<sup>-1</sup>, and higher N°<sub>Nod</sub> than the rest. These responses were 97% and 67% higher, on average, at D70 and D191, respectively (Additional material 1). Biom<sub>Total</sub> ranged from 287 to 149 mg pl<sup>-1</sup>, with D156, D155, and D191 showing the highest values (233-221 mg pl<sup>-1</sup>), *i.e.* 6% higher than the Biom<sub>Total</sub> of D70 with the four cultivars (Additional material 1).

Based on these results, greenhouse experiments included strain D156 selected for biomass production ability, D191 for nodulation behavior, and D192 and D193 for fast initial growth (not shown) as compared to the reference strain D70.

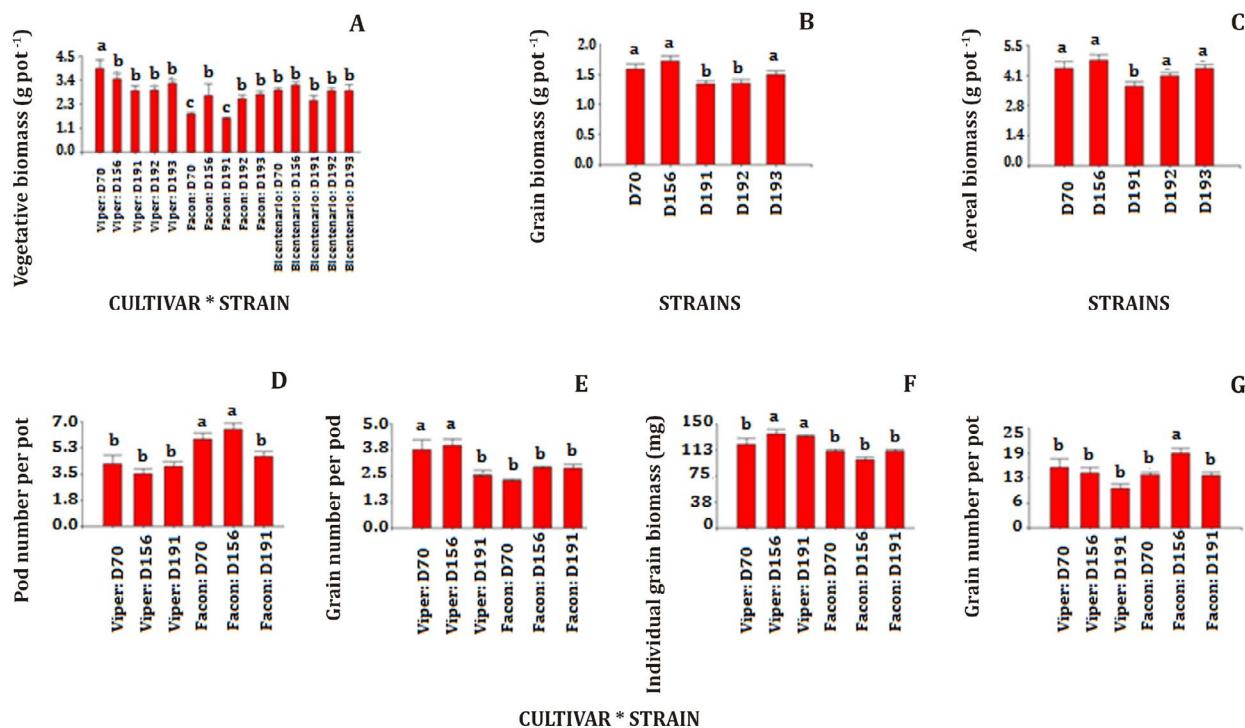
### Greenhouse experiment

Strains D70, D156, D191, D192, and D193 were evaluated in the greenhouse experiment. At 56 DAP, a highly significant interaction between strain and cultivar was detected in all variables, except root biomass. Strain D192 had the best behavior in  $Leng_{Root}$  (358 mm pl<sup>-1</sup>) followed by D156 and D193 (322 mm pl<sup>-1</sup>, on average) with cultivar Facón, with 28 and 15% differences in comparison to D70 and D191. D192 and D193 showed better  $Leng_{Aerial}$  values, with 235 mm pl<sup>-1</sup>, followed by D156 and D70 (206 mm pl<sup>-1</sup>), all with cultivar Bicentenario, being 66% for 192 and D193 and 46% for D156 and D70. Instead, strain D193 had higher  $Leng_{Plant}$  (515 mm pl<sup>-1</sup>), followed by D192 (508 mm pl<sup>-1</sup>) with Bicentenario and Facón. Likewise, strains D192 and D193 had higher  $Biom_{Plant}$  (473 mg pl<sup>-1</sup>) with Bicentenario, with significant differences with the rest of 55%. These strains also had the highest  $Biom_{Aerial}$  values (271 mg pl<sup>-1</sup>), followed by D156 and D70 (214 mg pl<sup>-1</sup>), all with Bicentenario. In contrast, regarding nodule variables, D70 and D156 showed higher  $N^o_{Nod}$  (32 nod pl<sup>-1</sup> on average) with Viper, 100% higher than the rest, as well as higher  $Biom_{Nod}$  (9 mg pl<sup>-1</sup> on average), 141% higher than the rest, and highest  $Biom_{Nod-1}$  (0.44 mg nod<sup>-1</sup> in average), 144% higher than the rest, all with Bicentenario.

At 78 DAP, a significant interaction between strain and cultivar was observed in the nodulation variables, except in individual nodule biomass. Strain D70 had the highest  $N^o_{Nod}$  together with strains D192 and D193, which were better associated with Bicentenario (109, 129, and 110 nod pot<sup>-1</sup>, respectively). Also, strain D70 showed better  $Biom_{Nod}$  with Viper (40 mg pot<sup>-1</sup>) and Bicentenario (38 mg pot<sup>-1</sup>), and the best  $Biom_{Nod-1}$  (0.53 mg nod<sup>-1</sup>) concerning other strains (75%). Differently, plant growth variables showed significant differences among strains. Strain D191 showed the lowest values of  $Biom_{Aerial}$  (2.0 g pot<sup>-1</sup>) and  $Biom_{Root}$  (0.7 g pot<sup>-1</sup>) with respect to the rest (40 and 39%, respectively).

At harvest (103 DAP), the five strains were evaluated for  $Biom_{Veg}$ ,  $Biom_{Grain}$ , and  $Biom_{Aerial}$ . Only  $Biom_{Veg}$  showed cultivar and strain interaction. All plant yield components (*i.e.*  $N^o_{pod}$ ,  $N^o_{Grain}$ ,  $N^o_{Grain-pod}$  and  $Biom_{Grain}$ ) showed significant interaction between both factors, although only measured in Facón and Viper with strains D70, D156, and D191. Strain D70 produced 3.9 g pot<sup>-1</sup> of  $Biom_{Veg}$  with Viper cultivar, significantly higher than the rest (45% on average), followed by D156 (3.5 g pot<sup>-1</sup>) also with Viper cultivar. In contrast, strain D191 showed the worst performance (1.6 g pot<sup>-1</sup>) with Facón (figure 1 A, page XXX). Regarding  $Biom_{Grain}$ , D156, D70 and D193 had higher values (1.7, 1.6, and 1.5 g pot<sup>-1</sup>, respectively) than D191 and D192 strains (figure 1B, page XXX). Regarding  $Biom_{Aerial}$ , D191 (3.6 g pot<sup>-1</sup>) showed significantly lower values (22%) than other strains (figure 1C, page XXX). Considering yield components, strain D156, followed by D70, threw the best results compared to D191, except in  $Biom_{Grain-1}$  where D191 had the second-best weight after D156. In addition, D156 strain had 6.5 pods pot<sup>-1</sup>, followed by D70 strain (5.8 pods pot<sup>-1</sup>), both with Facón cultivar having more pod number per pot ( $N^o_{Pod}$ ) than the rest (figure 1D, page XXX). Regarding grain number per pod ( $N^o_{Grain-pod}$ ), D156 also performed better with Facón (4.0 grains pod<sup>-1</sup>) and D70 with Viper (3.8 grains pod<sup>-1</sup>) (figure 1E, page XXX). Likewise, D156 followed by D191, had the highest individual grain biomasses ( $Biom_{Grain-1}$ ) with 136 and 132 mg grain<sup>-1</sup> respectively, with Facón (figure 1F, page XXX). D156 with Facón had higher  $N^o_{Grain}$  (18.8 grains pot<sup>-1</sup>) than the rest (44%) (figure 1G, page XXX).

Total N (%N<sub>upt</sub>) and δ<sub>15</sub>N (‰) contents of whole plants were determined at 92 DAP for Facón and Viper inoculated with D70, D156, and D191 (Additional material 3). D156 (2.2%) and D70 (2.1%) strains had higher biomass <sub>upt</sub>%N<sub>upt</sub> than D191 ( $p < 0.01$ ). The mean β value was -0.66‰, where D70 had lower depletion of δ<sub>15</sub>N (-0.45‰), without differences with D156 (-0.55‰), being both over D191 (-0.88‰) with a significance level of  $p = 0.06$  (Additional material 3).



**A)** vegetative weight (g), **B)** grain weight (g), **C)** aerial biomass (g), **D)** pod number, **E)** grain per pod, **F)** individual grain biomass (mg) and **G)** grain number per pod.

Different letters indicate significant differences between treatments at  $p \leq 0.05$ . Results shown in panel D correspond to cultivars Facón and Viper average values.

**A)** biomasa de grano (g), **B)** biomasa vegetativa (g) y **C)** biomasa aérea total (g), **D)** número de vainas por maceta, **E)** número de granos por vaina, **F)** biomasa individual del grano (mg) y **G)** número de granos por maceta.

Letras diferentes indican diferencias significativas entre tratamientos con valor  $p \leq 0.05$ . Los resultados mostrados en el panel D corresponden al promedio de los cultivos Facón y Viper.

**Figure 1.** Plant variables and yield components per pot at harvest (103 days after planting) as dry matter.

**Figura 1.** Variables de la planta y componentes del rendimiento por maceta a la cosecha (103 días después de la siembra).

### Field experiment

In the field experiment, only nodule number had significant interaction between factors. Regarding strain behavior significant differences were found for Biom<sub>Veg</sub>, Biom<sub>Aerial</sub>, and Biom<sub>Nod-l</sub>.

Strain D191 behaved differently than in the greenhouse regarding Biom<sub>Veg</sub>, Yield<sub>Grain</sub>, Biom<sub>Aerial</sub> and N<sub>upt</sub>. Likewise, strain D191 strain had better behavior in Biom<sub>Veg</sub> ( $3.3 \text{ Mg ha}^{-1}$ ) and Biom<sub>Aerial</sub> ( $6.7 \text{ Mg ha}^{-1}$ ) than D156 (15% in both variables) and D70 (33 and 25%, respectively, table 1, page XXX). In addition, D191 strain had higher N content in aerial biomass ( $212 \text{ kg N ha}^{-1}$ ) than D156 (8%) and D70 (16%). Average Yield<sub>Grain</sub> was  $3.5 \text{ Mg ha}^{-1}$ , %N<sub>FBN</sub> was 70.5% and N°<sub>FBN</sub> was  $139 \text{ kg N ha}^{-1}$  (table 1, page XXX). Instead, regarding %N<sub>upt</sub> D156 (3.43%) and D70 (3.41%) showed higher values than D191 (8 and 7%, respectively), maintaining the same behavior as in the greenhouse (table 1, page XXX). Strain D191 had the highest N°<sub>Nod</sub> per plant ( $10.5 \text{ nod pl}^{-1}$ ) differing 105% with the rest. Instead, D156 and D70 had highly significant differences ( $p \leq 0.01$ ) in Biom<sub>Nod-l</sub> (2.5 and  $1.7 \text{ mg nod}^{-1}$ , respectively) with respect to D191 (162 and 85%, respectively), but with no differences in Biom<sub>Nod</sub> (table 1, page XXX).

**Table 1.** Nodulation at 60 DAP, nitrogen plant variables at 95 DAP and plant harvest at 108 DAP.**Tabla 1.** Nodulación a los 60 DDP, de las variables de nitrógeno en planta (95 DDS) y rendimiento de granos a cosecha (108 DDS).

| Strain / Variable                                    | Plant variables (95 DAP)                   |   |   | Nodulation (60 DAP)       |                             |
|--|--|---|---|---------------------------|-----------------------------|
|  | Biom <sub>Veg</sub><br>Mg ha <sup>-1</sup> | Yield <sub>Grain</sub> *<br>Mg ha <sup>-1</sup> | Biom <sub>Aerial</sub><br>Mg ha <sup>-1</sup> | Biom <sub>Nod</sub><br>mg | Biom <sub>Nod-I</sub><br>mg |
| <b>D70</b>   | 2.5 b                                      | 3.3 a   | 5.3 b   | 97.2 a                    | 1.74 a                      |
| <b>D156</b>  | 2.9 b                                      | 3.3 a   | 5.8 b   | 104.3 a                   | 2.46 a                      |
| <b>D191</b>  | 3.3 a                                      | 3.8 a   | 6.7 a   | 67.3 a                    | 0.94 b                      |
| <i>p</i> -value                                      | 0.006                                      | 0.09  | 0.004   | 0.56                      | 0.008                       |
| <b>Nitrogen plant variables at harvest (108 DAP)</b> |  |   |   |                           |                             |
| Strain / Variable                                    | N <sub>upt</sub><br>kg ha <sup>-1</sup>    | %N <sub>FBN</sub><br>%                          | N <sub>FBN</sub><br>kg ha <sup>-1</sup>       | %N <sub>above</sub><br>%  |                             |
|  |  |   |   |                           |                             |
| <b>D70</b>   | 182 b                                      | 72 a  | 132 a   | 3.43 a                    |                             |
| <b>D156</b>  | 196 b                                      | 73 a  | 143 a   | 3.41 a                    |                             |
| <b>D191</b>  | 212 a                                      | 67 a  | 142 a   | 3.18 b                    |                             |
| <i>p</i> -value                                      | 0.03                                       | 0.25  | 0.51  | 0.02                      |                             |

\*Adjusted to 0.135 kg H<sub>2</sub>O kg grain<sup>-1</sup> grain moisture. Values followed by different letters in columns indicate significant differences at p ≤ 0.05.

\*Ajustado a una humedad de grano de 0,135 kg H<sub>2</sub>O kg grano<sup>-1</sup>. Los valores seguidos de letras diferentes en la columna indican diferencias significativas en p ≤ 0,05.

### Molecular characterization

Molecular identification of D70, D156, and D191 strains via partial sequencing of the 16S rRNA gene showed 100% identity with *Rhizobium leguminosarum*, and deposited in the same database under accession numbers KU933357, KX 066064, and KX346599, respectively. Sequences were then processed to obtain multiple alignments then concatenated for the construction of a phylogenetic tree. In turn, strains Az39 (*Azospirillum argentinense*) and E109 (*Bradyrhizobium japonicum*) were incorporated as members of other groups. Results of this analysis showed that D70 and D156 are more closely related than D191.

### DISCUSSION

Our results showed that nodulation ability of both native and naturalized isolates of rhizobia in field pea was similar to that observed by other authors (3, 8, 23, 38). For example, Ballard *et al.* (2004) found that 67% (22 of 33) of naturalized populations of rhizobia (applied to pea plants as soil suspensions) caused early and abundant nodulation in one cultivar of field pea, while our work found that 44-55% of native isolates of Argentina or introduced strains kept at the IMYZA-INTA collection were infective in four field pea cultivars. Likewise, Boivin *et al.* (2020b) observed that *Rhizobium leguminosarum* sv *viciae* are all potential symbionts of Fabaceae hosts (field pea, vetch, and lens) but display variable competitiveness to form root nodules. However, when Fabaceae legumes are exposed to natural soil bacterial populations, symbiotic efficiency is often suboptimal. In a recent study, Boivin *et al.* (2020a) determined that small genetic differences in *nodD* genes allowed observing a higher specificity degree among *R. leguminosarum* sv *viciae* in pea, faba bean, and lentil. In the present study, we observed that rhizobia nodule isolates from *Lathyrus* sp and *Lens* sp were the most infective, followed by *Vicia* sp. Instead, isolates from pea plants did not ensure infection in all cultivars of the same species (*Pisum sativum*), since only 1 of 12 isolates/strains caused nodules in the four cultivars evaluated. Therefore, field pea

cultivars could have genetic differences (2, 36), which, associated with the rhizobial genome, may influence competitiveness for nodulation as well as rhizosphere colonization, decisive steps in the competition for nodule occupancy by *R. leguminosarum* in soil populations (23).

The elite strain D70, isolated from *Vicia* sp, is most used in inoculant formulations. This strain has high nodulation ability and BNF contribution. Enrico *et al.* (2020) determined an average of 134 kg ha<sup>-1</sup> of N<sub>FBN</sub> in field pea (8 experiments, average). In this work, D70 had a contribution of 131 kg N ha<sup>-1</sup>, while D156, also isolated from *Vicia* sp, showed a similar and better BNF behavior in both greenhouse and field experiments (*i.e.* 143 kg N ha<sup>-1</sup>). Instead, the high nodulation ability observed in D191 (obtained from *Lens* sp) in the growth chamber, then resulted the least effective regarding yield variables in the greenhouse experiment. However, this strain performed better in the field experiment regarding biomass production (6.7 Mg ha<sup>-1</sup>), grain yield (3.8 Mg ha<sup>-1</sup>) and N absorbed (212 kg ha<sup>-1</sup>), considering the other strains. Table 2 shows BNF contributions from different studies, indicating the selected strains had high N contribution. Likewise, D156 and D70 had better N content in biomass (%N<sub>upt</sub>), which, according to Laguerre *et al.* (2007), depends on BNF efficiency of rhizobia derived from a robust nodular system.

**Table 2.** Contribution of biological nitrogen fixation (BNF) on field pea.

**Tabla 2.** Contribución de la fijación biológica de nitrógeno (FBN) en arveja sobre experimentos de campo.

| BNF contribution (%) | N derived from BNF kg N ha <sup>-1</sup> | References                             |
|----------------------|--|--|
| 72                   | 131                                      | D70 strain (this work)                 |
| 73                   | 143                                      | D156 strain (this work)                |
| 67                   | 142                                      | D191 strain (this work)                |
| 69-71                | 166-286                                  | Kumar and Goh 2000                     |
| 84                   | 238                                      | Voisin <i>et al.</i> , 2002            |
| 47-81                | 64-81                                    | Hauggaard-Nielsen <i>et al.</i> , 2009 |
| 87-91                | 64-130                                   | Abi-Ghanem <i>et al.</i> , 2011        |
| 37-85                | 45-106                                   | Ruisi <i>et al.</i> , 2012             |
| 41-45                | -  | Keneni <i>et al.</i> , 2013            |
| 43-62                | 58-69                                    | Hossain <i>et al.</i> , 2016           |
| 35-65                | -  | Yang <i>et al.</i> , 2017              |
| 55                   | 63                                       | Liu <i>et al.</i> , 2019               |
| 33-83                | 11-71                                    | Enrico <i>et al.</i> , 2020            |

In the present study, strains D70 and D191 had high nodulation ability in controlled conditions (growth chamber and greenhouse). In contrast, both D156 and D70 had higher individual nodule biomass than D191, similar to controlled conditions. This behavior may be due to differences observed by the 16S genes concerning D70 or the larger number of specific genes that control nodulation competitiveness (8). This behavior can also be due to plant energetic regulation in association since, according to Rainbird *et al.* (1984), sustaining nodular system involves 22% of total energy, and nitrogenase activity (BNF) consumes an additional 52% (in soybean). Likewise, Ryle *et al.* (1986) determined that the energy consumption of white clover nodules was 22-27%. Therefore, nodule numbers adapt to plant possibility to maintain a symbiotic system benefiting yield.

Early growth effects in the growth chamber (D156) and greenhouse experiment (D156, D192, and D193 strains) could be associated with strain-dependant phytohormone effects, a fact not addressed in this study. Therefore, strains tending to increase auxins would be associated with stem and root elongation during post-emerging growth (6, 27, 33). In addition, cytokine balance in the early nodulation stages may be associated with a larger number of nodules. Also, final number of nodules may depend on ethylene production (4, 15). Strains D192, D193 and D156 stood out in stem and root elongation and nodule number and

could have an adequate profile of phytohormones helping plant implantation. Then, at an advanced stage (78 DAP), the most relevant aspect would be symbiotic functionality, where the contribution of biological N translates into greater biomass (11, 34). Both strains D156 and D70 showed differences with D191, possibly due to symbiotic effectiveness. Differences were also observed in grain yield when the only N source was biological. Likewise, the only source of  $^{15}\text{N}$  abundance in biomass resulted to be the biological source ( $\beta$  value), adjusting the contribution of BNF in field conditions. These results are consistent with those obtained by Unkovich *et al.* (1994) in clovers under different environments and with those by López-Bellido *et al.* (2010) in chickpea and faba beans between selected and natural soil strains. For non-different nodule structures (*i.e.* the same cultivar), differences in  $^{15}\text{N}$  abundance in the plant would be associated with strain genotype, generating a distinctive behavior (23). This study confirms the importance of *Rhizobium* strains in  $^{15}\text{N}$  isotopic discrimination in field pea, due to isolate origin, where adaptive or microevolutionary changes would be expected after genetic divergences (20, 28).

In our field experiment, aerial biomass ( $\text{Biom}_{\text{Aerial}}$ ) and yield ( $\text{Yield}_{\text{Grain}}$ ) had no interaction (same as in the greenhouse). Yields obtained from the field experiment ( $3.2 \text{ Mg ha}^{-1}$  for D70,  $3.3 \text{ Mg ha}^{-1}$  for D156, and  $3.8 \text{ Mg ha}^{-1}$  for D191;  $3.5 \text{ Mg ha}^{-1}$  on average) were higher than those obtained in the 2014 crop cycle by Prieto *et al.* (2015) ( $2.7 \text{ Mg ha}^{-1}$ ) and Enrico *et al.* (2020) ( $1.3 \text{ Mg ha}^{-1}$ ) in eight experiments performed in different environments of Argentina between 2012 and 2017. Unlike that observed in the greenhouse, inoculation with D191 showed the best behavior in aerial and vegetative biomass and nodule number per plant. In the field experiment, D156 and D70 had the same behavior on the specific variables derived from the *Rhizobium* strain, such as  $\%N_{\text{BNF}}$  and/or the contribution of  $N_{\text{BNF}}$ , and maintained the efficiency demonstrated in the greenhouse (23). In this study, in the field experiment, we observed 70.5% (range 67 to 73%) of  $\%N_{\text{BNF}}$  on average with 67% for D91, 72% for D70, and 73% for D156, all higher values than those established by Enrico *et al.* (2020), who found 59% inoculated with D70, but within the interquartile range obtained for field pea (33 to 83%).

The incorporation of a winter legume such as field pea (inoculated with efficient N-fixing strains) preceding a summer crop (*e.g.* maize) may constitute a valid agronomic management. Enrico *et al.* (2020) observed a positive effect on maize yield when the previous crop was inoculated (field pea with strain D70). Having rich in N and low C:N ratio stubble allowed rapid soil N availability. This higher availability of N considerably reduced the need for chemical nitrogen fertilization.

## CONCLUSIONS

Most of the isolates that were infective and effective for *Pisum sativum* cultivars here evaluated came from species other than field pea.

Strain D70 maintained good behavior in the growth chamber, greenhouse, and field experiments concerning nodule, nitrogen, biomass, and yield variables, whereas, D156 had similar or better behavior in the greenhouse and field experiments. In contrast, D191 had only better behavior in the field experiment.

The  $\beta$  value calculation of  $^{15}\text{N}$  natural abundance model to determine  $\%N_{\text{FBN}}$  was key to adjusting  $N_{\text{FBN}}$  contribution. Determining differences in strain behavior results an essential requirement and would allow a more precise adjustment of N input.

Finally, strains selected for BNF efficiency should implicitly carry infectivity ability in all commercial cultivars. This will allow certainty in their recommendation for the formulation of inoculants. If new evaluations are required in different agroecological environments, strain D156 could be a good candidate for bacterial single-strain inoculants, as well as for a microbial consortium for *Pisum sativum*.

## SUPPLEMENTARY MATERIAL

Schematic figure of the dual-choice olfactometer utilized in choice test with stored grain pests:  
[https://drive.google.com/drive/folders/17vBzpdAKT\\_WP2l\\_SdnicLF\\_EH-puPzCy?usp=sharing](https://drive.google.com/drive/folders/17vBzpdAKT_WP2l_SdnicLF_EH-puPzCy?usp=sharing)

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