



## Induction of signals in *Rhizobium leguminosarum* bv. *viciae* and its biological activity in pea (*Pisum sativum* L.)

### Inducción de señales en *Rhizobium leguminosarum* bv. *viciae* y su actividad biológica en arveja (*Pisum sativum* L.)

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**ABSTRACT:** Pea is a versatile plant, used in different ways and for different purposes, due to its high nutritional value. Its symbiotic interaction with bacteria of the rhizobia family guarantees significant amounts of nitrogen, from biological fixation, which allows it to grow in environments lacking this element. The agronomic and environmental importance of this symbiosis has required technology improvement for the production and inoculant application, so this work set out to evaluate the induction effect of a *Rhizobium leguminosarum* bv. *viciae* strain in the generation of signals in the inoculant and its biological activity on pea plants. Daidzein was used as an inducer at a final concentration of 5 µM. The lipidic fraction in the inocula was extracted with n-butanol and analyzed by high performance liquid chromatography and gas chromatography coupled to a mass spectrometer. Biological activity was evaluated in one experiment under controlled conditions, two field experiments, and one extensive trial. The results showed that in the inoculant induced with the flavonoid, a higher quantity and diversity of molecules related to the symbiotic interaction was detected. Similarly, the induced inocula showed a higher positive effect on nodulation (14.8 vs 8.7) and the yield of pea plants, with 468 and 125 kg ha<sup>-1</sup> of seeds above the inoculated treatment without inducing in the experiments in plots and 746 kg ha<sup>-1</sup> in the extensive trial.

**Key words:** biological fixation, nodulation, communication, legumes.

**RESUMEN:** La arveja es una planta versátil, utilizada de diferentes formas y para distintos propósitos, por su elevado valor nutritivo. Su interacción simbiótica con bacterias de la familia de los rizobios garantiza importantes cantidades de nitrógeno, a partir de la fijación biológica, lo que le permite crecer en ambientes carentes de este elemento. La importancia agronómica y ambiental de esta simbiosis ha exigido el perfeccionamiento de las tecnologías de producción y aplicación de inoculantes, por lo que este trabajo se propuso evaluar el efecto de la inducción de una cepa de *Rhizobium leguminosarum* bv. *viciae* en la generación de señales en el inoculante y su actividad biológica sobre plantas de arveja. Se utilizó como inductor la daidzeína a una concentración final de 5 µM. La fracción lipídica en los inóculos fue extraída con n-butanol y analizada por cromatografía líquida de alta resolución y cromatografía gaseosa acoplada a un espectrómetro de masas. La actividad biológica se evaluó en un experimento en condiciones controladas, dos experimentos en campo y un ensayo extensivo. Los resultados mostraron que en los inóculos inducidos con el flavonoide se detectó una cantidad y diversidad superior de moléculas relacionadas con la interacción simbiótica. De manera similar, los inóculos inducidos mostraron un efecto positivo superior en la nodulación (14,8 vs 8,7) y el rendimiento de plantas de arveja, con 468 y 125 kg ha<sup>-1</sup> de semillas por encima del tratamiento inoculado sin inducir en los experimentos en parcelas y 746 kg ha<sup>-1</sup> en el ensayo extensivo.

**Palabras clave:** fijación biológica, nodulación, comunicación, legumbres.

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## INTRODUCTION

Pea (*Pisum sativum* L.) is a grain legume, characterized by its high nutritional value and its use in human and animal nutrition. Its seeds contain between 18 and 20 % of dry matter, of which 10 to 12 % are carbohydrates and between 5 and 8 % are proteins (1). Pea beans can be used for immediate consumption or canned for long-term storage (2). This crop can also be used as green fodder, hay, silage or flour and as green manure (3). As a legume, the pea has another important characteristic, related to the ability to fix atmospheric nitrogen in symbiosis with bacteria of the rhizobia family (4), specifically with the species *R. leguminosarum* bv. *viciae* (5). This symbiosis provides significant amounts of N to the soil for successor crops and it is suggested that inoculation with these bacteria can produce similar yields to the application of 200 kg of N ha<sup>-1</sup> (2).

The application of large amounts of inorganic fertilizers in intensive production systems (6,7) represents a polluting burden for the environment and high costs that many countries cannot afford. Achieving similar or higher yields with low inputs and organic products represents a challenge for sustainable agriculture. Symbiotic fixation of N is a promising alternative in this regard (8,9).

The inoculation technologies of various beneficial microorganisms in seeds constitute an economical agronomic practice, recommended in agriculture. Successful inoculation will depend on several factors, including the ability of the introduced strain to compete with the existing native population and achieve greater infection, nodulation, and N contribution (10).

The symbiosis established between legume plants and rhizobia responds to an intense exchange of signals from the beginning of the interaction, which allows communication between both symbionts: the macro and the microorganism (11). Seeds release various compounds, including flavonoids, which cause the expression of *nod* genes in bacteria, which induces the synthesis of Nod factors, essential molecules in the interaction success (12). These compounds have also been related to the chemo-attraction exerted by legumes on rhizobia and with the expression of other genes in these bacteria that regulate the synthesis of exopolysaccharides and the type III secretion system, related to the defensive responses in the host and protein export in nodulation, respectively (13).

High molecular weight fatty acids are structural components of lipoquitoligosaccharides associated with nodulation. Nod factors consist of a backbone of three to five molecules of N-acetyl-glucosamine, which at the amino group of the non-reduced end is acylated with a fatty acid 16-20 C atoms long (C16-C20) (14).

The use of inoculants induced in the synthesis of these signals that govern communication, has made it possible to obtain not only better nodulation and plant performance (15), but also tolerance to stress conditions in the environment (16). For this reason, the present work aimed to evaluate the induction effect of a *Rhizobium leguminosarum* bv. *viciae* strain in the generation of signals in the inoculant and its biological activity in pea plants.

## MATERIALS AND METHODS

### Bacterial culture. Induction with daidzein

A strain of *Rhizobium leguminosarum* bv. *viciae* was used, known for its symbiosis with the pea. A preinoculum was produced in 50 mL of mannitol yeast extract medium (17) at pH 6.8; to inoculate 600 mL of the same medium without and with daidzein (Sigma), which was added to a final concentration of 5 µM. The inocula were obtained after keeping the flasks at 150 rpm for 48 h, in an orbital shaker at 28 ± 2 °C of temperature. For each case, the procedure was repeated three times and included three samples from each treatment (uninduced and daidzein-induced).

### Extraction of the lipidic fraction

Taking into account that most of signals recognized by their biological activity in the rhizobia-legume interaction are lipids, a selective extraction of these molecules was carried out from the inoculum treated with daidzein (Induced) or not (Control), using 180 mL of n-butanol in each sample. They were placed on an orbital shaker at 150 rpm for 15 min and kept overnight under dark conditions at a room temperature of 25 ± 2 °C. Then the organic phase in each sample was extracted, centrifuged at 12000 g, 10 °C, for 10 minutes. All samples were concentrated by rotary evaporation at 50-80 °C until 2 mL of each were obtained, which were used for the detection of signals produced by the bacteria.

### Signal detection. Structural characterization of main components in the lipidic fraction

### High performance liquid chromatography analysis, reversed phase

To evaluate the presence of nodulation factors among the metabolites, 10 µL of all lipidic extracts were analyzed by HPLC, using a Waters Symmetry C-18 reversed phase column (46 x 250 mm) of 5 µ particle size, installed on a Waters Alliance HPLC System. The flow rate was 1 mL min<sup>-1</sup> and water (A) and acetonitrile (B) were used as solvents, with a gradient: 0-10 min 18 % B, 10-30 min 60 % B, 30-35 min 95 % B, 35-45 min 18 % B. A UV-Waters spectrophotometric detector was used at a wavelength of 214 nm.

### Gas chromatography coupled to a mass spectrometer

The volatile fatty acid derivatives were prepared by silylation, using BSTFA (N, O-Bis trimethylsilyl trifluoroacetamide) as a reagent in combination with trimethylchlorosilane (TMCS) (BSTFA+TMCS Kit, Supelco). For the analysis by GC-MS, a gas chromatograph coupled to a Shimadzu GC-MS QP-2010 mass spectrometer was used; system equipped with AOC-20i autoinjector, AOC-20s automatic injector and a direct insertion system controlled by the "GC-MS Solution" software. An Optima 5 MS column (30 µm × 0.25 mm ID, 0.25 µm film thickness) was used.

The conditions of the chromatographic analysis were: injector temperature 310 °C, oven temperature 100 °C for six minutes. Subsequently, it was increased to 320 °C at a rate of 20 °C min<sup>-1</sup> and held for 5 minutes. The injection volume was 1 µL and the column flow was 0.75 mL min<sup>-1</sup>, using helium as a stripping gas.

### Effect on the pea plant nodulation, under controlled conditions

From the biopreparations obtained, 2 mL were inoculated for every 500 g of Facon variety seeds, which were sown in a sterile soil/vermiculite mixture (1:1), without the application of other nutrients. The plants were grown in a growth chamber, with a photoperiod of 16/8 hours of light/dark, at 28 °C and with 60 % relative humidity, for 35 days. Irrigation was carried out by capillarity with deionized water, maintaining the water regime at approximately 90 % of the field capacity. Nine plants were used per treatment to evaluate the number and dry mass of nodules (mg) per plant.

### Effect on plant performance, under field conditions

This study was carried out in experimental fields of the localities Ferré (34°09'S, 61°09'W, altitude 93 m, typical black Mollisol soil, which corresponds to a Feozem soil (18)) and Pergamino (33° 53'S, 60 ° 34'W, altitude 56 m, typical Argiudol soil, loamy-silty (Argic Feozem), Buenos Aires province, in Argentina, during the months of July-October 2015. Two of the trials were carried out in plots (in both localities) and an extensive one in Ferré. The data from the soil analysis in Pergamino were: pH (1: 2.5) = 5.6; N-NO<sub>3</sub> (ppm) = 4; P Bray I (ppm) = 19.2; Org. M. (%) = 2.2; Total N (%) = 0.111; Carbon (%) = 1.3. Temperatures (°C) between July-October: 16.4-20.1-20.4-25.0 and rainfall (mm): 33.0-8.0-60.0-111.0.

For Ferré, the data from the soil analysis were: pH (1: 2.5) = 5.6; N-NO<sub>3</sub> (ppm) = 13.7; P Bray I (ppm) = 23.6; Org. M. (%) = 2.7; Total N (%) = 0.134; Carbon (%) = 1.6. Temperatures between July-October: 16.3-21.0-20.7-24.8 °C and rainfall: 3.0-4.0-68.0-105.0 mm, respectively.

Seeds of Facon variety were inoculated at a dose of 4 mL per 1000 g of seeds, with a sowing density of 25 seeds m<sup>-1</sup> and a direct sowing system (without tillage) in Ferré and conventional sowing (previous tillage of the soil) in Pergamino. As a predecessor crop, in Ferré soybeans were cultivated, while in Pergamino it was pastures. In all the experiments, the following was used as fertilization: 80 kg ha<sup>-1</sup> of Mixture (8-38-0-8 S). At the time of harvest, the yield (kg ha<sup>-1</sup>) was determined.

### Experimental design and statistical processing

The growth chamber experiment was repeated three times. A completely randomized design was used and the normality and homogeneity of the variance was checked in the data, which were analyzed according to Tukey  $p < 0.05$ .

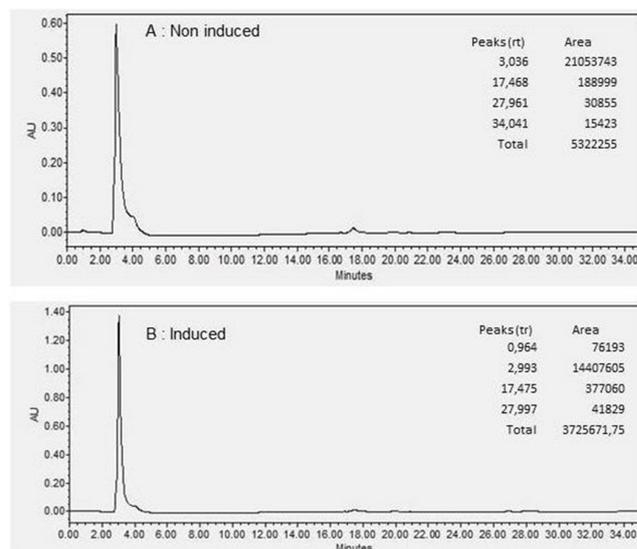
In the plot experiments a bifactorial analysis was used, taking into account the inoculant factors and localities,

with three and two levels, respectively. To discriminate the differences between the means, results were compared using Duncan's Multiple Range Test  $p < 0.05$ . The Statgraphics Plus program, version 5.1, was used.

## RESULTS AND DISCUSSION

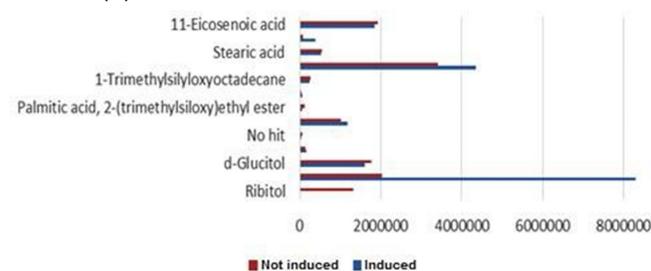
### Signal detection. Structural characterization of main components in the lipid fraction

The extract composition analysis in n-butanol, corresponding to three independent biological replicates for each treatment, carried out by HPLC and GC-MS is presented in Figures 1 and 2, respectively.



Data represents the average of three replications

**Figure 1.** Chromatograms obtained by HPLC, retention time (rt) and area of the separated peaks, when analyzing the samples of the non-induced inoculant (A) and the inoculant induced with daidzein (B).



Data represents the average of three replications

**Figure 2.** Area of peaks corresponding to the chromatograms obtained by GC-MS in the inoculant not induced and induced with daidzein and representing fatty acids and other molecules.

Differences are observed between the induced and non-induced treatments in the peaks obtained and the area (concentration), which was higher when the biopreparation was induced with daidzein (Figure 1 A and B), whose total area was 372 567 175, with respect to non-induced treatment, which was 5 322 255.

When the samples were analyzed by gas chromatography coupled to a mass spectrometer (Figure 2), several peaks were identified, corresponding to high molecular weight fatty acids such as ecosenoic and stearic acid and other molecules synthesized by the microorganism.

The results show an increase in the area of these peaks with the induced treatment. This means that daidzein promotes not only the production of nodulation factors in bacteria, but also other components of a lipid nature, such as fatty acids and other compounds.

In previous works, other authors also found an increase in these high molecular weight fatty acids in the Nod factors produced by symbiotic strains of beans and chickpeas, in the presence of *nod* gene inducers (15,19). The biological role of nodulation factors is well documented. However, a possible function of the rest of the compounds detected in the nodulation process is unknown. In fact, high molecular weight fatty acids have been reported to exhibit antimicrobial activity and play a role in *Rhizobium* growth and stress adaptation (20). These fatty acid structures are also components of plant cell membranes and have been shown to accumulate in roots of soybean plants colonized by *Bradyrhizobium japonicum* (21). Some high molecular weight fatty acids are precursors of the synthesis of jasmonic acid, which is essential in the plant's responses to biotic and abiotic stress (22).

Through the methods used to characterize lipid compounds and detect the presence of Nod factors in inoculant samples, these structures were identified to a greater extent in the daidzein-induced inoculant.

### Effect on nodulation under controlled conditions

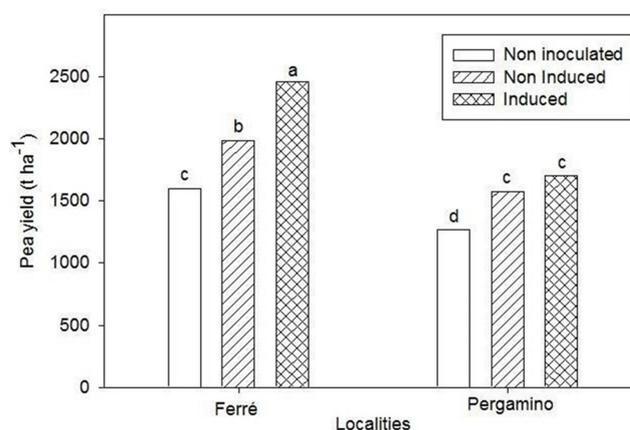
The non-inoculated plants did not form nodules, but regarding the effect of the induced and non-induced treatments on nodulation, a superior effect of the treatment inoculated with the induced inoculant was observed on the number of nodules formed in the main and total roots and also in its mass (Table 1).

These results indicate that the presence of a greater quantity and diversity of signals in the induced inoculant (Figures 1 and 2), stimulated the formation of nodules in the plants. It has been suggested that the perception of signals, such as Nod factors, activate the biosynthetic pathways required for nodulation (23) and that a greater number of these structures means greater biological fixation, as well as

a greater nodular mass could indicate more nodular tissue, more bacteroides and more fixation; therefore, greater supply of N to the plant (24).

### Effect of inoculants on pea yield, under field conditions

The analysis in plots showed interaction between the factors: inoculants (control, not induced and induced) and localities (Ferré and Pergamino). Higher yields were obtained in the biofertilized treatments (Figure 3), which demonstrates the role of these bacteria in the nitrogen nutrition of the culture. Similar results were obtained by other authors (25).



Means with similar letters indicate non-significant differences Duncan  $p < 0.05$ . ES X = 54.67

**Figure 3.** Effect of non-inoculated, induced and non-induced treatments on pea yield in two locations.

Yields were higher in Ferré, with around 1000 t ha<sup>-1</sup> more, compared to Pergamino. Taking into account that the same variety, treatments and cultural attentions were used in both experiments and that the characteristics of the soil and the climate were not significantly different, the difference in yield obtained for both locations could be related to the previous crop and to the type of seeding used. The positive effect of legumes on crop rotation is known (26,27), due to residual rhizobia populations with positive attributes in promoting plant growth and their ability to fix atmospheric nitrogen (28), by facilitating the circulation of soil nutrients and water retention (29) and by reducing nutrient runoff and greenhouse gas emissions (30).

**Table 1.** Number of nodules and nodule dry mass (mg) in non-inoculated control plants (Control) and plants inoculated with the induced or non-induced biopreparation, 35 days after sowing.

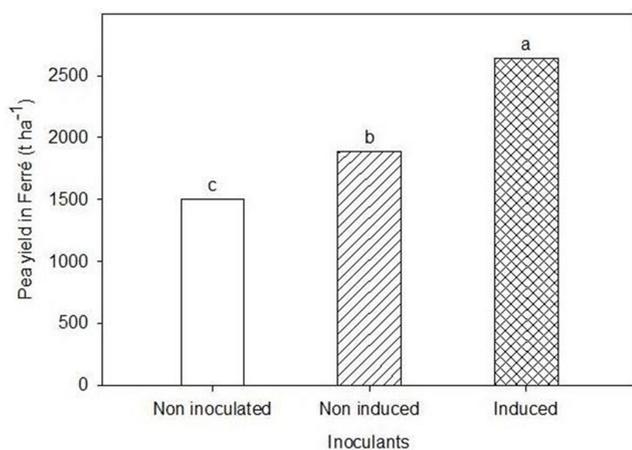
Treatment	Nodules number Principal Root pl <sup>-1</sup>	Nodules dry mass Principal Root pl <sup>-1</sup>	Number of nodules Total pl <sup>-1</sup>	Total nodule dry mass Total pl <sup>-1</sup>
Induced	3.56 a	7.67 a	14.89 a	19.56 a
Not Induced	0.36 b	0.73 b	8.79 b	13.21 b
Control	0.0 b	0.0 b	0.0 c	0.0 c
SE x	0.35	0.89	1.24	1.02

The data represents the average of three repetitions  
Similar letters indicate that there are no differences between the treatments, according to Tukey  $p < 0.05$ ; n = 9

Regarding advantages of direct sowing over conventional soil preparation methods, there are increases in the number of established seedlings (31), water savings (32) and increases in crop yields and quality (33).

When inoculated treatments were compared, a superior yield of the legume was observed in both places when the induced inoculant was used, with 468 and 125 kg ha<sup>-1</sup> of seeds, above the inoculated treatment without inducing, for Ferré and Pergamino, respectively. However, significant differences between these inoculated treatments were only observed in Ferré.

In the extensive trial, similar results were found to the experiments in plots, with higher yields in the treatment inoculated with the induced bacteria (2637 kg ha<sup>-1</sup>), followed by the non-induced treatment, but inoculated (1891 kg ha<sup>-1</sup>). The control showed the lowest yield value (1501 kg ha<sup>-1</sup>) (Figure 4).



Means with similar letters indicate non-significant differences, according to Duncan  $p < 0.05$ . ES X = 22.16

**Figure 4.** Effect of non-inoculated (control), induced and non-induced treatments on pea yield, under extensive field conditions in Ferré.

The positive effect found in the nodulation variables (Table 1) translated into higher crop yields under different conditions. Similar results were obtained by other authors for the *Bradyrhizobium elkani*-soybean, *Rhizobium leguminosarum*-bean and *Mesorhizobium ciceri*-chickpea interaction, when the inocula were also induced. The analysis of the chromatographic profiles obtained in these investigations also showed positive differences in the number of peaks and their area; results that corresponded with the number of nodules and later with the yield of these cultures (15,19,34).

The importance of Nod factors in the interaction with legumes is not only related to nodulation and the efficiency in the biological fixation of N under normal conditions and abiotic stress. The similarity of its structure with the chitooligosaccharides derived from the cell wall of fungi and defense activators (35), as well as direct evidence of its role in reducing diseases (36) and other indirect evidence in the

activation of defense enzymes (37), represent a participation of these molecules in certain immune responses to invasion by pathogens (38).

## CONCLUSIONS

- The induction of nodulation genes in *Rhizobium leguminosarum* bv. *viciae*, as part of the improvement in the inoculants and their dialogue with the plants, causes the synthesis and excretion of different compounds by the bacteria, which has an impact on greater nodulation and yield in the pea crop.
- The development of inoculants and more complex formulations such as those discussed here, allow to improve the symbiosis and guarantee higher levels of N fixation.

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