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SIGNALS PRODUCED BY *Rhizobium leguminosarum* IN THE INTERACTION WITH COMMON BEAN (*Phaseolus vulgaris* L.)

Señales producidas por *Rhizobium leguminosarum* en la interacción con frijol común (*Phaseolus vulgaris* L.)

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ABSTRACT. Legume-*Rhizobium* interaction depends on a complex signal exchange that continues throughout the entire symbiotic process, out of which just the correct combination will give rise to an efficient symbiosis. These plants secrete flavonoids that are recognized by compatible bacteria inducing their *nod* genes, which encode proteins that synthesize and export lipochitooligosaccharides called Nod factors. These factors activate infection process and initiate cell division in the root until making up the nodule and also participate in nitrogen biological fixation. There is evidence that the use of *nod* gene inducers increases some legume nodulation. The objective of this work was to study the production of some signal molecules induced by genistein isoflavon in a *R. leguminosarum* strain as well as to evaluate induction impact on inoculum effect upon common bean plants. Inoculum lipid fraction was isolated with n-butanol and analyzed by thin layer chromatography, high performance liquid chromatography and gas chromatography coupled to a mass spectrometer. Regarding the inocula induced with genistein, a higher amount of lipooligosaccharides (Nod factors) and high-molecular-weight fatty acids were detected, showing significant differences with non-induced controls. Concerning such signal molecule enrichment, genistein-induced inocula had a positive effect on "Cubacueto 25-9" bean plants, with a higher amount of nodules and chlorophyll content than non-inoculated plants (control).

Key words: Nod factors, nodulation, symbiosis, legumes

RESUMEN. La interacción *Rhizobium*-leguminosas depende de un complejo intercambio de señales, que se mantiene durante todo el proceso simbiótico y de las que solamente una combinación correcta permitirá una simbiosis eficiente. Estas plantas secretan flavonoides, reconocidos por bacterias compatibles y que inducen sus genes *nod*. Estos codifican las proteínas que sintetizan y exportan lipoquitooligosacáridos conocidos como factores Nod. Los factores Nod activan los procesos de infección e inician la división celular en la raíz, hasta la formación del nódulo y participan también en la fijación biológica del nitrógeno. Existen evidencias de que el uso de inductores de los genes *nod* incrementa la nodulación en algunas leguminosas. El objetivo de este trabajo fue estudiar la producción de algunas moléculas señales, inducidas por la isoflavona genisteína en una cepa de *R. leguminosarum* y evaluar el impacto de esa inducción en el efecto del inóculo sobre plantas de frijol común. La fracción lipídica en los inóculos fue extraída con n-butanol y analizada por cromatografía de capa fina, cromatografía líquida de alta resolución y cromatografía gaseosa acoplada a un espectrómetro de masas. En los inóculos inducidos con genisteína se detectó una cantidad superior de lipooligosacáridos (factores de nodulación) y de ácidos grasos de alto peso molecular, con diferencias significativas respecto a los controles sin inducir. En relación con ese enriquecimiento en moléculas señales, los inóculos inducidos con genisteína, mostraron un efecto positivo en las plantas de frijol de la variedad Cubacueto 25-9, con mayor número de nódulos y contenido de clorofila que las plantas no inoculadas (control).

Palabras clave: factores Nod, nodulación, simbiosis, leguminosa

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INTRODUCTION

Among the leguminous group having edible seeds, common bean (*Phaseolus vulgaris* L.) corresponds to one of the most important plants. This crop was domesticated in Mesoamerica and the Andean region several thousand years ago (1). At present, this crop is distributed across five continents and it represents the most significant legume in human diet, providing proteins and carbohydrates to more than 300 million people, especially in Latin America, Caribbean and Africa[A]. As a legume, *P. vulgaris* may be associated to soil nitrogen-fixing bacteria, which allows them to grow under low N conditions (2, 3).

In recent years, various investigations have enabled to know and understand communication at the molecular level governing rhizobia-legume symbiosis (4, 5). Nod factors or lipochitooligosaccharides have been the best molecules studied between those signals, with an essential role on nodule formation and its operation (6, 7). Other authors have shown that these molecules may play a role in germination, photosynthesis, growth and yield of several crops, as well as under biotic and abiotic stress conditions (8, 9).

Great efforts have been made to improve this relationship, since this symbiosis is largely responsible for providing the nitrogen required in agriculture all over the world. This contribution has been focused on optimizing bacterial physiologic state to produce determining symbiotic signals; thus, the aim of this work was to study the production of some signal molecules, induced by genistein isoflavonoid in a *R. leguminosarum* strain and to evaluate such induction impact on inoculum effect upon common bean plants.

MATERIALS AND METHODS

Bacterial crop. Lipid fraction extraction

R. leguminosarum CF1 strain was used, which is known for its symbiosis with bean and coming from the Institute of Soils, Ministry of Agriculture, Havana, Cuba. A pre-inoculum was obtained from it in 50 mL of Mannitol extract yeast medium (10) at pH 6,8 to inoculate 600 mL of this medium and other 600 mL by adding genistein (Sigma) at a final concentration of 5 µM (5 micromoles of genistein per liter of medium). Inocula were got after keeping bottles at 150 rpm for 48 hours on an orbital shaker at 28 ± 2 °C temperature.

For each case, the procedure was repeated three times; thus, reaching three samples of each treatment (non-induced and induced with genistein).

Considering that most signals recognized by its biological activity in *Rhizobium-legume* interaction are of a lipid nature, a selective extraction of these inoculum molecules was performed, treated with genistein (induced) or not (control) by using a high specificity solvent for such type of components. Therefore, 180 mL of n-butanol were used in each sample. They were stirred on an orbital shaker at 150 rpm for 15 minutes and rested overnight in the dark at a room temperature of 25 ± 2 °C. The organic phase was extracted in each sample, centrifuged at 12,000 g, 10 °C for 10 min. All samples were concentrated by rotoevaporation at 50-80 °C up to achieve 2 mL of each, which were used to detect bacterial signals.

Signal detection

Thin layer chromatography analysis (TLC)

Mixture components were separated by TLC using silica gel plates (Merck 60 GF-254), using n-propanol: water: concentrated ammonium 7:2:1, v:v:v as solvent. Spots were visualized through a developer on a basis of sulfuric acid in methanol.

High performance liquid chromatography analysis (HPLC-C18), reverse phase

In order to evaluate the presence of nodulation factors among metabolites, 10 µL of all samples were analyzed by HPLC using a Waters Symmetry C-18 (46 x 250 mm) reverse phase column of 5 µ particle size, installed in a HPLC Waters Alliance system. Flow speed was 1 mL min^{-1} and water (A) as well as acetonitrile (B) were employed 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. An UV-Waters spectrophotometric detector was used at a wavelength of 214 nm.

Gas chromatography-Mass spectrometry analysis (GC-MS)

Volatile fatty acid derivatives were prepared by silylation using BSTFA (N, O-Bis (trimethylsilyl) trifluoroacetamide) as reagent combined with trimethyl chlorosilane (TMCS) (BSTFA + TMCS Kit, Supelco). For GC-MS analysis, Shimadzu GC-MS QP-2010 gas chromatograph coupled to a mass spectrometer

[A] CGIAR. *Common bean* [en línea]. [Consultado: 15 de febrero de 2016], Disponible en: <http://www.cgiar.org/our-strategy/crop-factsheets/beans/>.

was used; an AOC-20i autoinjector-equipped system, AOC-20s autosampler and a direct insertion system, controlled by GC-MS solution software. A 5 MS optimal column (30 m × 0,25 mm ID, 0,25 µm film thickness) was employed. Chromatographic analysis conditions were injector temperature 310 °C; oven temperature 100 °C for six minutes. Subsequently, it rose to 320 °C at a rate of 20 °C min^{-1} and stayed for five minutes. Injection volume was 1 µL and column flow of 0,75 mL min^{-1} using helium as dragging gas.

Effect on nodulation and plant growth

"Cubacueto 25-9" bean variety was used. Seeds were inoculated with 0,5 mL of a 5,4 x 108 UFC mL^{-1} bacterial suspension and sown on a typical eutric Lixiviated Red Ferralitic soil (11). Plants were kept under growth chamber conditions with 12-hour-light photoperiod at 24 ± 2 °C and daily watered with tap water. After 35 days, total nodule number, aerial dry mass (subsequent drying at 65 °C for five days) and chlorophyll content were determined by SPAD. A non-inoculated control and an inoculated treatment with induced biopreparation were employed.

Data were processed by a single analysis of variance. Tukey mean comparison test for $\alpha<0,05$ was used, with the aim of discriminating differences between means. All figures were drawn in Microsoft Excel 2010 program.

RESULTS AND DISCUSSION

Signal detection

The separation profile by TLC on a silica gel support (normal phase) of each replicate of induced and control treatments is shown in Figure 1.

While this method is relatively not so specific to analyze and separate complex mixtures, it is a useful tool to quickly estimate sample complexity and identify differences in its composition (12), which becomes evident in the greater number of spots obtained for induced treatments compared to the control.

Figure 2a and b shows the characteristic chromatographic profile of control treatment mean and genistein-induced extract mean.

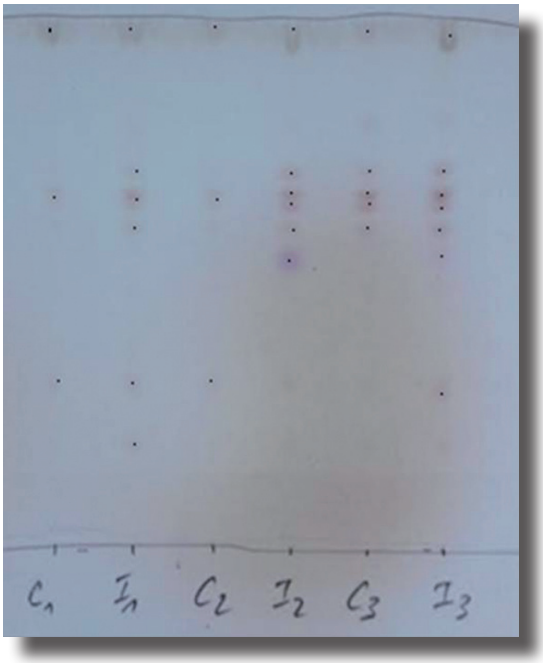


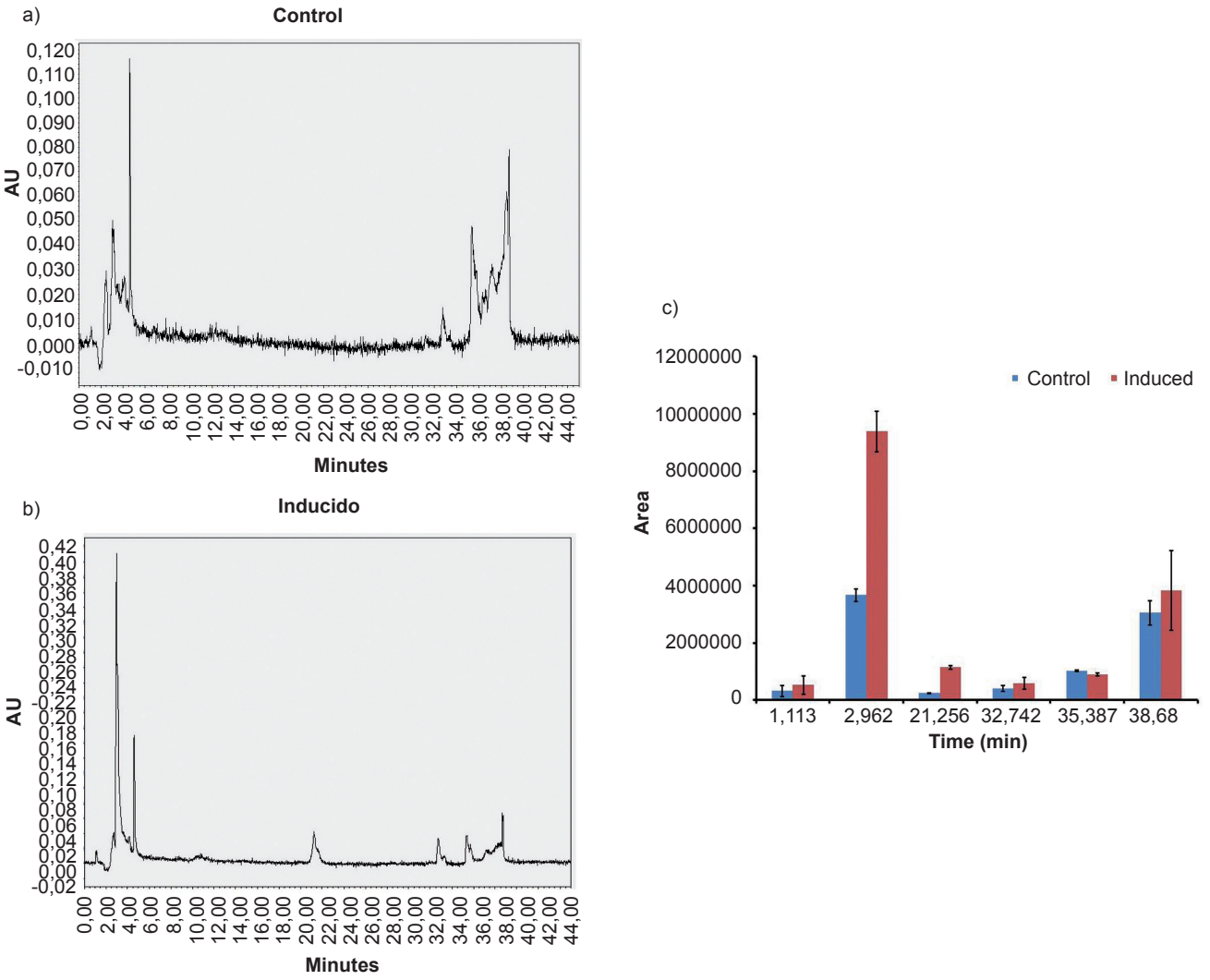
Figure 1. Spots resulting in thin layer chromatography when analyzing non-induced inoculant samples (C) and genistein-induced inoculant (I)

In addition, the average area is presented for each peak, considering each treatment mean (Figure 2c).

In peaks corresponding to 2 and 21 minutes, a difference in the area (concentration) is evident between non-induced and induced treatments, such compound concentration being higher when induced. Peaks obtained at 1, 32, 35 and 38 minutes showed no significant differences between treatments. The second peak area (t 2 min) corresponding to induced sample is quite higher than the other peaks obtained.

Taking into account that there are not available commercial standards of nodulation factors, it is difficult to say with certainty that these peaks correspond to such structures. However, this analysis is undeniably evident of the notable metabolite profile difference produced by bacteria in the presence of genistein.

When analyzing samples by gas chromatography coupled to a mass spectrometer (Figure 3), four different peaks corresponding to high-molecular-weight fatty acids (C16: palmitic acid, C18: oleic acid and C20: eicosenoic acid) were identified.



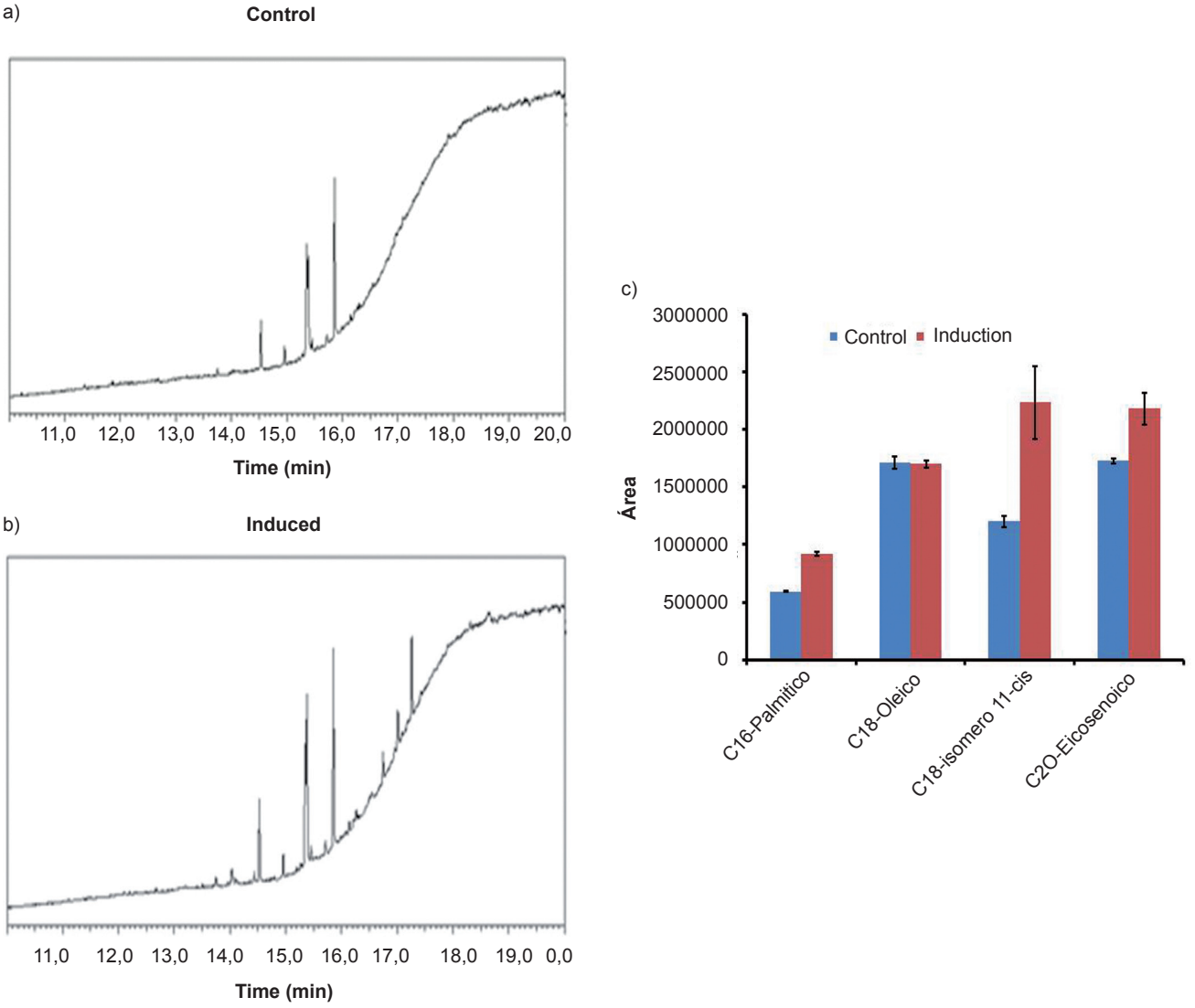
Data represent the mean of three replicates whereas interval bars indicate differences, according to Tukey for p≤0,05

Figure 2. Chromatograms obtained by HPLC (a, b) and separated peak area (c) when analyzing non-induced inoculant samples (control) and genistein-induced inoculant

Results show in every case, except for oleic acid, that there are not differences between both treatments, but an increase in the peak area with induced treatment (Figure 3c). This means that genistein promotes not only nodulation factor production in bacteria, but also other lipid nature components, such as fatty acids.

High-molecular-weight fatty acids are structural components of lipooligosaccharides associated to nodulation. Nod factors consist of a skeleton made up by 3-5 molecules of N-acetyl-glucosamine, which is acylated with a fatty acid of 16-20 carbon atoms long (C16-C20) in the amino group of non-reduced end (13). Other authors have also found an increase of these fatty acids in Nod factors produced by bean symbiont strains in the presence of *nod* gene inducers under abiotic stress conditions (14).

The biological function of nodulation factors is well documented; however, a possible role of fatty acids is unknown in the nodulation process. Nevertheless, high-molecular-weight fatty acids are described to exhibit antimicrobial activity at *in vitro* assays, so that a possible role could be defensive molecules in the presence of soil pathogens (15). These fatty acid structures are also cell membrane components of plants and its accumulation has been proved in roots of soybean plants colonized by *Bradyrhizobium japonicum* (16). Some high-molecular-weight fatty acids are synthetic precursors of jasmonic acid, which is essential in plant responses to biotic and abiotic stresses (17). It is interesting that by incubating *B. japonicum* with jasmonate or its methylated derivative induces *nod* gene expression and consequently it increases nodulation and nitrogen fixation (18).



Data represent the mean of three replicates whereas interval bars indicate differences according to Tukey p≤ 0,05

Figure 3. Chromatograms obtained by GC-MS (a, b) and separated peak area (c) corresponding to fatty acids when analyzing non-induced inoculant samples (control) and genistein-induced inoculant

At the induced sample chromatogram (Figure 3b), it is observed the presence of several peaks with retention times between 17 and 18 minutes, which do not appear in the control sample. The structure of these compounds is not reported in the database connected to GC-MS, so that its structural elucidation requires additional analysis. Thus, genistein is inducing the production of other molecules different to those already identified (nodulation factors and fatty acids) in the bacteria.

Through the three methods used to characterize lipid compounds and detect the presence of Nod factors in inoculant samples, a greater amount of these structures were identified in genistein-induced inoculant. This isoflavonoid induces *nod* gene transcription in several rhizobial species, resulting in nodulation factor production from bacteria, which in turn induce legume nodule formation (19).

Similar results were found in *Bradyrhizobium elkanii* ICA 8001 soybean symbiont strain, when induced in Nod factor synthesis and excretion. The chromatographic profile analysis obtained by HPLC and TLC showed positive differences in the number of peaks, their area and spot number, respectively, when compared to non-induced control (20). These results were directly correlated with nodulation response of soybean plants.

Effect on nodulation and plant growth

When analyzing the effect of both treatments on common bean plants (Table I), a greater effect was observed on the inoculated treatment with induced bioproduct upon the number of nodules formed over the control. Aerial dry mass showed no significant differences between both treatments; however, chlorophyll content showed the positive effect of inoculation.

It is important to highlight the fact that control plants also nodulated. This is because a non-sterilized soil was used, so it is quite possible that there were rhizobial populations related to this legume, which are able to establish an efficient symbiotic interaction. In addition to form plenty of nodules, they had a positive effect on dry mass of plants. However, the presence of increased number and diversity of signals at the induced inoculant (Figures 1, 2 and 3) conferred a higher quality to this treatment, which was notable for the number of nodules formed in plants and chlorophyll content. This indicator is an indirect measure of nitrogen fixation, performed in such root structures. It is stated that Nod factor perception activates biosynthetic pathways required for nodulation and the one involved in calcium flow, which are important for infection efficiency (21).

The highest nodulation values, together with increased chlorophyll and related to N content, indicate that induction had a positive effect on nitrogen fixation. Common bean produces and translocates N in the form of ureides, allantoin and allantoate (22, 23). Ureides are considered more vigorously favorable to transfer N from nodes to leaves (24). It has been shown that they only require half of ATP needed to produce amides in their synthesis and less carbon, which gives advantages to these ureidic legumes compared to amidic ones[B].

Other results show that by using induced inoculants in nodulation factor production with *B. elkanii* ICA 8001 strain, it indirectly acts on ureide catabolism of soybean plants (25).

The significance of Nod factors with legume interaction is not only related to nodulation and N biological fixation efficiency under normal and abiotic stress conditions. The similarity of its structure with chitooligosaccharides, derived from fungal cell wall and defense activators (26), as well as direct evidences on disease reduction (27) and indirect ones on defense enzyme activation (28) suppose these molecules are involved in certain immune responses to pathogen invasion.

Compared to other legumes, common bean is not a plant that can fix nitrogen more efficiently (29); however, there are some varieties of *P. vulgaris* and rhizobial strains related to them, which exhibit high fixing ranges (30). At present, new researches are trying to improve bean NBF, including the use of more competitive and efficient strains, the search for varieties (31, 32) and the development of inoculants and more complex formulations. This study has been chosen to induce inoculant synthesis and metabolite excretion of special interest and role in this interaction.

In future studies, it will be interesting to compare the effect of induced and non-induced inocula on bean plants. Nevertheless, other tests with bean and soybean crops confirm positive results by using induced inoculants on non-induced inocula and non-inoculated control (9, 33).

[B] Kabahuma, M. K. *Enhancing biological nitrogen fixation in common bean (Phaseolus vulgaris L)* [en línea]. Graduate Theses and Dissertations, Iowa State University, 2013, 81 p., [Consultado: 12 de enero de 2016], Disponible en: <http://lib.dr.iastate.edu/etd/13162>.

Total number of nodules, aerial dry mass and total chlorophyll content for control plants and those inoculated with the induced bioproduct 35 days after experimental seeding

Treatments	Total number of nodules plant^{-1}	Aerial dry mass plant^{-1} (mg)	Total chlorophyll plant^{-1}
Control	50,0 b	369,5 a	31,1 b
Induced	63,8 a	372,6 a	34,4 a
SE	4,4	37,5	0,92

*Common letters are not significantly different (Tukey, α=0,05) n= 5

CONCLUSIONS

- The use of an exogenous inducer such as genistein to prepare *R. leguminosarum* inoculants enhances a higher signal production by bacteria.
- The use of this induced inoculant on common bean plants improves nodulation and nitrogen supply to the plant.

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