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INDUCTION OF SIGNALS IN *Mesorhizobium cicerii* - *Cicer arietinum* L. INTERACTION

Inducción de señales en la interacción *Mesorhizobium cicerii* - *Cicer arietinum* L.

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ABSTRACT. Chickpea is a grain legume of exceptionally high nutritive value and most versatile food used. It is mostly grown under rainfed conditions in arid and semi-arid areas around the world. Chickpea is the third most cultivated grain legume in the world. Like most legumes, it is associated in symbiosis with bacteria from the rhizobia family, which guarantee much of the nitrogen it needs for its growth and development. In early stages of the symbiosis, a complex molecular dialogue takes place, involving Nod factors synthesized by the bacterium and flavonoids released by legume roots, so that both partners can recognize each other and initiate nodulation. This work aimed to evaluate the production of the induced signals in *Mesorhizobium cicerii* strain and the impact of that induction on chickpea nodulation and yield. The lipid fraction in the inocula was extracted with n-butanol and analyzed by high performance liquid chromatography and gas chromatography coupled to a mass spectrometer. A higher amount of *lipochitooligosaccharides* (nodulation factors) and high molecular weight fatty acids were detected in daidzein-induced inocula. Similarly, the inoculums induced with this flavonoid showed a positive effect on nodulation and yield of chickpea in semicontrolled and field conditions, respectively.

Key words: communication, Nod factors, legumes, symbiosis

RESUMEN. El garbanzo es una leguminosa de grano con un valor nutritivo excepcionalmente alto que permite producir gran variedad de alimentos. Se cultiva principalmente en condiciones de secano en las zonas áridas y semiáridas de todo el mundo, donde llega a considerarse la tercera leguminosa de grano más cultivada. Como la mayor parte de las leguminosas, se asocia en simbiosis con bacterias de la familia de los rizobios, que le garantizan parte del nitrógeno que necesita para su crecimiento y desarrollo. En las primeras etapas de la simbiosis, se genera un complejo diálogo molecular que involucra a los factores Nod sintetizados por la bacteria y los flavonoides liberados por la semilla, de modo que ambos simbiontes pueden reconocerse mutuamente e iniciar todo el proceso de la nodulación. Este trabajo tuvo como objetivo evaluar la producción de moléculas señales inducidas en la cepa *Mesorhizobium cicerii* y su impacto sobre la nodulación y el rendimiento del garbanzo. 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. En los inóculos inducidos con daidzeína se detectó una cantidad superior de *lipoquitooligosacáridos* (factores de nodulación) y de ácidos grasos de alto peso molecular. De manera similar, los inóculos inducidos con este flavonoide mostraron un efecto positivo en la nodulación y el rendimiento de garbanzo en condiciones semicontroladas y de campo, respectivamente.

Palabras clave: comunicación, factores Nod, legumbres, simbiosis

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a legume of great importance in the human diet, especially in the Middle East, the Mediterranean and India, where it is the second most widely cultivated legume. The global production of chickpeas has been increasing and data offered by the FAO show 13.7 Mt produced in 2014.

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India is one of the largest producers with approximately 70 % of world production (1).

This legume fixes atmospheric nitrogen (N) by symbiosis with effective strains of *Mesorhizobium*. It is proposed that it can reach up to 141 kg N year^{-1} (2), which improves soil fertility, as well as the productivity of subsequent crops, in which it reduces dependence on N (3).

The inoculation of seeds with appropriate rhizobia at the time of sowing is an agronomic practice recommended in legume production technology. Successful inoculation depends on several factors, including the ability of the strain introduced to compete with the existing native population and achieve greater infection, nodulation and N intake (4).

In the early stages of the symbiosis, a molecular exchange occurs that allows the communication of both symbionts. (5). The flavonoids released by the seed, including daidzein, induce the expression of the *nod* genes in the bacteria, which synthesize the Nod factors, essential molecules in the success of the interaction (6). These compounds have also been related to the chemoattraction exerted by legumes on rhizobia and the expression of other genes in said bacteria that regulate the synthesis of exopolysaccharides and the type III secretion system, related to defensive responses in the host and with the export of proteins in the nodulation, respectively (7). Other compounds derived from the microorganism determine the symbiosis: lipopolysaccharides (LPS), capsular polysaccharides (CPS), exopolysaccharides (EPS) and cyclic glucans, as well as secretion proteins (8).

The use of inoculants induced in the synthesis of these signals that govern communication, has allowed obtaining not only a better nodulation and yield of the plants, but a tolerance to stressful conditions in the environment (9).

The agronomic and environmental importance of the rhizobia-legume symbiosis has demanded the improvement of the technologies of production and application of inoculants, where it is taken into account from the selection of strains to the use of protectors and conditions that improve the process more and more of infection and formation of active nodules, which then translate into greater fixation of N (10).

In this sense, the present work tried to modify the composition of chemical signals in an inoculant based on *M. cicerii* and to evaluate the effect of these biopreparations on nodulation and chickpea yield.

MATERIALS AND METHODS

BACTERIAL CULTURE. INDUCTION WITH DAIDZEIN

A strain of *M. cicerii* was used, from the strain of the laboratory of Rhizobacteria, INCA, known for its symbiosis with the chickpea (11). From there, a pre-inoculum was produced in 50 mL of Mannitol Yeast Extract medium, at pH 6.8; with which 600 mL of the same medium and another 600 mL of this medium were inoculated, to which daidzein (Sigma) was added at a final concentration of 5 μM (micromolar). The inocula were obtained after keeping the bottles at 150 rpm for 56 h in an orbital shaker, at 28 ± 2 ºC of temperature.

For each case, the procedure was repeated three times, which included three samples of each treatment (without inducing and induced with daidzein).

EXTRACTION OF LIPID FRACTION

Taking into account that most of the signals recognized by their biological activity in the Rhizobium-legume interaction are of a lipid nature, a selective extraction of these molecules of the inoculum treated with daidzein (Induced) or not (Control) was performed, using a high specificity solvent for this type of components. For this, 180 mL of n-butanol was used in each sample. They were shaken in an orbital shaker at 150 rpm for 15 min and kept overnight at rest and darkness at room temperature of 25 ± 2 °C. The organic phase was extracted in each sample, centrifuged at 12000 g, 10 °C, and 10 minutes. All the samples were concentrated by rotoevaporation at 50-80 ºC until 2 mL of each were obtained; these were used for the detection of signals produced by the bacteria.

SIGNAL DETECTION STRUCTURAL CHARACTERIZATION OF MAJOR COMPONENTS IN THE LIPID FRACTION

Analysis by high performance liquid chromatography, reverse phase (HPLC-C18, High Performance Liquid Chromatography)

In order to evaluate the presence of nodulation factors between the metabolites, 10 μL of all lipid extracts were analyzed by HPLC, using a Waters Symmetry C-18 reverse phase column (46 x 250 mm) of 5 μm in size of particles, installed in a Waters Alliance HPLC system. The flow velocity was 1 mL min^{-1} 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.

Analysis by gas chromatography coupled to a mass spectrometer (GC-MS, from the English Gas Chromatography - Mass Spectrometry)

The volatile derivatives of the fatty acids were prepared by silylation, using BSTFA (N, O-Bis (trimethylsilyl) trifluoroacetamide) in combination with trimethylchlorosilane (TMCS) (Kit BSTFA + TMCS, Supelco) as reagent. For GC-MS analysis, a gas chromatograph coupled to a mass spectrometer brand Shimadzu GC-MS QP-2010 was used; system equipped with AOC-20i autoinjector, AOC-20s autosampler and a direct insertion system controlled by the 'GC-MS solution' software. An Optima 5 MS column (30 m × 0.25 mm ID, 0.25 µm film thickness) was used. The conditions of the chromatographic analyzes 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^{-1} and maintained for 5 minutes. The injection volume was 1 µL and the flow in the 0.75 mL min^{-1} column, using helium as the entraining gas.

Effect on the nodulation of chickpea plants, under controlled conditions

From the biopreparations obtained, 4 mL per 1000 g of seeds were inoculated. The plants were grown in a growth chamber with a photoperiod of 16/8 hours light / dark at 28 °C and 60 % relative humidity for 35 days. Irrigation was performed by capillarity with deionized water, maintaining the water regime in approximately 90% of the field capacity. Nine plants per treatment were used to evaluate number and dry mass of nodules (mg) per plant.

Effect on the performance of plants, under field conditions

The trial was conducted in the town of Ferré, Argentina, during the 2015 campaign, using the Chañarito variety of chickpea. The seeds were inoculated at a rate of 4 mL per 1000 g of seeds and the direct seeding system was used. At the time of harvest the yield was determined (kg ha^{-1}).

Design and statistical processing

The experiment in the growth chamber was repeated three times, the data of the number of total nodules were transformed according to $\sqrt{x}$. A completely randomized design was used and the data were subjected to the test of normality and homogeneity of variance. The field trial followed a randomized block design, with a planting density of 25 seeds m^{-1}. In both cases the data were analyzed according to Tukey for $p \leq 0.05$.

RESULTS AND DISCUSSION

Signal detection Structural characterization of major components in the lipid fraction

The analysis of the composition of the extracts in n-butanol, corresponding to three independent biological replicas for each treatment, performed by HPLC and GC-MS is shown in Figures 1 and 2, respectively.

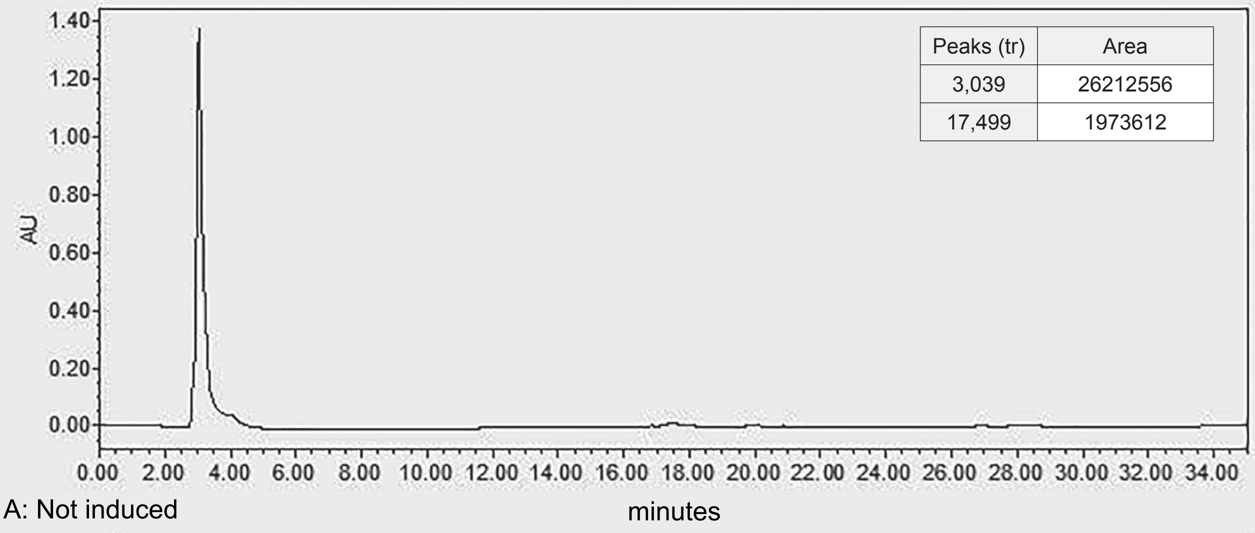
The chromatographic profile characteristic of the average of the control treatments and the average of the extracts induced with daidzein, as well as the average area for each peak is represented in Figure 1 (A and B).

There is a difference between the treatments without inducing and induced in the number and area (concentration) of the peaks, being higher when the biopreparation is induced with daidzein.

Taking into account that commercial standards of nodulation factors are not available, it is difficult to affirm with certainty that these peaks correspond to said structures. However, this analysis constitutes unquestionable evidence about the marked difference in the profile of metabolites produced by the bacteria in the presence of daidzein.

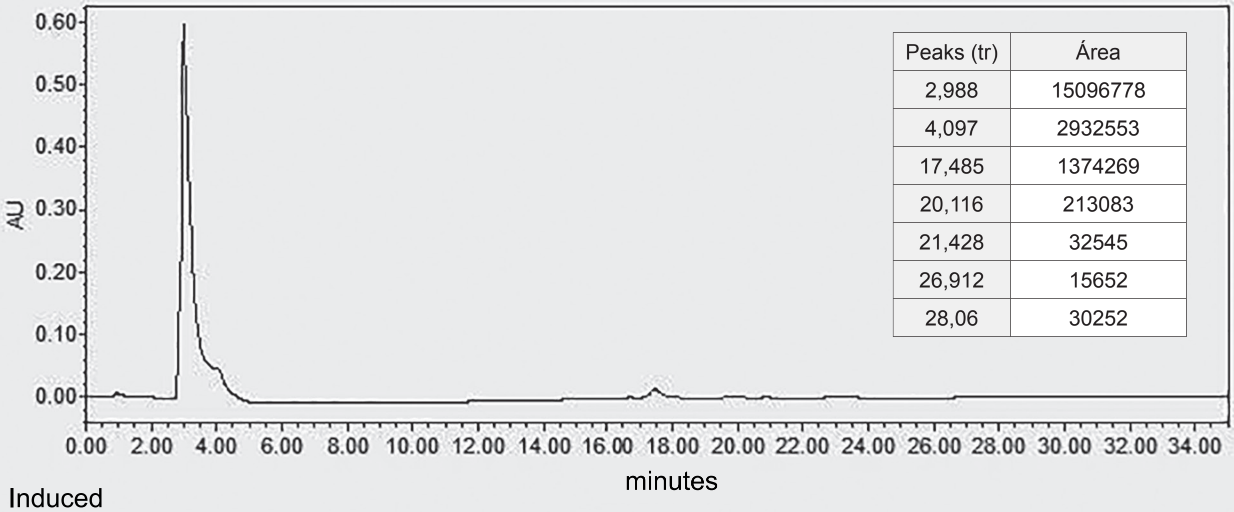
When analyzing the samples by gas chromatography coupled to a mass spectrometer (Figure 2), four different peaks corresponding to high molecular weight fatty acids (palmitic acid, eicosenoic acid, Cis-eicosenoic acid and oleic acid) were identified. Oleic acid was only present in the induced sample. The results show in all cases an increase in the area of these peaks with the induced treatment (Figure 2C). This means that daidzein promotes in the bacterium not only the production of nodulation factors, but also other components of lipid nature such as fatty acids.

The high molecular weight fatty acids are structural components of the lipo-quito oligosaccharides associated with nodulation. The Nod factors consist of a skeleton of 3-5 molecules of N-acetyl-glucosamine, which in the amino group of the non-reduced end is acylated with a fatty acid of 16-20 C atoms long (C16-C20) (12). Other authors have also found an increase of these fatty acids in Nod factors produced by symbiotic bean strains in the presence of *nod* gene inducers and in conditions of abiotic stress (13).



Peaks (tr)	Area
3,039	26212556
17,499	1973612

A: Not induced

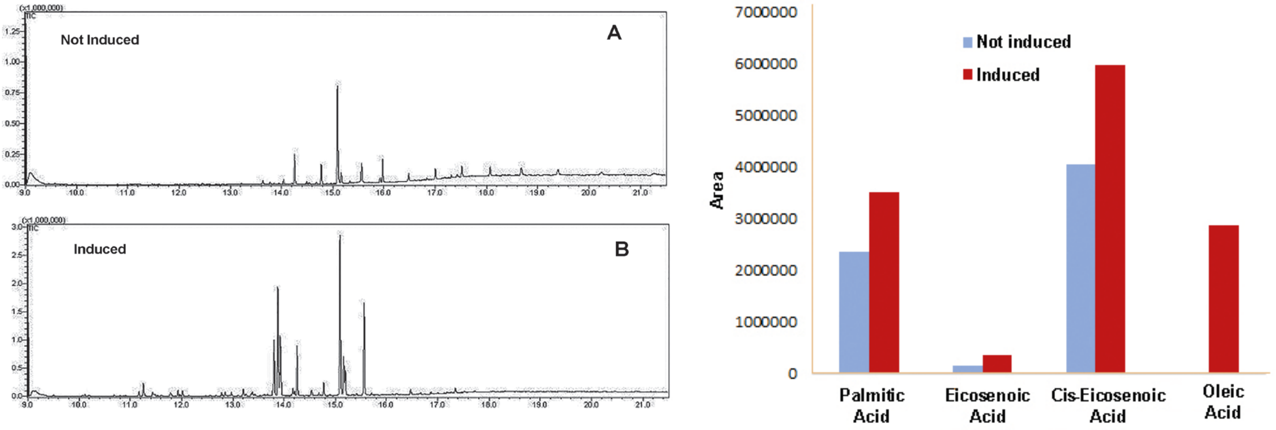


Peaks (tr)	Área
2,988	15096778
4,097	2932553
17,485	1374269
20,116	213083
21,428	32545
26,912	15652
28,06	30252

B: Induced

The data represents the average of three replicas

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



The data represents the average of three replicas

Figure 2. Chromatograms obtained by GC-MS (A and B) and area of the separated peaks (C), corresponding to the fatty acids, by analyzing the samples of the uninduced inoculant (Not induced) and the inoculant induced with daidzein (Induced)

The biological function of the nodulation factors is well documented. However, a possible role of fatty acids in the nodulation process is unknown. However, it has been described that high molecular weight fatty acids exhibit antimicrobial activity in *in vitro* tests, so they could act as defensive molecules, in the presence of pathogens in the soil (14). These structures of fatty acids are also components of the cell membranes of plants and their accumulation in the roots of soybean plants colonized by *Bradyrhizobium japonicum* has been demonstrated (15).

Some fatty acids of high molecular weight are precursors of the synthesis of jasmonic acid, which is fundamental in the responses of the plant to biotic and abiotic stresses (16). Interestingly, incubation of *B. japonicum* with jasmonate or its methylated derivative induces the expression of *nod* genes, and consequently increases nodulation and nitrogen fixation (17).

By means of the methods used to characterize the lipid compounds and detect the presence of Nod factors in the samples of inoculants, these structures were identified in greater quantity in the inoculant induced with daidzein. This isoflavonoid induces the transcription of the nod genes in several species of rhizobia, which results in the production of nodulation factors from the bacteria, which in turn induce the formation of nodules in the legume (18).

EFFECT ON NODULATION UNDER CONTROLLED CONDITIONS

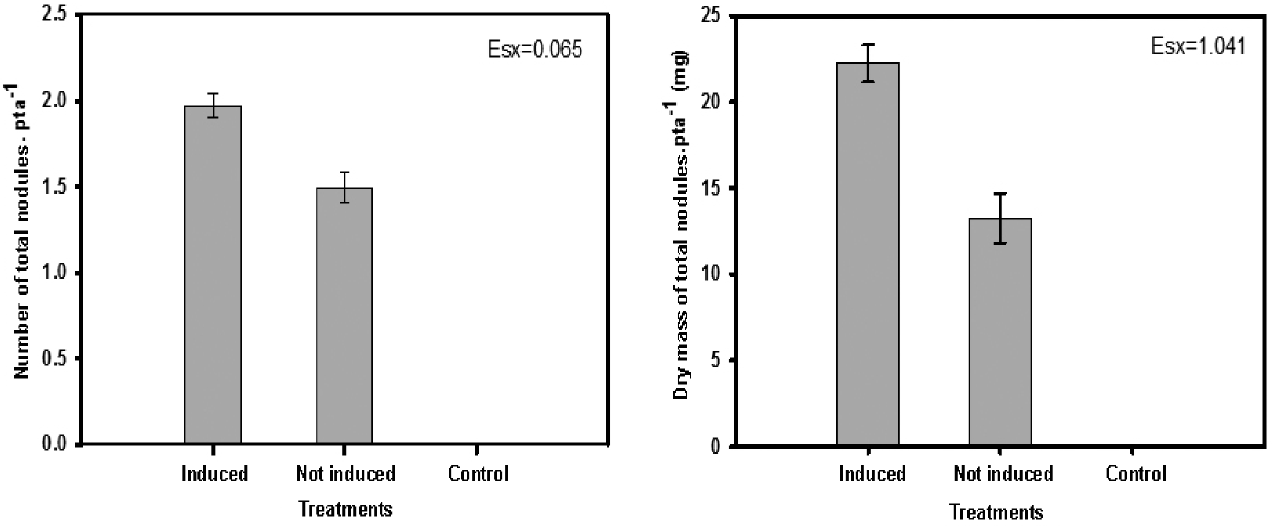
The non-inoculated plants did not form nodules (Figure 3), which indicates that in the soil used there were no populations of rhizobia or were not compatible with the chickpea.

When analyzing the effect of the induced and non-induced treatments on the nodulation, a superior effect of the treatment inoculated with the biopreparation induced on the number of formed nodules and their mass could be observed. These results suggest that the presence of greater quantity and diversity of signals in the induced inoculant (Figures 1 and 2) stimulated the formation of nodules on the plants. It is proposed that the perception of Nod factors activates the biosynthetic pathways required for nodulation (19).

EFFECT ON THE PERFORMANCE OF THE CHICKPEA, IN FIELD CONDITIONS

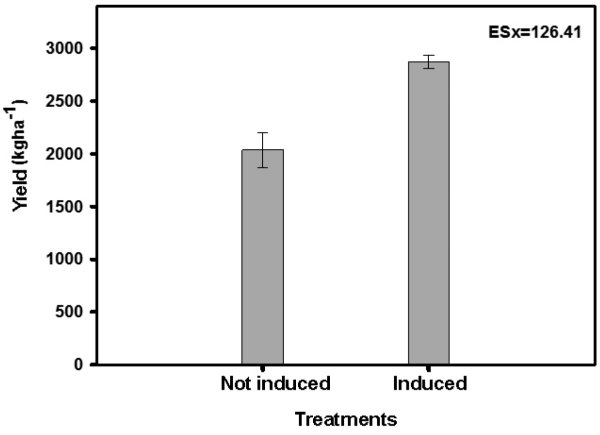
The results under field conditions showed a higher yield when the induced inoculant was used, with 833 kg ha^{-1} of chickpea seeds above the inoculated treatment without inducing.

The greater number of nodules and greater mass of these structures (Figure 4) suggests a greater number of bacteroides in their interior and therefore a greater activity in the Nitrogen fixation or that would result in higher crop yields.



The data represent the mean of three repetitions and the interval bars indicate differences according to Tukey for p≤ 0.05; n = 9

Figure 3. Number of total nodules (√x transformed data) and dry mass of total nodules (mg) in the control plants without inoculation (Control) and the plants inoculated with the biopreparation induced or not induced, 35 days after planting the experiment



The data represent the mean of three replicas and the interval bars indicate differences according to Tukey for p≤ 0.05

Figure 4. Effect of Non-induced and Induced Inoculant treatments on chickpea yield under field conditions

Results similar to those obtained in this investigation were found in symbiotic strains of soybeans and beans, when induced in the synthesis and excretion of Nod factors. The analysis of chromatographic profiles obtained showed positive differences in the number of peaks and their area; results that corresponded to the number of nodules and the yield of both cultures (20,21).

The importance of the Nod Factors in the interaction with the legumes is not only related to the nodulation and the efficiency in the biological fixation of the N in normal conditions and of abiotic stress. The similarity of its structure with the quitooligosaccharides, derivatives of the fungal cell wall and defense activators (22), as well as direct evidence on the reduction of diseases (23) and indirect evidence on the activation of defense enzymes (24), suppose a participation of these molecules in certain responses of immunity to invasion by pathogens.

Current production systems are based on the use of large quantities of inorganic fertilizers (25,26), which represents a threat to the environment. Achieving high yields with the use of fewer of these chemicals means a challenge for sustainable agriculture. The symbiotic fixation of N is a promising alternative in this regard (27). Attempts to improve fixation values in chickpeas as in other legumes include from the use of more competitive and efficient strains, the search of varieties to the development of inoculants and more complex formulations (28,29). In this study, we have chosen to induce in the inoculant the synthesis and excretion of metabolites of special interest and function in this interaction.

CONCLUSIONS

The use of an exogenous inductor such as daidzein in the preparation of inocula based on *M. cicceri*, generates a greater signal production by the bacteria. The use of this inoculant induced on chickpea plants, causes greater nodulation and crop yield.

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