

# CARACTERIZATION OF RIZOBIA ISOLATES FROM SOYBEAN NODULES (*Glycine max* (L.) Merrill) WITH POTENCIALITY TO PROMOTE THE PLANT GROWTH

## Caracterización de aislados de rizobios provenientes de nódulos de soya (*Glycine max* (L.) Merrill) con potencialidades en la promoción del crecimiento vegetal

Ionel Hernández Forte<sup>✉</sup>, María C. Nápoles García and Belkis Morales Mena

**Abstract.** The objective of this work was to characterize four rhizobia and isolated from soybean nodules about three positive attributes which characterize these promoting growth plant rizobacteria. The siderophores and indol acetic acid (AIA) production and the specific activity of 1-aminocyclopropane-1-carboxylate (ACC) desaminase enzyme, were determined. Siderophore was produced only by S11 isolated. All bacterial isolates and the strain produced IAA, but S11 produced the highest concentrations. All isolates, except S11, showed ACC deaminase enzyme activity when they were cultivated with the ACC precursor. The characterization allowed to know beneficial attributes, which represent advantages in the competitiveness of these bacterias in the crop rhizosphere, which together with their ability to perform the biological nitrogen fixation, could promote soybean plants growth.

Key words: siderophores, *Rhizobium*, rhizobacteria

**RESUMEN.** El objetivo del presente trabajo fue caracterizar cuatro aislados de rizobios y la cepa *Bradyrhizobium elkanii* ICA 8001 provenientes de nódulos de plantas de soya, en cuanto a su capacidad para exhibir tres atributos positivos que caracterizan estas rizobacterias promotoras del crecimiento vegetal. Se determinó su capacidad de producir sideróforos; AIA (ácido 3-indolacético) y la actividad específica de la enzima 1-aminociclopropano-1-ácido carboxílico (ACC) desaminasa. Solo el aislado S11 produjo sideróforos en el medio de cultivo. Aunque todos los aislados y la cepa produjeron AIA, S11 produjo las mayores concentraciones de esta fitohormona. Todos los aislados, excepto S11, mostraron actividad de la enzima ACC desaminasa cuando se cultivaron en presencia del precursor 1-aminociclopropano-1-ácido carboxílico. La caracterización realizada permitió conocer atributos beneficiosos que brindan ventajas en la competitividad de estas bacterias en la rizosfera del cultivo, los que en conjunto con su capacidad de realizar la Fijación Biológica del Nitrógeno (FBN), pudieran incrementar el crecimiento de plantas de soya.

Palabras clave: sideróforos, *Rhizobium*, rizobacterias

## INTRODUCTION

Plant growth promoting bacteria (PGPB), following its acronym in English, associate to several plant species present in most ecosystems (1). This bacterial group has the ability to increase nutrients availability, transform them into assimilable forms by the plant, produce growth promoting substances

or phytohormones, and serve as biological control of pathogens, then receiving the name of biocontrol-PGPB (2). Within the first of these bacterial groups, rhizobium are found, they are microorganisms symbiotically associated to leguminous plants hosting inside special structures called nodules. These organs are the ideal habitat where the Biological Nitrogen Fixation (BNF) is done (3).

The BNF, together with the production of siderophores, of 3-indolacetic acid (IAA) and the production of the, -aminocyclopropane-1-carboxylate (ACC) desaminase enzyme, are some of the direct mechanisms in plant growth promotion (4, 5).

Instituto Nacional de Ciencias Agrícolas (INCA), gaveta postal 1, San José de las Lajas. Mayabeque, CP 32700, Cuba.

✉ ionel@inca.edu.cu

Some rhizobia strains have the ability of producing siderophores, biomolecules that act as specific iron chelating agents, many times not available for living organisms and essential for vital functions as the DNA synthesis, respiration, photosynthesis, and BNF (6). Likewise, siderophores are used by rhizobium to reduce the iron concentrations available in the soil and so inhibit the dissemination of pathogens (7).

On the other hand, 3-indol acetic acid is an auxin that regulates plants growth since it activates different responses in the plant cell at the biochemical, physiological and morphological levels. Such an effect is seen in the cell division, elongation, and differentiation, in the formation of lateral roots and root hairs as well as in the apoptosis and morphogenesis (8).

Ethylene, a phytohormone produced by plants, inhibits the progress of the infection caused by rhizobium in the symbiotic process with leguminous plants. For comparative reasons, some rhizobia produce the enzyme ACC desaminase, which reduces ethylene concentration and its inhibiting effects on nodule formation (9).

Despite it is well backed up by the literature, the beneficial effect caused by these metabolites produced by PGPB in plant growth promotion, research dealing with the production of these compounds is still limited. Hence, the objective of this study has been characterizing four rhizobium isolates from soybean nodules and the strain *B. elkanii* ICA 8001, as to their capability of producing siderophores, thype AIA metabolites in the presence of the enzyme ACC desaminase as positive attributes that characterize these PGPB.

## MATERIALS AND METHODS

The study was conducted at the Biology Department of the University of Waterloo, Canada, and the Plant Physiology and Biochemistry Department from the National Research Institute on Agricultural Sciences (INCA), Cuba.

### MICROBIAL MATERIAL

Four rhizobia isolates S1, S10, S11, Snb and the strain *Bradyrhizobium elkanii* ICA 8001 (10) were used. All these microorganism come from soybean plants (*Glyxine max*) and now they are part of the isolate collection of the Bacteriology Lab attached to the Department of Plant Physiology and Biochemistry of INCA. According to previous studies where conventional methods of identifying rhizobia, multiplication speed and the production of the acid-base were applied, S11 belongs to the *Rhizobium* genus and the rest of the isolates to the *Bradyrhizobium* genus<sup>A</sup>.

<sup>A</sup> García, D. Aislamiento y selección de especies de rizobios asociados al cultivo de la soya (*Glyxine max* (L.) Merrill). [Tesis de Maestría]. Instituto Nacional de Ciencias Agrícolas. 2000. 100 pp.

Three trials were conducted in which the production of siderophores, metabolites of the IAA type and the activity of the enzyme ACC desaminase, were determined. Cultures of different bacterial isolates were prepared in Erlenmeyers of 25 mL volume containing 5 mL of liquid medium Yeast Extract Manitol (YEM) (11). Media were inoculated with a portion of rhizobium isolate previously preserved at 4 °C in essay pipes with the same solid culture medium. Liquid cultures were incubated at 30 °C, under agitating conditions at 150 rpm min<sup>-1</sup> Optic ivimen system-Comecta during 24 hours for the isolate S11 and 72 hours for the rest. In the specific case of determining the production of type IAA metabolites, culture media were supplemented with two concentrations of triptophane 200 and 500 µg mL<sup>-1</sup> at the time of preparation. As control, culture media without triptophane were used.

### DETERMINATION OF SIDEROPHORES PRODUCTION

Three aliquots of 10 µL from bacterial cultures were taken and placed on Petri dishes that contained the solid medium Chromo Azurol Sulphonate CAS Agar (12). Petri dishes were incubated at a WiseCube-Daihan-brand from three to five days at 28 °C. Finally, the production of siderophores was evaluated as orange halos around the microbial growth were observed (13). Aliquotes of 10 µL of sterile culture medium were placed in dishes with the medium CAS Agar, and were used as control for the trial.

### METABOLYTE PRODUCTION OF 3-INDOL ACETIC ACID (IAA) TYPE

Induced bacterial cultures were centrifuged at 8000 rpm min<sup>-1</sup> and at 4°C during 10 min Centrifuge 5804R, Eppendorf. One milliliter of each supernatant were added to 4 mL of the reagent Salkowski (14) and were incubated for 20 minutes at room temperature. The production of metabolites of the IAA type was detected through an spectrophotometer Genesys 6, Thermospectronic at 535 nm. The IAA concentration was determined through a calibration curve with a concentration range from 10 to 400 µg mL<sup>-1</sup> of IAA (SIGMA) and the results were expressed in µg mL<sup>-1</sup>.

### DETERMINATION OF THE ENZIMATIC ACTIVITY OF THE ACC DESAMINASE

Bacterial cultures were centrifuged at 8000 rpm min<sup>-1</sup> and 4 °C for 10 min Centrifuge 5804R, Eppendorf. In order to induce the synthesis of the ACC desaminase, cell sediments were washed twice with 5 mL of the minimum sterile saline medium M9 (15). Then they were resuspended with 7.5 mL of the same sterile medium supplemented with 0,05 Mol L<sup>-1</sup> of 1-aminociclopropane<sup>-1</sup>-carboxylic acid (ACC) (SIGMA), as the only nitrogen source. Isolates were then cultured at 150 rpm min<sup>-1</sup> Optic ivimen system-Comecta and

at an incubation temperature from 28 to 30 °C during 24 hours for the isolate S11 and 72 hours for the rest. Immediately after, the ACC activity of the enzyme was determined by quantifying the production of  $\alpha$ -cetobutirate generated from ACC (16).

Simultaneously, to calculate the specific activity of the ACC enzyme desaminase, the concentration of total proteins in the bacterial extract according to Bradford's method (17). Bovine serum albumin (SIGMA) was used as the standard of the calibration curve with a concentration range of 10-100  $\mu\text{g mL}^{-1}$ . The specific activity of the ACC enzyme desaminase was expressed in  $\mu\text{g}$  of  $\alpha$ -cetobutirate  $\mu\text{g}^{-1}$  protein  $\text{h}^{-1}$ .

### DESIGN AND STATISTICAL ANALYSIS

Data from trials where the production of metabolites of the IIA type and the activity of the enzyme ACC desaminase were evaluated, were subjected to the Bartlett test and the analysis of variance test of Kormogorov- Smirnov; the analysis of variance of simple classification was also used, using the Tuckey's Test for comparing mean values,  $p < 0.05$  and discriminate differences among mean values (18). Data were also graphically represented using the software SigmaPlot 2001. In each case, including the trial to determine siderophores, four replicates per treatment were made.

## RESULTS AND DISCUSSION

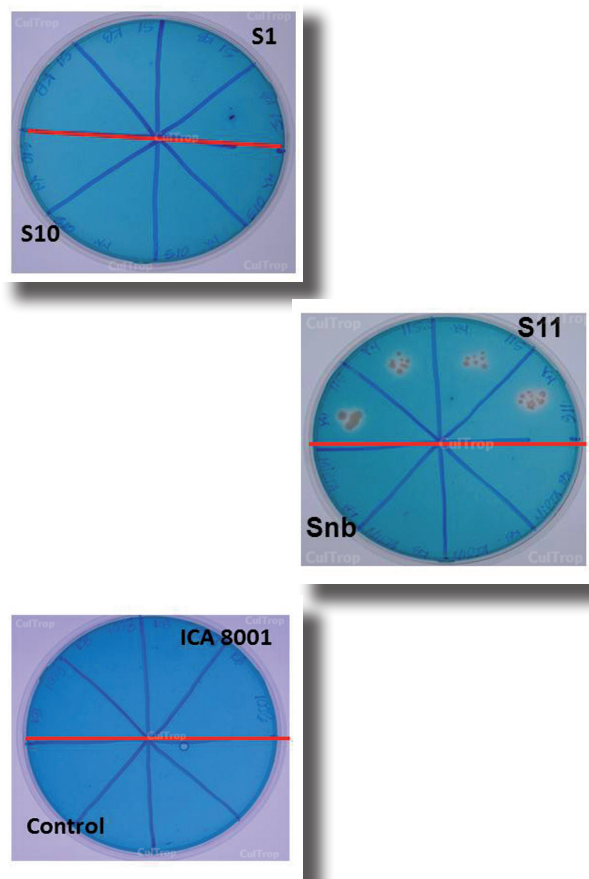
### SIDEROPHORE PRODUCTION BY RHIZOBIUM ISOLATES

Out of the four rhizobium isolates used in this study and the strain *B. elkanii* ICA 8001, only S11 had the ability of producing siderophores (Figure 1).

Dark orange halos were observed around the colonies produced by this isolate, as compared to the rest of studied microorganisms that were not multiplied in this medium. This culture medium, with a blue color at the time of preparing it, turned into orange in those areas where microorganisms grew up with the ability of producing siderophores (13).

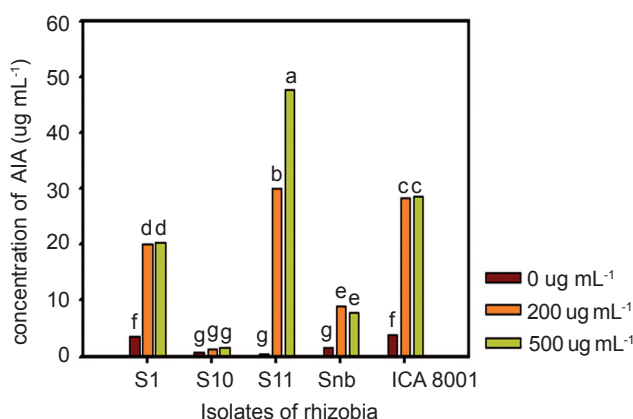
Similar results to this trial's were observed in studies with ten isolates of the Rhizobium genus from chick pea (*Cicer arietinum*), where in six of them orange halos with 1,0 – 2,2. mm of diameter were found (19).

The culture medium Chromo Azurol Sulphonate is usually used for the qualitative determination of siderophores, since it shows low iron concentrations (1 mM  $\text{FeCl}_2 \cdot 6\text{H}_2\text{O}$ ). IIA values recorded in media supplemented with triptophane, approximately ranged from 3  $\mu\text{g mL}^{-1}$  (for S10) and 47  $\mu\text{g mL}^{-1}$  for S11 (Figure 2). These concentrations were the essential condition to induce siderophore synthesis in those microorganisms with ability to produce them (20).



ICA 8001: isolate *Bradyrhizobium elkanii* ICA 8001, control: Aliquotes of 10  $\mu\text{L}$  of sterile culture medium

**Figure 1. Production of siderophores by different isolates on the medium Chromo Azurol Sulfonate Agar**



Common letters do not differ significantly according to (Duncan  $p < 0,05$ ).  $ES^{***} = 0,314$ ,  $n=4$

**Figure 2. Production of metabolites of the 3-indol acetic acid type ( $\mu\text{g mL}^{-1}$ ) by rhizobium isolates in the YEM medium without triptophane and supplemented with 200 and 500  $\mu\text{g mL}^{-1}$  of this amonoacid**

Under such conditions, Fe<sup>2+</sup> present inside the microbial cell, is dissociated from repressive proteins adhered to DNA to prevent the siderophore synthesis in view to the high iron concentrations. The PGPR producing siderophores use the complex Fe<sup>3+</sup>-siderophores to incorporate this metal to the inner cell mediated by a specific receptor located at the bacterial membrane. This kidnap of iron reduces its availability in the medium for other microorganisms that lack this system and that occasionally are pathogens to the plants (13).

It is known that rhizobium has the ability of producing different types of siderophores; however, some of them have been structurally characterized. Bacteria of the Rhizobium genus living freely on the rhizosphere, siderophores of the hydroxamate type have been found (21). Still to be determined are the mechanisms used by bacterioids inside the root nodule to incorporate iron, since approximately 44 % of the iron present in soybean plants is found in these structures (22).

The importance of uptaking iron in the FBN process that rhizobium does in the symbiosis with leguminous plants has been shown. Nitrogenase, essential metalloenzyme in this process representing 10<sup>-1</sup> 2 % of total proteins of rhizobium cells, catalyzes the reduction of molecular nitrogen to ammonium through redox reactions dependent on iron. Indirectly, this metal contributes to FBN because it is an important part of cytochromes, essential molecules in the cell respiration process, which the necessary energy for nitrogen fixing process is taken from (23). Trials made with Sinorhizobium meliloti showed evidences of the important role iron uptake plays for rhizobium in symbiosis with leguminous plants, where a reduction of nodule occupation by mutant isolates with deficient siderophore synthesis was observed as compared to wild isolates (24).

### PRODUCTION OF METABOLITES OF THE 3-INDOL ACETIC ACID TYPE BY RHIZOBIUM ISOLATES

All bacterial isolates and the strain *B. elkanii* ICA 8001 produced IAA though with differences en S1 S10 S11 Snb ICA 8001.

In rhizobium isolates and the strain *B. elkanii* ICA 8001, except S11, there was not a direct proportional relation between triptophane concentration and the concentration of IAA produced, because the increased triptophane in the culture medium did not mean an increased IAA concentration. From the biochemical point of view, there are several causes that could explain this phenomenon, mainly those related to enzymes involved in each of the metabolic pathways. In order to make this clear, it would be necessary certain isoenzymatic analysis, an even genetic analysis that allow identifying the actual cause of this behavior in the different studied microorganisms.

In all studied microorganisms, except S10 isolate, an induction in the synthesis of IAA is observed when they were cultured in triptophane supplemented media.

This forerunner is commonly used to induce IAA synthesis since it is known that the formation of this phytohormone comes from certain metabolic routes whose forerunner is this aminoacid. Four ways depending on the triptophane present in the rhizobacteria have been described: the indol-3-pyruvic acid (IPyA), the triptamine way (TAM), the indol-3-acetonitril (IAN) way and the indol-3-acetamide (IAM) way. A fifth way of synthesis of IAA irrespective of the triptophane has been mainly described in plants, where the indol-3-glycerol phosphate (27) is presumed the forerunner. The results of this study indicate that isolate S10, though in very low concentrations, had the ability of producing IAA in the absence of triptophane. Similar results were reached with rhizobium isolates native from amazonian soils in which increased triptophane concentrations in the culture medium did not increase IAA production by these bacterial isolates. (25).

Studies done with certain *Azospirillum* isolates conclude that the independent way triptophane turns into a greater IAA source occurs when bacteria are grown in media without forerunners (28). There are references from *Streptomyces* sp. and *Flavobacterium* sp. isolates where it has been confirmed that ammonium is the forerunner of the IAA synthesis since in the absence of this molecule, isolates are not able to synthesize this auxin. It is presumed to be due to the competence of this molecule and triptophane by the active sites of certain enzymes in the biosynthetic process of IAA (29). Such results could suggest the existence of independent ways of triptophane for IAA synthesis in S10 isolate. Moderate IAA levels recorded in S1 and Snb isolates and also in the isolate ICA 8001, grown without triptophane, could also support this hypothesis.

Such as has been stated, several studies have focused on the quantitative determination of the IAA produced by rhizobium isolates from the Rhizobium genus. However, in specimens of the *Bradyrhizobium* genus, most of the microorganisms studied in this research, the effect of this auxin has been mainly on the tolerance to certain types of abiotic stress and therefore on their survival increase in the rhizosphere (30).

The IAA also plays an important role in the process of FBN taking place among leguminos plants and rhizobia. Through this metabolic process, the nitrogenase enzyme present in these microorganisms catalyzes the reduction of nitrogen to molecular ammonium. Ammonium has been found as one of the forerunners of the IAA synthesis, but very little studies have been done to clear up the role of this molecule. It has been observed that Rhizobium isolates have not only the ability of producing IAA when they live freely on the soil, but also within nodules of soybean plants

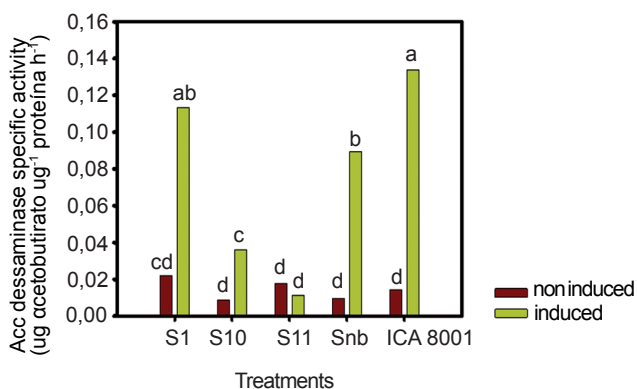
as bacterioids, once symbiosis is established (31). Rhizobium, within the root nodule could use part of the  $\text{NH}_4^+$ , due to the FBN process for the IAA synthesis.

Studies performed with hyperproducing mutant isolates of *B. japonicum* IIA in soybean, showed an increase of the volume and the number of effective root nodules in the FBN, which brings about a positive effect on yields (32). Similarly, in other trials with soybean plants, the number of nodules has been lower when isolates with the inability of producing IAA were inoculated AIA (33).

Phytohormones synthesis as IAA by soil microorganisms, that also have the ability of fixing atmospheric nitrogen is an additional benefit that permits promoting the growth of leguminous plants and then increase yield and harvest quality.

### ACTIVITY OF THE ENZYME 1-AMINOCICLOPROPANO-1-CARBOXILATE (ACC) DESAMINASE

When analyzing the enzymatic activity of the ACC desaminase of the different bacterial extracts, all isolates, except S11, showed activity of this enzyme when they were culture in the presence of an inductor. A similar behavior was seen when there was a significant increase in the activity of the enzyme ACC desaminase, of the induced media as compared to the control of these isolates (Figure 3).



Common letters do not differ significantly according to (Duncan,  $p < 0,05$ ) ES\*\*\* = 0,007,  $n = 4$

**Figure 3. Activity of the enzyme 1-aminociclopropano-1-carboxilato (ACC) desaminase in rhizobia isolates cultured in induced and non-induced media with  $0,05 \text{ mol L}^{-1}$  of ACC**

The activity of the enzyme ACC desaminase in the studied isolates ranged from 0,01 (S11) to 0,13 (ICA 8001)  $\alpha$ -acetobutirato  $\mu\text{g}^{-1}$  protein  $\text{h}^{-1}$ . The presence of the enzyme ACC desaminase in rhizobium has been widely described in the literature (9). However, the activity of this enzyme even varies among isolates of the same genus. It is due to the fact that nucleotids

forming gen *acdS* (that codifies for ACC desaminase) is very variable among bacterial genus, even among species of the same genus (34). It could explain the differences found in the values of the enzymatic activity in the isolates S1, S10, Snb and the strain ICA 8001, all of the *Bradyrhizobium* genus.

Phylogenetic studies suggest that gen *acdS* is not always found in the microbial chromosome, but it has also been detected with certain stability in bacterial plasmids. Moreover, it has been stated that these genomic packages can be transferred horizontally bringing about –to some degree– the loss of the ability to synthesize the enzyme ACC desaminase (35). It could be a possible explanation to the low levels of enzymatic activity reached in the S11 isolate, even in the medium supplemented with the inductor. In this regard, molecular biology techniques have been used to promote the production of ACC desaminase in rhizobium and create more competitive isolates in the nodulation process (36). This technology could be used in the preparation of more effective inoculants for leguminous plants that positively contribute to achieve higher agricultural yields taking into account the role of this enzyme in reducing negative effects of certain biotic and abiotic stresses (34, 37).

The enzyme ACC desaminase degrades the ACC, forerunner of ethylene, in  $\alpha$ -acetobutirato and ammonium, very common metabolites in plants and other organisms (38). It is known that plant growth promotion is encouraged not only by a reduction in the ethylene content, but also for the generation of ammonium from ACC carried out by rhizobacteria as rhizobia (35). Under this strategy, plants have an extra source of nitrogen and associated microorganisms have another alternative for surviving at the rhizosphere. Thus, PGPR with the ability to produce the enzyme ACC desaminase, increase its proliferation at colonization sites of roots and they are favored with the competence of other microorganisms. In the particular case of the symbiotic interaction rhizobium-leguminous, it has been observed that ethylene determines the specific site where the nodular primordium will be formed since mutants from non-sensitive leguminous to this phytohormone, show a higher number of nodular primordia than wild plants (39). In this way, rhizobium degrades the ACC exudated by the root reducing ethylene concentrations in the plant and with it reducing too its inhibiting effects on nodulation (9).

In order to explain the promoting effect on growth done by PGPR two types of mechanisms have been identified, indirect ones or biocontrol and direct ones that promote plant growth. The first one is characterized by the existence of an antagonistic activity between the microorganism of interest and a plant pathogen, causing reduced harmful effects of the latter on the plant (40). The competition for mineral

nutrients and space, detoxification of virulent factors of pathogens, the induction of systemic resistance to the plant and the synthesis of metabolites as antibiotics and siderophores, are some of these mechanisms (41). Direct growth-promoting mechanisms show up in the absence of other microorganisms and includes, in addition to FBN, the production of enzymes like the ACC desaminase, solubilization of minerals like phosphorus and the synthesis of phytohormones as gibberellins, cytokinins and the IAAIA (42). The above classification has only served to study each of them deeper, since it is known that the promoting activity of plant growth done by the PGPB as rhizobia, is the result of the simultaneous action of several of these mechanisms.

Both direct and indirect mechanisms can function helping each other and contributing to the execution of beneficial processes to the plant like FBN. Iron uptake by rhizobia through siderophores, favors the synthesis of nitrogenase as well as of other molecules involved in the generation of the necessary energy to perform fixation. The ammonium generated in this process, and that produced from ACC degradation by the ACC desaminase and triptophane present in root cells, could be used by microorganisms to synthesize IAA, that together with the IAA produced by the plant, encourage cell proliferation and elongation. The IAA, in turn, induces ACC synthase synthesis, which catalyzes the formation of ACC (43) and also stimulates the production of ACC desaminase (39), which some processes favorably interconnect.

When the ACC on the root is increased, there is degradation by the ACC desaminase produced by rhizobia, reducing the concentration of ethylene and favoring a higher quantity of sites for nodular proliferation. In this way, it could be thought on a complex molecular dialogue between rhizobia and the leguminous plant, from its approach to symbiosis and during it, assuring a higher effectiveness in the nitrogen fixing process and in promoting plant growth.

The characterization done in this research of four rhizobia isolates and the strain *B. elkanii* ICA 8001, taken from soybean nodules, allowed knowing that all of them produced IAA while S11 isolate was the only one with the ability of producing siderophores without the activity of the enzyme ACC desaminase. These positive attributes, particularly present in bacteria, have different sensitive effects on the plant and are advantages in the competition with other microorganisms that also colonize the rhizosphere. The contribution of hormones to plant growth, the possibility of assimilating insoluble forms of nutrients like iron and tolerate biotic and abiotic stresses, without mentioning those indirectly involved in so important processes like FBN, are some of the direct benefits of this contribution.

These results, together with other studies on characterization of these isolates and its interaction

with crops, are a contribution to the selection of isolates that could positively contribute to nutrition, protection, and development of crops of interest.

## CONCLUSIONS

The four isolates and the strain of *B. elkanii* ICA 8001, in addition to fixing nitrogen, show and least two of the evaluated characters: production of IAA, siderophores and activity of the enzyme ACC desaminase; features that make them attractive for their use as possible biofertilizers that help promoting growth and yield in soybean.

## BIBLIOGRAPHY

1. Martínez-Viveros, O.; Jorquera, M. A.; Crowley, D. E. *et al.*. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *J. Soil. Sci. Plant. Nutr.*, 2010, vol. 10, pp. 293-319. ISSN 1522-2624.
2. Bashan, Y. y Holguin, G. Proposal for the division of Plant Growth-Promoting Rhizobacteria into two classifications: biocontrol-PGPB (Plant Growth-Promoting Bacteria) and PGPB. *Soil Biol. Biochem.*, 1998, vol. 30, no. 8-9, pp. 1225-1228. ISSN 0038-0717.
3. Verma, J. P.; Yadav, J.; Tiwari, K. N. *et al.*. Impact of plant growth promoting rhizobacteria on crop production. *Int. J. Agric. Res.*, 2010, vol. 5, no. 11, pp. 954-983. ISSN 0976-058X.
4. Bashan, Y. y de-Bashan, L. E. How the plant growth-promoting bacterium *Azospirillum* promotes plant growth-a critical assessment. Chapter two. *Adv. Agron.*, 2010. vol. 108, pp. 77-136. ISSN 0065-2113.
5. Machado, R. G.; Saccol de Sá, E. L.; Bruxel, M.; Giongo, A.; da Silva Santos, N. y Schönhoffen, A. Indoleacetic acid producing rhizobia promote growth of Tanzania grass (*Panicum maximum*) and Pensacola grass (*Paspalum sauriae*). *Int. J. Agric. Biol.*, 2013, vol. 15, pp. 827-834. ISSN 15608530, 18149596.
6. Arif, K.; Archana, G. y Desai Anjana, J. Engineering heterologous iron siderophore complex utilization in rhizobia: Effect on growth of peanut and pigeon pea plants. *Applied Soil Ecology*, 2012, vol. 53, pp. 65-73. ISSN 0929-1393.
7. Maksimov, I. V.; Abizgil'dina, R. R. y Pusenkova, L. I. Plant growth promoting rhizobacteria as alternative to chemical crop protectors from pathogens (Review). *Appl Biochem Microbiol.*, 2011, vol. 47, pp. 333-345. ISSN 1608-3024.
8. Takahashi, K.; Hayashi, K. I. y Kinoshita, T. Auxin activates the plasma membrane H<sup>+</sup>-ATPase by phosphorylation during hypocotyl elongation in *Arabidopsis*. *Plant physiology*, 2012, vol. 159, no. 2, pp. 632-641. ISSN 1532-2548.
9. Desbrosses, G.; Contesto, C. y Varoquaux, F. PGPR-Arabidopsis interactions is a useful system to study signalling pathways involved in plant developmental control. *Plant Signal Behav.*, 2009, vol. 4, pp. 321-323. ISSN 1559-2324.

10. Nápoles, M. C.; Martínez, J.; Costales, D. *et al.*. Avances en la reidentificación de la cepa ICA 8001 (*Bradyrhizobium japonicum*) como perteneciente a *Bradyrhizobium elkanii*. *Revista Biología*, 2006, vol. 20, no. 1-2, pp. 43-46. ISSN 1336-9563.
11. Vincent, J. M. A manual for the practical study of root-nodule bacteria /J. M. Vincent. En: International Programme Handbook. No. 15. Blackwele scientific publications, Oxford, England, 1970.
12. Verma, V.; Joshi, K. y Mazumdar, B. Study of Siderophore Formation in Nodule-Forming Bacterial Species. *Research Journal of Chemical Sciences*, 2012, vol. 2, no. 11, pp. 26-29. ISSN 2231-606X.
13. Aguado, G. A.; Moreno, B.; Jiménez, B. *et al.*. Impacto de los sideróforos microbianos en la asimilación del hierro por las plantas: una síntesis. *Rev. Fitotec. Mex.*, 2012, vol. 35, no. 1, pp. 9-21. ISSN 0187-7380.
14. Salkowski, E. Über das Verhalten der skatolcarbonsäure im organismus. *Physiol Chem.*, 1885, vol. 9, pp. 23-33. DOI:10.1515/bchm.1.1885.9.1.23.
15. Sambrook, J.; Fritsch, E. F. y Maniatis, T. Molecular Cloning. En: Crimi, M. y Cremonese, G. Molecular Biology. 2nd Ed. New York: Plainview: Cold Spring Harbor Lab. 1989. pp. 3-5.
16. Penrose, D. M. y Glick, B. R. Methods for isolating and characterizing ACC deaminase containing plant growth-promoting rhizobacteria. *Physiol. Plant.*, 2003, vol. 118, pp. 10-15. ISSN 0031-9317.
17. Bradford, M. M. A. Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.*, 1976, vol. 72, pp. 248-254. ISSN 0003-2697.
18. Sigarrosa, A. Biometría y Diseño Experimental. Primera parte. Editorial Pueblo y Educación. Ciudad de La Habana, Cuba, 1985. pp. 319-328.
19. Pooja, A. Molecular identification of root nodule bacteria from *Cicer arietinum*. *Research Journal of Biotechnology*, 2012, vol. 7, no. 1, pp. 73-79. ISSN 2278-4535.
20. Bhattacharya, A. Siderophore mediated metal uptake by *Pseudomonas fluorescens* and its comparison to iron (III) quelation. *Cey. J. Sci. Bio. Sci.*, 2010, vol. 39, no. 2, pp. 147-155. ISSN 0069-2379.
21. Wright, W.; Little, J.; Liu, F. and Chakraborty, R. Isolation and structural identification of the trihydroxamate siderophore vicibactin and its degradative products from *Rhizobium leguminosarum* ATCC 14479 bv. trifolii. *BioMetals*, 2013. vol. 26, no. 2, pp. 271-283. DOI: 10.1007/s10534-013-9609-3.
22. Brear, E. M.; Day, D. A. y Smith, P. M. C. Iron: an essential micronutrient for the legume-rhizobium symbiosis. *Front Plant Sci.*, 2013, vol. 4, no. 359, pp. 1-15. ISSN 1664-462X.
23. Kobayashi, T. y Nishizawa, N. K. Iron uptake, translocation, and regulation in higher plants. *Annual review of plant biology*, 2012, vol. 63, pp. 131-152. ISSN 1543-5008.
24. Battistoni, F.; Platero, R.; Noya, F. *et al.*. Intracellular Fe content influences nodulation competitiveness of *Sinorhizobium meliloti* strains as inocula of alfalfa. *Soil Biol. Biochem.*, 2002, vol. 34, pp. 593-597. ISSN 0038-0717.
25. Dubler, M. R.; Sacool da Sá, E. L.; Goulart, R. *et al.*. Caracterización e influencia de rizobios aislados de alfalfa en la germinación y crecimiento de plántulas de arroz. *Ciencia Rural, Santa María*, 2011, vol. 41, no. 10, pp. 1738-1743. ISSN 0103-8478.
26. Kumar, P.; Saha, P.; Mayilraj, S. *et al.*. Role of IAA metabolizing enzymes on production of IAA in root, nodule of *Cajanus cajan* and its PGP *Rhizobium* sp. *Biocatalysis and Agricultural Biotechnology*, 2013, vol. 2, no. 3, pp. 234-239. ISSN 1878-8181.
27. Patten, C. L.; Blakney, A. J. y Coulson, T. J. Activity, distribution and function of indole-3-acetic acid biosynthetic pathways in bacteria. *Critical reviews in microbiology*, 2013, vol. 39, no. 4, pp. 395-415. ISSN 1549-7828.
28. Princen, E.; Costacurta, A.; Michiels, K.; Vanserleyden, J. y Van Onckelen, H. *Azospirillum basillense* indole-3-acetic acid biosynthesis: evidence for a non-tryptophan dependent pathway. *Mol Plant-Microb Interact*, 1993, vol. 6, pp. 609-615. ISSN 0894-0282.
29. Merzaeva, O. V. y Shirokikh, I. G. The production of auxins by the endophytic bacteria of winter rye. *Applied Biochemistry and Microbiology*, 2010, vol. 46, no. 1, pp. 44-50. ISSN 1608-3024.
30. Donati, A. J.; Lee, H. I.; Leveau, J. H. J. *et al.*. Effects of Indole-3-Acetic Acid on the Transcriptional Activities and Stress Tolerance of *Bradyrhizobium japonicum*. *PLoS ONE.*, 2013, vol. 8, no. 10, pp. 1-11. ISSN 1932-6203.
31. Kumar, P. G.; Saha, P.; Mayilraj, S. *et al.*. Role of IAA metabolizing enzymes on production of IAA in root, nodule of *Cajanus cajan* and its PGP *Rhizobium* sp Original. *Biocatalysis and Agricultural Biotechnology*, 2013, no. 3, pp. 234-239. ISSN 1878-8181.
32. Kaneshiro, T. y Kwoleck, W. Stimulated nodulation of soybean by *Rhizobium japonicum* mutant (B-14075) that catabolizes the conversion of tryptophan to indol-3yl-acetic acid. *Plant Sci.*, 1985, vol. 42, pp. 141-146. ISSN 0168-9452.
33. Fukuhara, H.; Minakawa, Y.; Akao, S. *et al.*. The involvement of indole-3-acetic acid produced by *Bradyrhizobium elkanii* in nodule formation. *Plant Cell Physiol.*, 1994, vol. 35, pp. 1261-1265. ISSN 471-9053.
34. Esquivel, R.; Gavilanes, M.; Cruz, R. *et al.*. Importancia agrobiotecnológica de la enzima ACC desaminasa en rizobacterias, una revisión. *Rev. Fitotec. Mex.*, 2013, vol. 36, no. 13, pp. 251-258. ISSN 0187-7380.
35. Glick, B. R.; Todorovic, B.; Czarny, J. *et al.*. Promotion of plant growth by bacterial ACC deaminase. *Rev. Plant Sci.*, 2007, vol. 26, pp. 227-242. ISSN 0735-2689.
36. Conforte, V. P.; Echeverría, M.; Sánchez, C. *et al.*. Engineered ACC deaminase-expressing free-living cells of *Mesorhizobium loti* show increased nodulation efficiency and competitiveness on *Lotus* spp. *J. Gen. Appl. Microbiol.*, 2010, vol. 54, no. 4, pp. 331-338. ISSN 1349-8037.

37. Sharma, D.; Chauhan, S.; Kumar, G. *et al.*. Cadmium stabilization by plant growth promotory fluorescent pseudomonas in combination with Indian mustard var. Kranti, South Asean. *J. Exptl. Biol.*, 2012, vol. 3, no. 2, pp. 128-135. ISSN 1477-9129.
38. Glick, B. R. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiol.*, 2005, vol. 251, pp. 1-7. ISSN 1465-2080.
39. Glick, B. Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Special Issue on Plant Growth Promotion*, 2014, vol. 169, no. 1, pp. 30-39. ISSN 1435-8107.
40. Acebo, Y.; Hernández, A.; Rives, N. *et al.*. Perspectivas del uso de bacterias rizosféricas en el control de *Pyricularia grisea* (CookeSacc.) en el cultivo del arroz (*Oryza sativa* L.). *Rev Colomb Biotecnol*, 2011, vol. 13, no. 1, pp. 16-22. ISSN 1909-8758.
41. Hernández, A.; Rives, N.; Acebo, Y. *et al.*. Potencialidades de las bacterias diazotróficas asociativas en la promoción del crecimiento vegetal y el control de *Pyricularia oryzae* (Sacc.) en el cultivo del arroz (*Oryza sativa* L.). *Rev. Protección Veg.*, 2014, vol. 29, no. 1, pp. 1-10. ISSN 2224-4697.
42. Hernández, A.; Heydrich, M.; Diallo, B. *et al.*. Cell-free culture medium of Burkholderia cepacia improves seed germination and seedling growth in maize (*Zea mays*) and rice (*Oryza sativa*). *Plant Growth Regul.*, 2010, vol. 60, pp. 191-197. ISSN 1573-5087.
43. Hontzeas, N. J.; Zoidakis, B. R.; Glick, M. M. *et al.*. Expression and characterization of 1-aminocyclopropane-1-carboxylate deaminase from rhizobacterium *Pseudomonas putida* UW4: a key enzyme in bacterial plant growth promotion. *Bioch. Biophys.*, 2004, vol. 1703, pp. 11-19. ISSN 0006-291X.

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