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INFLUENCE OF CHICKPEA (*Cicer arietinum* L.) INTERACTIONAND THE INOCULATION OF SELECTING STRAINS OF *MESORHIZOBIUM* SPP.

Influencia de la interacción entre el cultivo del garbanzo (*Cicer arietinum* L.) y la inoculación con cepas seleccionadas de *Mesorhizobium* spp.

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ABSTRACT. The availability of microorganism strains with growth promoting effect on economically important species is one of the main aspects for elaborating bio-products, contributing to the sustainability of the production systems. The aim of this research was to select effective strains of *Mesorhizobium* sp to increase the yield of chickpea (*Cicer arietinum*. L), due to the variability shown by the INIFAT GR-1 strain over the last years, which is recommended in the technical instructions for this cultivar. Positive strains were purified and performed to conduct a morphological, physiological and biochemical characterization of the isolates, allowing the inclusion of two of them in the *Mesorhizobium* genre. The strain metabolic characterization showed its superiority with respect to INIFAT GR-1, as indicated by the increase in the phosphorus solubilization halo of 0,18 cm, and an increase of indol acetic acid (IAA) production ranging between 7-12 µgmL^{-1}. Its compatibility *in vitro* with *Azotobacter chroococcum, Bacillus megatherium var phosphaticum,* and *Bacillus subtilis*, as well as, with pesticides as Poncho, Celest Top, Gaucho FS-60, Gaucho MT, Yunta, Celest, TMTD, Cropstar and Apron Star was tested. Regarding the effects under field conditions, both strains promotes chickpea growth, development and yield (pods and grains), with values statistically superior to the pattern employed. They were growth in shaker conditions at 200 r.p.m. and 32 ^{0}C of temperature with a final concentration of 10^{11} UFC.mL^{-1}. The uses of the better strains produce the increase of Nacional-29 variety yield in 42 %, in comparison with no treated plants.

Key words: strategy, stimulation, increase

RESUMEN. Contar con cepas microbianas que permitan la estimulación del crecimiento de especies vegetales de interés económico, es uno de los aspectos fundamentales a tener en cuenta para la elaboración de bioproductos que contribuyan a la sustentabilidad de los sistemas de producción. El objetivo de este trabajo fue seleccionar cepas de *Mesorhizobium* sp, efectivas para el incremento de los rendimientos en el cultivo del garbanzo (*Cicer arietinum* L.), debido a la variabilidad que en los últimos años mostró la cepa INIFAT GR-1, recomendada en el Instructivo Técnico del mismo. Las cepas que resultaron positivas fueron purificadas y se les realizó una caracterización morfológica, fisiológica y bioquímica, mediante veinticuatro pruebas, que permitió la inclusión de dos de ellos dentro del género *Mesorhizobium*. La caracterización metabólica de estas cepas evidenció su superioridad con respecto a la INIFAT GR-1, teniendo en cuenta que presentan un aumento del halo de solubilización de fósforo de 0,18 cm y un incremento en la capacidad de producir ácido indol acético (AIA) entre 7 y 12 µgmL^{-1}. Demostró, en condiciones *in vitro,* su compatibilidad con *Azotobacter chroococcum, Bacillus megatherium var phosphaticum,* y *Bacillus subtilis*, y los plaguicidas Poncho, Celest Top, Gaucho FS-60, Gaucho MT, Yunta, Celest, TMTD, Cropstar y Apron Star. Respecto a los efectos bajo condiciones de campo ambas cepas propiciaron el crecimiento, desarrollo y rendimiento del garbanzo (vainas y granos) con valores estadísticos superior a los patrones empleados. Las cepas fueron crecidas en zaranda rotatoria a 200 r.p.m. de agitación a 32 ^{0}C de temperatura de las que se obtuvo una concentración final de 10^{11} UFC mL^{-1}, aproximadamente. El empleo de los mejores aislados provocó un efecto estimulador sobre el rendimiento de la variedad Nacional-29, superior al 42 %, con relación a las plantas controles.

Palabras clave: estrategia, estimulación, incremento

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INTRODUCTION

Knowing the physiological responses and studying the plant at the basic level, allows achieving an efficient interaction according to its genetic components that allow to increase the benefits of the microbial inoculants (1). Rhizobia are a group of microorganisms that establish interactions with leguminous plants, which allow for processes such as adhesion, deformation and curvature of the root hair, formation of infection cord, division of cortical cells, release of bacteria in the nodule in Formation, differentiation to bacteroid, final constitution of the nodule and finally the biological fixation process of dinitrogen (2). Nod genes are responsible for nodulation in different legumes, so they are present in each and every one of the interactions, a reason that defines the high specificity of the association (3).

The knowledge about the existence of symbiotic bacteria of this type goes back to 1888. In Cuba, these studies began in the 60's, mainly in forage legumes. In this decade, research was enriched and a program of microbial genetic resources was established for the first time in the country. Between 1985 and 1990, the production of inoculants to produce some crops (4) began at the artisanal scale.

The chickpea (*Cicer arietinum* L.) is among the most cultivated legumes after soybean (*Glycine max*) and beans (*Phaseolus* sp). It was introduced into Cuba by the Spanish and has been sown on a small scale since the colonial period. Numerous attempts to adapt this grain have been successful in the last 40 years in areas of Valle de Caujerí, Guantanamo, Banao and Sancti Spíritus (5), demonstrating that production is possible in the country.

In recent years, considerable areas have been devoted to sowing this crop, although the INIFAT GR-1 strain, which has been part of its technological package, has shown high variability in its behavior, and the expected effects have not been obtained with its application, in terms of crop response.

MATERIALS AND METHODS

The work corresponding to the isolation and characterization of the strains of *Mesorhizobium. sp* was carried out in the Laboratory of Agrobiotechnology, of the Institute of Fundamental Investigations in Tropical Agriculture "Alejandro de Humboldt" (INIFAT), located in Santiago de Las Vegas, municipality of Boyeros, Province of Havana, Cuba.

For the isolation, 20 chickpea plants from different parts of the country were used: Las Tunas, Artemisa and La Habana, from the National-27, Nacional-29 and Jamu-96 varieties. The nodules were disinfected with a 5 % sodium hypochlorite solution and washed twice with sterile distilled water. They were macerated in a test tube. The extract obtained was seeded on LMA medium with Congo Red[A]. The grown colonies were purified by depletion, setting conditions for their growth of 28-30 ° C temperature for 72 hours.

The isolates obtained were subjected to metabolic characterization, which included growth in LMA medium without Congo red, in 9 cm Petri dishes after 72 hours incubation at 30 ° C temperature (6). Microscopic characterization was determined by staining Gram and demonstrating the presence of capsule. The following tests were performed: presence of enzymes (oxidase, catalase); degradation of starch and proteins such as gelatin and casein. The citrate is used as carbon source production of Indole from tryptophan motility, Vogues Proskauer, Methyl Red. Also the use of sulfur from the growth in Kliger medium and utilization of sugars: mannitol, maltose, dextrose, glucose, fructose, sucrose and lactose. In addition, cellulose degradation and acid production were determined in the LMA[B] culture medium. All assays were assembled in triplicate.

For identification, the results obtained were compared with the characterization of the species according to the Manual of Systematics for Bacteria (Bergey).

The solubilization potential was determined under *in vitro* conditions, from bacterial growth on Pikosvkaya[A] medium. The medium was autoclaved for 30 minutes at 121 °C and 1,5 atmospheres. The 9 cm Petri dishes were maintained at 30 °C temperature for 48 hours to quantify the solubilization halo formed. Three replicates were made for each strain, which were planted by the Giant Colony Method. The experiment was repeated three times.

In addition, the production of indole acetic acid (IAA) was determined following the methodology described (7). The experiment was performed with three replicates per strain and the non-inoculated medium was used as the negative control. The compatibility of the strains determined by the Zonal Diffusion Method in Plates (6) was also evaluated

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[B] Martínez, V. R.; López, M.; Dibut, B.; Parra, C. y Rodríguez, J. *La fijación biológica del nitrógeno atmosférico en el medio tropical*. edit. MPPAT, Caracas, 2007, 190 p.

using the Mueller-Hinton Agar culture medium inoculated with the selected *Mesorhizobium* sp strains.

The wells of 7 mm in diameter were filled with products made from the fermentation of *Azotobacter chroococcum* (INIFAT-12 strain), *Bacillus megatherium* var *phosphaticum* (INIFAT Bmcub II) and *Bacillus subtilis* (INIFAT-101) Belonging to the Collection of Beneficial Microorganisms of the Agrobiotechnology Department of the Fundamental Investigations Institute in Tropical Agriculture “Alejandro de Humboldt” (INIFAT). After 48 hours of incubation at 30 °C temperature the inhibition halo dimensions present in the medium were determined. For the final result the value obtained in the three replicates was averaged. For the evaluation of the results, the proposed scale (4) was used, where a halo of 0-0,25 cm (compatible), of 0,26-0,50 cm (moderately compatible) and greater than 0, 50 cm (incompatible).

The experiments were carried out on Red Ferralitic soil (8) belonging to agricultural areas of the Fundamental Research Institute in Tropical Agriculture “Alejandro de Humboldt”, INIFAT, Santiago de las Vegas, during three campaigns between 2009-2012, with the National-29 variety. An experimental design of Blocks Random was used with four replicates, a plot size of 30 m^2, six grooves and four treatments, for the evaluation were discarded the edges.

The application of the product was performed from a suspension thereof in common water at 1:10 (v: v), soaking the seed for 10 minutes. After the aerating, it was sowed Plant height, total plant weight, shoot diameter and dry nodule weight were evaluated as growth indicators. The presence of nodules in the primary and secondary roots and the presence of leghemoglobin leghemoglobin (pink to red tones) were also qualitatively determined for the aspects of higher incidence in the production, the number and weight of the pods and the grains per plant (9).

The results of the experiments performed on *in vitro* laboratory conditions were processed taking into account a Completely Randomized Design used for its execution, while those obtained in the experimental phase executed under field conditions were processed considering the Design of Random Blocks. The significant differences among the means of the treatments for the analyzed variables were discriminated according to the Duncan Multiple Rank test at 5% significance. All information was processed using the STATGRAPHICS Plus Version 5.1 program (10).

RESULTS AND DISCUSSION

From the sampling of 20 chickpea plants (*Cicer arietinum* L.) belonging to different varieties, five isolates were tested that showed the typical characteristics of the genus *Mesorhizobium*. In this case they were grown in LMA medium (colonies between 0,1 and 0,3 cm in diameter at 48 hours of planting in the culture medium, incubated at 32 °C temperature) (6).

The objective of this work stage was to find heterogeneity in the selection, to design a strategy based on a wide genetic diversity of the crop, according to the edaphoclimatic conditions prevailing in each region, to obtain inoculants with greater spectrum of action (11).

Regarding the macromorphological description, the five isolates show by Gram stain test that all are short Gram negative bacilli and have capsule presence. All of them coincide with the characteristics described for the genus *Mezorhizobium*, so that, from the morphological point of view, none of them can be ruled out for use in biofertilization of the chickpea (*Cicer arietinum* L.) crop.

The physiological-biochemical characterization (Table I) allowed to differentiate the microorganisms and to eliminate the RM-2, RM-4 and RM-10 isolates because they present a positive starch reaction, a response that does not coincide with the characteristics to which the gender belongs (6).

In addition to these there are several distinctive features among the genres of rhizobia. For example *Azorhizobium* contains strains that form root and stem nodules and fix nitrogen under free living conditions. *Bradyrhizobium* grows slowly and produces alkali in YMA medium, while *Allorhizobiun*, *Rhizobium* and *Sinorhizobium* grow fast and produce acid in that culture medium. In the case that concerns us as the genus *Mesorhizobium*, it presents colonies of slow or moderate growth and produces acid in LMA^A medium. It includes bacteria that have a high degree of specificity, which only nodulate a very narrow range of plant species, which makes it necessary to have an adequate management strategy that allows strains to establish an efficient relationship (12).

From these results, the work continued only with the R-1 and R-3 strains, since they are those that meet the characteristics corresponding to the genus Mesorhizobium, and therefore, they are those that present possibilities in inducing the nodulation in the culture of the chickpea (*Cicer arietinum* L), given the specificity of this microorganism with its host (13).

Table I. Physiological-biochemical characterization of the five purified isolates

Isolate	Ox	Cat	Cit	Alm	Ind	Gel	Cas	Mot	VP	RM	Kli	Cel	YMA acid
RM-1	+	+	+	-	-	-	0.9	+	-	-	-	-	+
RM-2	+	+	+	0.6	-	-	0.2	+	-	-	-	-	-
RM-3	+	+	+	-	-	-	0.7	+	-	-	-	-	+
RM-4	+	+	+	0.7	-	-	0.2	+	-	-	-	-	-
RM-10	+	+	+	1.3	-	-	0.1	+	-	-	-	-	-

Ox: oxidase Cat: catalase Alm: starch Ind: indole production from tryptophan Gel: gelatin Cas: casein
Cit: use of citrate as carbon source Mot: motility VP: VoguesProskauer
Klig: use of sulfur from growth in Kliger medium M: Methyl Red Cel: Degradation of cellulose
Acid LMA: Production of acid in YMA medium

For these strains the physiological-biochemical characterization was completed by determining the use of some sugars as a carbon source such as (fructose, glucose, sucrose, lactose, maltose, dextrose and mannitol) all of them showed positive results[A]. For the rhizobia its taxonomy is defined on the basis of the phylogeny of the 16S rRNA genes, some phenotypic characteristics allow to distinguish among the different genera. The morphological and physiological study that is commonly carried out for the characterization of these microorganisms allows at least differentiate phenotypically very different genera, such as Bradyrhizobium, Mesorhizobium and the family *Rhizobiaceae* (12).

The R-1 and R-3 strains were subjected to a deeper characterization study, to determine their metabolic potential, and then to be able to evaluate the possibility of substituting INIFAT GR-1 for biofertilization with Cultivation of chickpea (*Cicer arietinum* L.). Phosphorus solubilization is a process of great importance for the cultures involved in the plant-microorganism interaction, since it is one of the main components of ribonucleic acid and deoxyribonucleic acid, besides being the energy carrier of the molecule adenosine triphosphate (ATP) and its two mono and diphosphate precursors (AMP and ADP). Its presence in the DNA molecules of the chromosomes allows the processes of storage, replication and together with the RNA, the transcription of genetic information. This element is a regulator of vegetation, so it is a factor that favors critical periods such as: fertilization, maturation and movement of plant reserves (14).

The presence of this nutrient is of great importance for the biological fixation of nitrogen during the rhizobia-leguminous interaction, affecting the lack of it, the correct functioning of the nodule, so that the estimation of the solubilizing activity is undoubtedly a tool for the Selection of promising materials for biofertilization, taking into account that with the solubilizing activity, microorganisms can contribute between 20-40 % of the nutrient requirements for the crop (12).

According to the results shown in Figure 1, the R-1 and R-3 strains have a solubilization halo greater than 0,25, even exceeding in a high percentage to the INIFAT GR-1 strain in the solubilization of phosphorus *in vitro*, so it can be inferred that they have the potential to increase the availability of this nutrient, taking into account the concordance of these tests with the behavior of microorganisms in their natural environment (15).

For rhizobia the ability to solubilize the phosphorus contained in the soil is described by the action of the enzymes phosphatases and organic acids, among other substances, as well as the *Pseudomonas*, *Bacillus* and *Enterobacter*. That the strains present the duality in the fixation of nitrogen and solubilization of phosphorus with appreciable tenors are of interest for the development of multipurpose products that benefit the crops, and it is, therefore, of particular importance in this work.

Another not less important process is the production of phytohormones by plant growth promoting bacteria, particularly indole acetic acid (IAA). By their release by the microorganisms, the growth of the cultures is stimulated from different physiological mechanisms (12).

It also allows the *Rhizobium* penetration by the exchange of different biochemical signals emitted by the macro and microsymbiont. This regulated and complex process, culminates with the formation of a highly organized structure in the plant: the nodule, which can be considered as a new organ of the same. In the same the bacterium will differentiate in its endosymbiont form fixer of atmospheric nitrogen (bacteroid). The transformation of the microorganism is so important that it can multiply in free life and fix nitrogen in special synthetic media, but not in the absence of the host, and there is often a marked specificity between both (4).

In this work the levels of release of this substance by the two strains of *Mesorhizobium* were evaluated, they show values of 20 µgmL^{-1} with a clear superiority with respect to the INIFAT GR-1 strain. For this group of microorganisms the presence of this hormone in its metabolic products is mentioned, and it is attributed to it, part of the stimulating effect of the growth that is obtained by its application. These results are related to those reported by other authors for the genre (16).

There are studies that demonstrate the possibility of applying rhizobia with other microorganisms with biofertilizer potential and demonstrate the effectiveness of the combined inoculation. This study shows that the two strains can be combined with *Azotobacter chroococcum* (INIFAT-12 strain), *Bacillus megatherium* var *phosphaticum* (INIFAT Bmcub II strain), and *Bacillus subtilis* (strain INIFAT-101), as well as INIFAT GR-1, since all are compatible to present inhibition halos of bacterial growth between 0,07-0,12 cm. This shows that in the future research could be carried out to exploit the benefits of co-inoculations of these microorganisms (6).

As part of the characterization to which the Mesorhizobium strains were subjected, they're *in vitro* relationship with chemical pesticides was included that can be applied in the management of the chickpea (*Cicer arietinum* L.) crop, considering that this crop is highly sensitive to diseases, mainly to the involvement of soil fungi and insects.

There is agreement with the response of Cropstar and ApronStar products, which were compatible for *Mesorhizobium* R-1 and R-3 strains. As well as Poncho, Celest Top Gaucho FS-60 and Celest as moderately compatible with results very similar to those that are evidenced in front of the INIFAT GR-1. The products Gaucho MT, Yunta and TMTD stand out for their toxicity. It is evident the aggressiveness of Thiram as an active substance that makes up two of these pesticides, so that the results obtained should be taken into account for subsequent studies with other crops and assess their specific compatibility with each cultivar, thus avoiding errors in agricultural management .

The influence of the concentration of the used products can condition the inhibitory action in some cases, taking into account that they were worked in a pure way. When decreasing its dose, the effects must be lighter on microorganisms. In addition, *in vitro* conditions imply a maximum interaction between the chemical and the bacteria, which also raises the likelihood of negative results. However, the sensitivity of plant growth promoting bacteria to the presence of fungicides is also described by other authors [C].

In general, it is considered that the R-1 and R-3 strains could be combined with the chemical pesticides, like the INIFAT GR-1 strain, which would undoubtedly be very useful in the search for an integral management proposal of the chickpea culture (*Cicer arietinum* L.).

In the Table II, it can appreciated the potential of both *Mesorhizobium* strains to stimulate different growth and culture develop indicators of chickpea, although the R-3 strain is highlighted with higher increasing in all the evaluated indicators.

The results obtained demonstrate an adequate development of the crop, as well as the establishment of a positive relation in the association plant and microorganism that determine the behavior of the vegetal species. In addition, they provide criteria to evaluate the development of the plant and its response to biological treatment; Hence the need for a balanced nutrition-biofertilization regime.

During the reproductive development phase, which is between 60 and 80 days, and which is one of the most important, coinciding with grain filling, requires greater supplies to compensate demand and achieve the expected yield, That biofertilization plays a very important role, as it places at the disposal of the plant, adequate nitrogen levels and the contribution of phosphorus through solubilization (14).

The nodules were located mainly in the main root, in the form of large clusters. In the secondary roots their presence was detected, characterized by a smaller volume in the same ones. In both cases, the presence of leghemoglobin was demonstrated, particularly for the variants inoculated with the R-1 and R-3 strains. This aspect is very important because it shows that the biological nitrogen fixation process is performed effectively, so the association between microorganisms and host is satisfactory (5).

This work allowed evaluating the advantage of using autochthonous strains with high competence, coming from nodules from different regions and showing their biological potential. Taking into account the effectiveness of the association, inferred from the increase in the growth indicators, development and performance evaluated, we can conclude that the strains used are able to compete with the native ones.

[C] Albretch, J.; Fontanetto, H. y Sillón, M. Informe sobre el cultivo del garbanzo. Red de Agricultura de Precisión. Fitopatología, Inst. Departamento de Producción Vegetal-FCA UNL INTA EEA, Córdoba, 2011, p. 15.

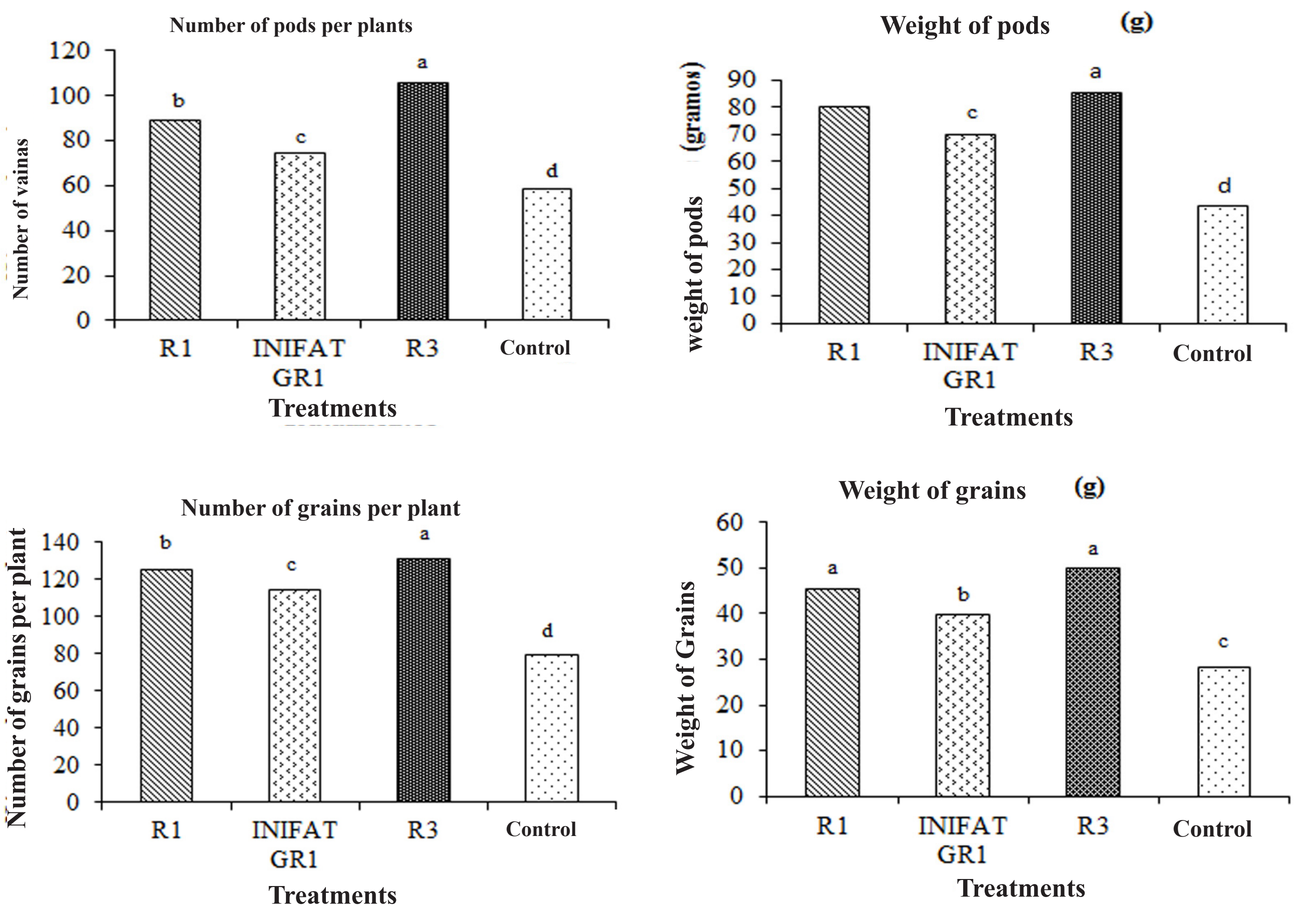
The importance of correct selection is evident, to provoke an adequate effect in the crop development stimulation. In the type of soil used and with the variety used, strain R-3 shows a clear superiority, which is why it is essential to perform similar experiments when discriminating among strains and selecting only those with greater potentialities.

The indicators evaluated, as components of yield, have a direct impact on crop production, which also showed increases due to the inoculation effect of Mesorhizobium strains (figure).

Table II. Study of the physiological parameters of growth and chickpea development (*Cicer arietinum* L.), National-29 variety

Variant	Plant height	Stem diameter (cm)	Total weight of plant (g plant^{-1})	Dry weight of nodules (g plant^{-1})
INIFAT GR-1	60,19 c	0,70c	112,381 c	0,38 c
Strain 1	67,80 b	0,75b	133,546 b	11,66 b
Strain 3	71,18 a	0,77a	137,745 a	14,02 a
Control	50,62 d	0,52d	63,293 d	--
EsX	3,199	0,60	0,132	0,168
CV (%)	10,11	0,89	2,99	2,87

Equal letters do not differ significantly from each other for a = 0,05



Equal letters do not differ significantly from each other for a = 0,05 Esx: 4.3091; 69.8175; 112.58; 1,511, for each indicator, respectively
CV (%): 5,27; 0,89; 0,87; 3,70, for each indicator, respectively

Behavior of productive indicators of the chickpea (*Cicer arietinum* L.) cultivar with the National-29 variety, inoculated with the *Mesorhizobium* strains

The similarity of the behavior of the R-1 and R-3 strains, and the superiority of both when comparing it with INIFAT GR-1 is evident, that´s why as main result of this work the same ones are selected to replace the latter in the biofertilization of the chickpea (*Cicer arietinum* L.) crop, mainly for the National-29 variety, due to the significantly superior response to the control (plants without *Mesorhizobium* application). In later works, it would be useful to evaluate the possibility of producing a multi-strain product that allows a greater effect of the inoculant in the different agroecosystems where the cultivation of chickpea (*Cicer arietinum* L.) is planted, as well as the possibility of combining these microorganisms with solubilizer species of phosphorus and chemical or biological pesticides in *in vivo* conditions, to propose an integral management of the crop. Taking into account that inoculation of legumes with rhizobia will be carried out for a long time, the best option for the management of this crop group is the continuous development of increasingly effective biofertilizers that should be a permanent work and vital importance for world agriculture and especially for Cuba.

CONCLUSIONS

- ♦ The R-1 and R-3 strains belong to the genus Mesorhizobium.
- ♦ Both strains of Mesorhizobium show potential for agricultural use, taking into account that they solubilize phosphorus, produce indole acetic acid at levels higher than INIFAT GR-1 and, like it, they are compatible with microorganisms of agricultural interest and chemical pesticides.
- ♦ The R-1 and R-3 strains stimulate the yield and nodulation of the chickpea (Cicer arietinum L) variety National-29, so they are promising to replace the INIFAT GR-1 strain in biofertilization.
- ♦ The R-3 strain stands out because of its superiority, since it shows increases in all the indicators evaluated.♦ Se destaca por su superioridad la cepa R-3 por mostrar incrementos en todos los indicadores evaluados.

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Note:

During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial