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EFFECT OF BIOACTIVE PRODUCTS IN BIOFERTILIZED BEAN (*Phaseolus vulgaris* L.) PLANTS

Efecto de productos bioactivos en plantas de frijol (*Phaseolus vulgaris* L.) biofertilizadas

Lisbel Martínez González✉, Yanelis Reyes Guerrero, Alejandro Falcón Rodríguez, María C. Nápoles García and Miriam de la C. Núñez Vázquez

ABSTRACT. Biofertilizers use in agriculture allow to ensure culture productivity, reducing chemical fertilizers and cost. Azofert® is a bioprepared composed by rizobia strains, with a positive effect on biological nitrogen fixation. Biobras-16 and Quitomax are natural bioactive products, considered as biostimulants which help plant to face various kinds of stresses and increase yields; however there is no information about the effect of the application of these biostimulants to seeds before biofertilizer inoculation on bean crop. The objective of this work was to determinate the Biobras 16 and Quitomax effect on nodulation and growth of common bean biofertilized with Azofert®. Two concentrations of Quitomax and Biobras-16 were applied on seeds the day before sowing and with Azofert® at the sowing moment, then they were put on Ferralític Red Lixiviated típic éutrico soil. 35 days after sowing were determined in ten plants by treatment: number and dry weight of nodules, shoot and root length and dry weight. Determination of leaf chlorophyll, nitrogen and phosphorus were also made. The results showed that application of specific concentration of Biobras-16 and Quitomax plus Azofer®t inoculation; stimulate, in a general way, the growth of plants; mainly with Biobras-16, which also increased leaf chlorophyll and nitrogen contents.

Key words: brassinosteroids, growth, leguminous, chitosan, rizobia

RESUMEN. El uso de biofertilizantes en la agricultura permite asegurar la productividad de los cultivos a un costo bajo y con un consumo mínimo de fertilizantes químicos. El Azofert® es un biopreparado a base de cepas de rizobios, que ejerce un efecto beneficioso en la fijación biológica del nitrógeno. Biobras-16 y Quitomax son bioestimulantes que ayudan a la planta a afrontar diversos tipos de estrés e incrementar los rendimientos; sin embargo, no existe información acerca del efecto de la aplicación de estos bioestimulantes a las semillas de frijol, previo a la inoculación con el biofertilizante. El objetivo de este trabajo fue determinar el efecto de Biobras-16 y Quitomax en la nodulación y el crecimiento de plantas de frijol, biofertilizadas con Azofert®. Un día antes de la siembra, las semillas fueron asperjadas con dos concentraciones de Quitomax y Biobras-16 y en el momento de la siembra, se inocularon con Azofert® y se colocaron en macetas con suelo Ferralítico Rojo Lixiviado típico, éutrico. A los 35 días se realizaron las siguientes evaluaciones a diez plantas por tratamiento: número de nódulos y masa seca de los mismos, longitud del tallo y la raíz, masa seca de ambos órganos y contenido de clorofilas, nitrógeno y fósforo en las hojas. Los resultados mostraron que la aspersión de las semillas, con determinadas concentraciones de Biobras-16 o Quitomax y la inoculación con Azofert® estimularon, de forma general, el crecimiento de las plantas; destacándose el efecto de Biobras-16, que también incrementó la concentración de clorofilas y nitrógeno en las hojas.

Palabras clave: brasinoesteroides, crecimiento, leguminosa, quitosana, rizobios

INTRODUCTION

Beans (*Phaseolus vulgaris*) is one of the most important foods in the daily diet in most Latin American countries, due to the high percentage of protein (20-28 %) and essential amino acids containing their grainy[A]. The level of production of beans in Latin America is relatively

Instituto Nacional de Ciencias Agrícolas (INCA), gaveta postal 1, San José de las Lajas, Mayabeque, Cuba, CP 32 700.

✉ lisbel@inca.edu.cu

low, no results expected harvest are obtained due to various factors affecting crop productivity, including drastic changes in climate, the presence of pests and diseases and deficiency nutrients in the soil.

In response to the excessive use of fertilizers and chemical synthesis to environmental problems they cause, it is stimulated increasingly implementing sustainable agriculture, allowing cultivate the soil, damaging the environment (1) as possible. In this context, special importance has been given to beneficial microorganisms (2,3). Being a legume, beans have the ability to associate with soil bacteria capable of forming nodules in plants, known as rhizobia (4, 5). These microorganisms provide to plant the nitrogen from biological fixation of atmospheric dinitrogen (BFN). The establishment of this symbiosis is a complex process that involves multiple coordinated exchanges of signals between the host plant and its microsymbiont (6).

Moreover, in agriculture there is a range of bioestimulantes with anti-stress and ability to increase yields (7-10) effect. The QuitoMax is a liquid formulation having as an active ingredient, chitosan polymers, which are obtained mainly from the exoskeleton of crab, shrimp or lobster (11, 12). Chitosan has been widely used in agriculture, mainly by having antimicrobial activity (13, 14), inducing resistance in plants (15), and promote plant growth (16). Biobras-16 is a formulation having as an active ingredient a spirostanic analogue of brassinosteroids, which has as main characteristics: increase yields, improve crop quality and increase tolerance to environmental stress conditions (water deficit, salinity in the soil, high temperatures). It is shown, moreover, that does not produce physiological damage, so it has been widely used in various crops such as rice, corn, soybean, tomato, among others (17).

However, the effect may exercise the prior application of these bioactive products bean seeds that will be inoculated at the time of planting, with biofertilizers as Azofert® is unknown.

The aim of this study was to determine the effect that the application of certain concentrations of Biobras-16 and QuitoMax exert in nodulation and growth of bean plants, Cuba-Cueto 25-9 variety biofertilized with Azofert®.

[A]*Common bean* [en línea]. CGIAR, 24 de marzo de 2016, [Consultado: 24 de marzo de 2016], Disponible en: <http://www.cgiar.org/our-strategy/crop-factsheets/beans/>.

MATERIALS AND METHODS

The experiment was conducted at the Department of Physiology and Plant Biochemistry of the National Institute of Agricultural Sciences (INCA). 50 bean seeds were sprayed cv. Cuba-Cueto 25,09 (18), 24 hours before sowing, with 3 x 10,3 L of two concentrations of Biobras-16 and two QuitoMax as each treatment. At the time of planting, each seed was inoculated with 1 mL of Azofert®, *Rhizobium leguminosarum* CF1strain, at a concentration of 5,4 x 108 colony forming units (CFU) ml^{-1}. They were used as controls without treatment products (untreated control) and treatment with only Azofert® inoculated. The treatments were conformed as follows:

1-Control Application
2-Azofert® without
3-Azofert® + QuitoMax (100 mg L^{-1})
4 Azofert® + QuitoMax (500 mg L^{-1})
5-Azofert® + Biobras -16 (0,005 mg L^{-1})
6-Azofert® + Biobras-16 (0,05 mg L^{-1}).

The seeds were planted in pots with 250 g of Ferralitic Red Typical Leaching, Eutric (19) and the same were placed in a room of lights, keeping in conditions of 24 ± 2 °C temperature, 12 h light photoperiod and 70 % relative humidity for 35 days. The plants were watered daily with tap water.

Agrochemical soil characteristics were determined in accordance with the provisions (20) and they are presented in Table I; while the microbiological characteristics related to the presence of rhizobia, are shown in Table II.

Table I. Agrochemical soil characteristics

pH	MO (%)	P (ppm)	K	Ca	Mg
				cmol kg-1	
6,1 Acid	3,79 slightly low	17 Medium	0,62 High	8,0 Very slow	5,0 Medium

Table II. Microbiological soil characteristics

Presence of rhizobia	Concentration (UFC.mL^{-1})
Rhizobium	5 x 10^3
Bradyrhizobium	1,8 x 10^4

At the end of the experiment (35 days after planting), ten plants were taken per treatment and underwent the following evaluations per plant: total number of nodules and nodular dry mass, length and dry weight of roots and aerial parts. Furthermore, the concentration of total chlorophyll in the leaves was estimated, using a computer Spad 502 and three samples of 0,2 g of leaves were taken for treatment to determine the content of nitrogen and phosphorus.

These determinations were performed by the colorimetric method using Nessler's reagent for the N and molybdenum blue for P (20).

The data were processed by analysis of variance of simple classification. the multiple comparison test of Duncan ranges α <0,05, in order to discriminate differences between means was used. All graphics were done with Microsoft Excel 2010 program.

RESULTS AND DISCUSSION

In Figure 1 the results of the effect of treatments on the total number of nodules (A) and dry mass of nodules (B), in the bean seedlings are presented.

In the number of nodules formed in Biobras-16 treatment, its highest concentration (T6) was the one that stood out, with no significant differences with treatment Azofert®. Similar results were obtained with the combination with the lowest concentration brassinosteroid (T5). The use of QuitoMax at concentrations of 100 and 500 mg L-1 decreased the number of nodules formed, no significant differences with absolute control (T1) (Figure 1A).

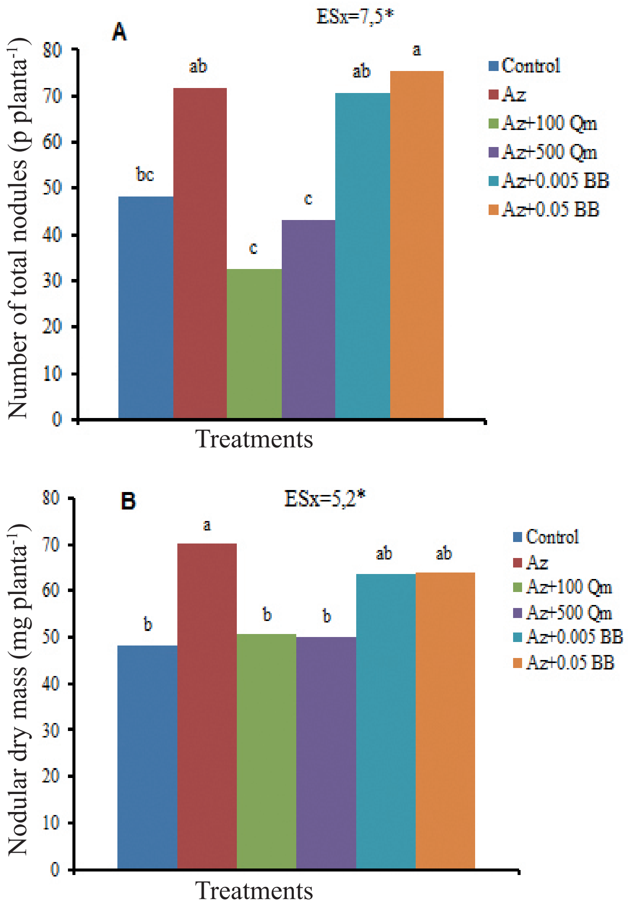
By analyzing the nodular dry mass, similarly the best results were obtained with the inoculated treatment (T2) and those further containing Biobras16 (T5 and T6) (Figure 1B), although the latter did not differ from the treatments QuitoMax (T3 and T4) and the control (T1).

In other studies conducted showed that immersion of soybeans for one hour in a solution of Biobras-16 (0.05 mg L-1), improved response *Bradyrhizobium*-soybean symbiotic interaction, increasing both the number of nodules and the fresh and dry masses of the same (21).

It has been observed, in works with bean plants cv. Arka Suvidha sprinkled with 0,1, 5 μM homobrassinolide (HBL) or epibrassinolide (EBL) in the flowering stage, that brassinosteroids stimulated the number of nodules, nodular mass of roots and root length, highlighting the EBL to 5 μM concentration as the most effective treatment (22).

As for the effect of quitosaccharides in the *in vitro* nodulation of soybean seedlings inoculated with *Bradyrhizobium elkanii*[B] found that concentrations up to 100 mg L^{-1} did not change the number of nodules, while concentrations of 500 and 1000 mg L^{-1} significantly reduced the occurrence of these structures in the roots.

[B] Costales, M. D. *Quitosácaridos en la nodulación y el crecimiento in vitro de soya* (*Glycine max* L. Merrill) *inoculada con Bradyrhizobium elkanii*. Tesis de Maestría, Instituto Nacional de Ciencias Agrícolas, La Habana, Cuba, 2010, 56 p.



Equal letters do not differ statistically according to test ranges Duncan multiple p≤0,05
Az (Azofert), Qm (QuitoMax), BB (Biobras-16) (n = 10)

Figure 1. Effect of Biobras-16 and QuitoMax on the total number of nodes (A) and nodular dry mass (B) of bean plants var. Cuba-Cueto 25-9 whose seeds were inoculated with Azofert®

There are several factors that affect nodulation and biological nitrogen fixation in beans. Including the wide variation that occurs between genotypes and its ability to symbiotic fixation, where indeterminate habit climber cultivars are more efficient. Other environmental factors such as acidity, toxicity of aluminum and manganese, the availability of phosphorus in the soil, ambient temperature and competition with native strains, also significantly affect the ability of nodulation and biological nitrogen fixation by the legume (23, 24).

By analyzing the length of the aerial part it was observed that only foliar spray with 0,05 mg L^{-1} of BB-16 was able to significantly increase this indicator plants inoculated with the biofertilizer.

Moreover, it should be noted that this indicator Azofert® significantly stimulated compared to control treatment plants (Figure 2A). In root length, also treatment Azofert® + 0.05 mg L-1 BB-16 was that the superior results showed (Figure 2B).

Aerial dry mass was greater in the T5 (Azofert® + BB-16 0,005 mg L^{-1}) and less treatment for control and inoculated treatments only (Figure 2C). While radical dry mass (Figure 2D) was favored with the combination of Azofert® + QuitoMax (500 mg L^{-1}) and Azofert® + Biobras-16 (0,05 mg L^{-1}).

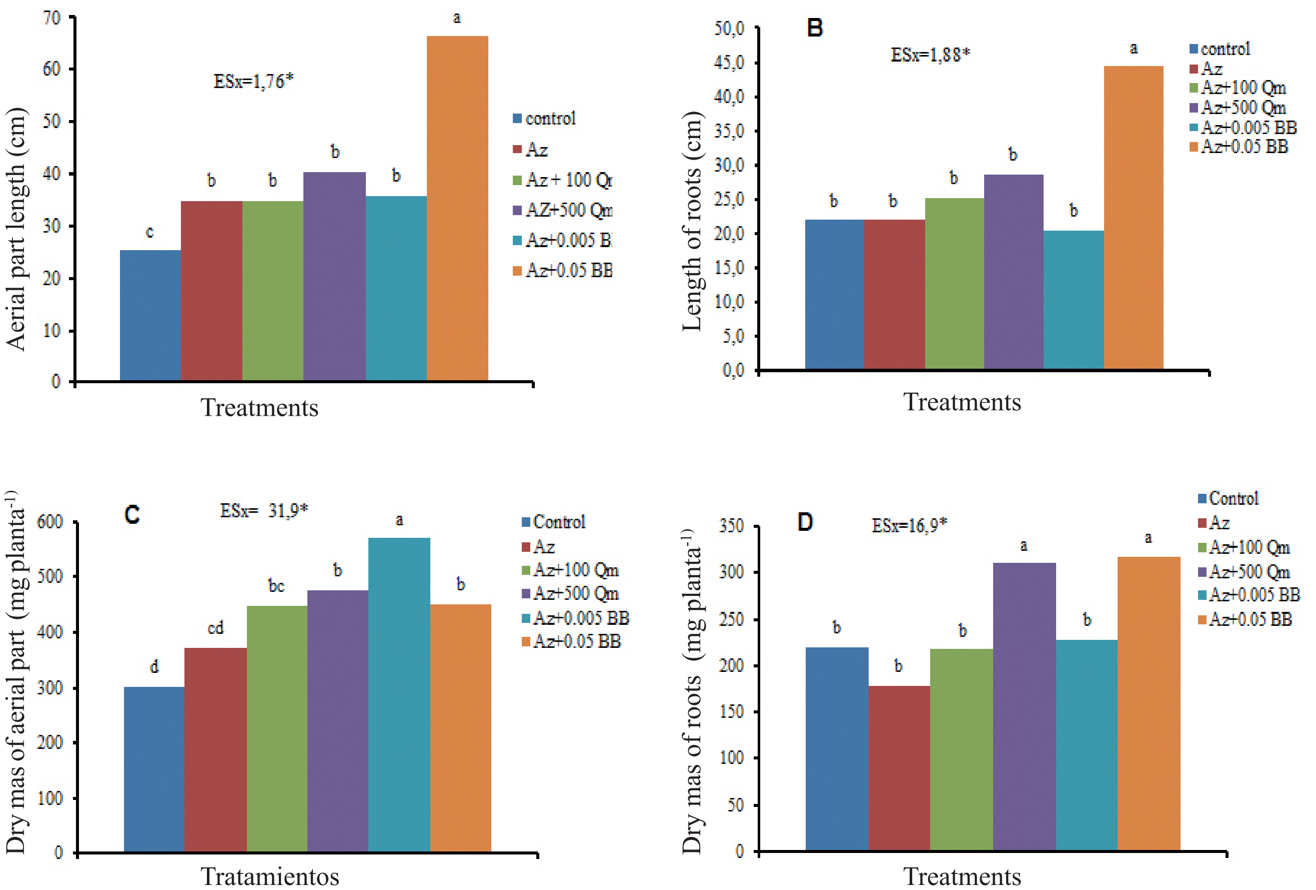
It has been reported that treatment of bean seeds cv. Cuba-Cueto 25-9 with rhizobia, increases plant height relative to uninoculated plants (25). In this research, although all exceeded the control treatments, the best results, in general, were obtained when the seeds with Biobras-16 (0,05 mg L^{-1}) were sprayed and subsequently inoculated with Azofert®. Several authors have shown that brassinosteroids are capable of stimulating the growth of plant organs (26).

As for the stimulation of rooting, it has been found that immersion cuttings guava (*Psidium guajava* L.), for 15 minutes in a solution of BB-16 0,05 mg L^{-1} induced rooting and growth stimulated of the positions (17).

Two species of orchids, repeated foliar sprays with BB-16 (0,05 mg L^{-1}) increased both the number of pseudobulbs or stems as the number of roots and would enhance the color of plants and the quality of flowers (27).

In soybeans, foliar spray Biobras-16 (20 mg ha^{-1}) in three varieties plants inoculated with *Bradyrhizobium japonicum* and *Glomus clarum*, yielded satisfactory results (28).

Other authors have reported that treatment of bean seeds with 24-epibrassinolide (EBL) for six hours, it is able to stimulate the length of seedlings (roots and aerial part) eight days (29).



Equal letters do not differ statistically according to test ranges Duncan multiple p≤0,05
Az (Azofert), Qm (QuitoMax), BB (Biobras-16) (n = 10)

Figure 2. Effect of various products over the length of the aerial part (A) and roots (B), the dry weight of the aerial part (C) and roots (D), bean plants var. Cuba-Cueto 25-9, 35 days after planting

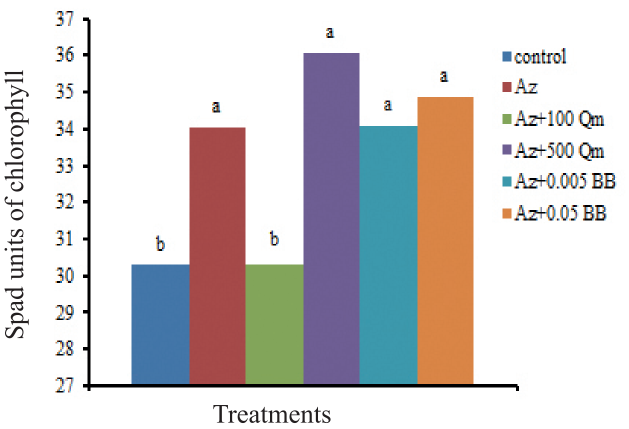
It has also been found that spraying to bean seeds cv. Bronco with EBL (5 PM), significantly increased the length of the stems and roots, leaf number and leaf area per plant and the dry mass of the same (30).

As for chitosan, several studies report that stimulates the growth and development of plants (31-33). Recently it was found that the application of polymer cucumber plants increased biomass production and yield. Has been reported soybean seeds coated with 1000 mg L^{-1} of chitosan solution for six hours, increases the length and fresh weight of the roots (33).

It could also observe the effect of treating seeds ispaghul (*Plantago ovata Forsk*) with different concentrations of chitosan (0,01, 0,05, 0,1, 0,2 and 0,5 %) produced an increase the length and the dry mass of the roots and stems, the most effective concentrations were 0,2 and 0,5 % (34). In this work presented it was not necessary to use high concentrations of chitosan as to favor the dry mass of the roots, which may be related to the presence of *Rhizobium*.

Foliar application with chitosan in two stages of the crop cycle of mung bean (*Vigna radiata* (L.) Wilzeck) favored the dry mass of the plants; however, effective concentrations depended on how the plants were grown, if it was in pots or in field conditions, with emphasis on 25, 50, 75 and 100 mg L^{-1} in the first case and 75 and 100 mg L^{-1} in the second (35).

An analysis of each treatment and their contribution to chlorophyll content shown in Figure 3. As can be seen, all treatments except of QuitoMax (100 mg L^{-1}) + Azofert® were significantly superior to the control treatment without application.



Equal letters do not differ statistically according to test ranges Duncan multiple p≤0,05
Az (Azofert), Qm (QuitoMax), BB (Biobras-16) (n = 10)

Figure 3. Units SPAD chlorophyll in leaves of bean var. Cuba-Cueto 25-9, at 35 days after planting

It has been shown that seeds of mung bean (*Vigna radiata* L. Wilczek) cv. T-44, inoculated with Rhizobium and treated with homobrassinolide (HBL) 0,01 µM, ten days after planting, increased leaf area, relative water content and leaf water potential and the activity of enzymes nitrate reductase and carbonic anhydrase, carboxylation efficiency and chlorophyll content (36).

In other plant species it has been reported that the application of certain concentrations of chitosan stimulates concentration chlorophyll in the leaves (37, 38).

As shown in Table III, Azofert® inoculation with increased concentration of nitrogen and phosphorus in the leaves, with respect to the control plants. The application of bioestimulants not influences the concentration of foliar phosphorus; however, in treatments where Biobras-16 was applied it was significantly increased nitrogen concentration in leaves of bean plants at 35 days after planting.

Table III. Influence of different bioactive products on the content of nitrogen and phosphorus foliar biofertilized bean plants, 35 days after sowing (n = 6)

Treatments	Elements	
	N (%)	P (%)
Control	4,17 c	0,05 c
Az	4,48 b	0,07 ab
Az+Qm 100	4,40 bc	0,07 ab
Az+ Qm 500	4,24 bc	0,08 ab
Az+BB 0.005	4,79 a	0,085 a
Az+BB 0.05	4,80 a	0,08 ab
SEx	0,08*	0,008*

Equal letters do not differ statistically according to the Test Duncan's multiple range for p≤0,05Az (Azofert®), Qm (QuitoMax) and BB (Biobras-16)

Nitrogen and phosphorus are considered among the elements that influence production of crops (39); however, the high cost of fertilizers limit their use, so that currently used alternatives capable of knowing the needs of crops without harming the environment (40).

Treating the seeds with Biobras-16 and the subsequent inoculation with Azofert® showed the best results in the nitrogen content in the leaves, so it could be an alternative to the addition of this element to the plant.

The Biobras-16 and QuitoMax products did not increase nodulation bean cultivar used. However, the sprinkling QuitoMax significantly stimulated the dry mass of the plants; while foliar spray the seeds with Biobras-16 had a positive effect on all indicators evaluated growth as well as on the content of chlorophylls and N in the leaves.

CONCLUSIONS

The use of biofertilizers in agriculture is an alternative to ensure the sustainability and productivity of crops. The need for higher agricultural yields while preserving the environment is linked to the widespread use of these products. Combination with other bioactives as Biobras-16 or

QuitoMax, in appropriate concentrations, can increase the positive results biofertilizers produced in legumes.

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