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Original Article

Response of peanut (*Arachis hypogaea* L.) to combined application of microbial and non-microbial biostimulants

Respuesta del maní (*Arachis hypogaea* L.) a la aplicación combinada de bioestimulantes microbianos y no microbianos

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ABSTRACT: In Cuba, peanut planting and yield are low and produced with limited inputs. The use of biostimulants in cultivation could constitute an innovative alternative to counteract this problem. In the country, there is little research that addresses the issue. The aim of this work was to evaluate the effect of the combined application of microbial and non-microbial biostimulants on peanut nodulation and growth. Inoculation tests were carried out under controlled conditions where inoculants of two strains of rhizobia were applied as well as formulations based on these strains and Pectimorf®. A completely randomized design was used and 45 days after sowing, nodulation, biochemical and growth variables were evaluated in peanut plants. The results showed that the application of inoculants based on the *Rhizobium* sp. C145 in combination with Pectimorf® increased the dry mass of the nodules, the relative content of total chlorophylls, the stomatal conductance, the content of nitrogen and phosphorus, as well as the height of the plants, the root length and the number of flowers . This research is the first evidence in Cuba that proves the positive effect of the combination of bacterial inoculants with Pectimorf® in the cultivation of peanuts.

Keywords: inoculation, bioproducts, nodulation, *Rhizobium*.

RESUMEN: En Cuba, la siembra y el rendimiento del maní son bajos y se producen con insumos limitados. El uso de bioestimulantes en el cultivo pudiera constituir una alternativa innovadora para contrarrestar esta problemática. En el país existen escasas investigaciones que abordan el tema. El objetivo de este trabajo fue evaluar el efecto de la aplicación combinada de bioestimulantes microbianos y no microbianos en la nodulación y el crecimiento del maní. Se realizaron ensayos de inoculación en condiciones controladas donde se aplicaron inoculantes de dos cepas de rizobios, así como formulados a base de estas cepas y de Pectimorf®. Se utilizó un diseño completamente aleatorizado y, a los 45 días de la siembra, se evaluaron variables de nodulación, bioquímicas y de crecimiento en las plantas de maní. Los resultados demostraron que la aplicación de inoculantes a base de la cepa *Rhizobium* sp. C145 en combinación con el Pectimorf® incrementó la masa seca de los nódulos, el contenido relativo de clorofilas totales, la conductancia estomática, el contenido de nitrógeno y fósforo, así como la altura de las plantas, el largo de la raíz y el número de flores. Esta investigación es la primera evidencia en Cuba que comprueba el efecto positivo de la combinación de inoculantes bacterianos con el Pectimorf® en el cultivo del maní.

Palabras clave: inoculación, bioestimulantes, nodulación, *Rhizobium*.

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INTRODUCTION

The peanut (*Arachis hypogaea* L.) is one of the most important leguminous plants because it is the sixth most important oilseed and economic crop in the world; it also stands out for its high nutritional value and generates employment and income (1). World peanut production is expanding and exceeds 35 million tons, generating 6 million tons of oil (2).

In Cuba, peanut production does not exceed 2 t ha^{-1} and is mainly produced by small producers with low inputs (3, 4). Strategies to increase crop yields would have a positive impact on Cuban society and economy. The use of microbial and non-microbial biostimulants can constitute an innovative alternative for this purpose.

Biostimulants are microorganisms or substances that favor plant nutrition, confer tolerance to abiotic stress and increase crop yield and quality. They also act directly on plant physiology and metabolism (5). The use of these natural products makes it possible to reduce the use of mineral fertilizers, which have a negative impact on the environment and health (6).

Bioproducts based on Plant Growth Promoting Bacteria (PGB) are part of the microbial biostimulants. Rhizobia are bacteria that belong to this group and they are studied mainly for the symbiosis they establish with leguminous plants and perform Biological Nitrogen Fixation (BNF) (7). Previous studies prove a positive effect of rhizobia inoculation on nodulation and growth of peanut crops (8, 9). However, in Cuba there is only one antecedent of these results in leguminous plants (4). Azofert® is a Cuban commercial inoculant based on nodulation factors and rhizobial strains that enhances the growth and yield of economically important legumes (10).

On the other hand, within the non-microbial biostimulants, a series of products derived from oligosaccharins, polysaccharides and natural oligosaccharides that are part of the cell walls of plants can be distinguished. Pectimorf® is a commercial product based on oligogalacturonides (OGAs) and its use attenuates abiotic stress in plants, increases growth and yield of crops such as beans (*Phaseolus vulgaris* L.) and rice (*Oryza sativa* L.) (11, 12).

As part of the strategy to increase yields in priority crops and conserve the agroecosystem, biostimulant combinations are offered to enhance complementary mechanisms that improve nutrition, growth, disease resistance and crop yield (13). The application of Azofert® and Pectimorf® in beans causes increases in nodulation and growth (14). In Cuba, these studies do not exist for the peanut crop, in spite of its nutritional properties and its potential as an exportable crop. The objective of the present study was to evaluate the effect of the combined application of microbial and non-microbial biostimulants on peanut nodulation and growth.

MATERIALS AND METHODS

Inoculation trials were carried out on peanut plants cv. Cascajal Rosado, where inoculants of two rhizobial strains were applied: *Rhizobium* sp. PL and *Rhizobium* sp. C145, and formulations based on these bacteria and Pectimorf®. Both strains come from peanut plant nodules and are part of the bacterial collection of the Department of Plant Physiology and Biochemistry of the National Institute of Agricultural Sciences of Cuba.

Rhizobial inoculants were prepared from a roast of strains, preserved in solid Mannitol-Yeast Extract (LM) medium, which were inoculated in 100 mL Erlenmeyer flasks with 10 mL of the same liquid medium. The cultures were kept in agitation at 150 r min^{-1} and 30 ºC, for 16 h. The purity of inoculants was monitored by Gram staining. The inoculants presented a concentration of 1.9 x 10^9 CFU mL^{-1} and 4.2 x 10^7 of *Rhizobium* sp. PL and *Rhizobium* sp. C145 strains, respectively.

The formulations consisted of a mixture of the bacterial inoculants with Pectimorf® (Registration No. RCF 017/18 and patent No. 22859/2003), at a final concentration of the latter of 8 mg L^{-1}. Taking into account the above, the following treatments were established, as described in Table 1

Table 1. Treatments carried out in the inoculant application trials and formulations based on *Rhizobium* and Pectimorf® strains on peanut plants

No.	Treatment
1	Absolute control
2	*Rhizobium* sp. C145
3	*Rhizobium* sp. PL
4	Formulated 1 (*Rhizobium* sp. C145 + Pectimorf®)
5	Formulated 2 (*Rhizobium* sp. PL + Pectimorf®)

Peanut seeds were placed on a typical Ferrallitic Red Leached soil substrate (15) in 0.27 kg pots containing the same volume of substrate (Tabla 2).

After sowing, peanut seeds were inoculated with 1 mL of rhizobia inoculants. The formulations were applied by imbibing the peanut seeds for 30 min before sowing. The experimental control consisted of inoculating seeds with 1 mL of sterile LM medium. Two seeds per pot were sown

Table 2. Soil chemical characteristics (depth: 0-20 cm)

pH H_2O	OM (%)	P_2O_5 (mg 100 g^{-1})	Ca^{2+}	Mg^{2+}	K^+
				($cmol_c$ kg^{-1})	
6.7	4.16	4.72	13.3	2.8	0.20

pH (potentiometry); OM, organic matter (Walkley Black); assimilable phosphorus by extraction with H_2SO_4 0.1N (Oniani's method); exchangeable cations by extraction with NH_4Ac 1 mol L^{-1} at pH 7, by complexometry (Ca^{2+} and Mg^{2+}) and by flame photometry (Na^+ and K^+)

and seven days after inoculation, thinning was performed, leaving one plant per pot. Plants were grown under controlled conditions 16 h light/8 h dark, 25-27 ºC, 50-70 % relative humidity and watered every day with tap water.

Nodulation, biochemical and growth variables were determined 45 days after planting. In the first group, the number of nodules and the dry mass of primary and secondary root nodules (g) were determined. Dry mass was determined with an analytical balance (+ 0.1 mg) (Sartorius CPA 3245), after keeping the nodules for 72 h at 75 ºC in an oven (BINDER, USA).

The biochemical variables determined were: nitrogen and phosphorus content in plant leaves, from 0.2 g of dry mass per treatment. For this purpose, the samples were digested with sulfuric acid and color was developed with Nessler's reagents and molybdenum blue for N and P, respectively (16). The relative total chlorophyll content (SPAD) in the central unveined part of the third leaf midrib was also determined using the Spad 502 portable chlorophyll meter. Stomatic conductance (mmol m^{-2} s^{-1}) was determined at 11:00 am, with a diffusion porometer (Delta-T Devices model AP3) and measurements were made in the abaxial zone of the third trifoliate leaf.

Regarding plant growth, the following were determined: total leaf area (cm^2), using the AM-300 portable meter, aerial and root dry mass (g) with an analytical balance (+ 1 mg) (Sartorius CPA 3245), after 72 h at 75 ºC in an oven (BINDER, USA). In addition, the height (cm), from the base of the stem to the terminal leaf bud and the root length (cm), from the base of the collar to the main root cap, were determined with a graduated ruler (+ 1 mm); the number of leaflets and the number of flowers.

Statistical analysis

A completely randomized design with 20 plants per treatment was used. Data were tested for normality (Bartlett's test) and homogeneity of variance (Kormogorov-Smirnov test). Analysis of variance of simple classification was applied, with Tukey's mean comparison test with p<0.05; to determine differences between means. The data were processed in Statgraphic Plus version 5.0 and Microsoft Excel 2016 was used for their representation.

RESULTS

In this research, it was possible to verify the effect of the application of inoculants based on *Rhizobium* strains, as well as formulations of these products in combination with Pectimorf®, on variables related to nodulation, physiology and growth of peanut plants cv. Cascajal Rosado, at 45 days of growth. The results showed that the inoculation of the *Rhizobium* sp. C145 strain increased the dry mass of the secondary root nodules, with respect to the experimental control. None of treatments affected the number of nodules and the dry mass of nodules in the main root, nor the number of nodules in the secondary root (Table 3).

On the other hand, the use of *Rhizobium* sp. C145 strain inoculants increased the relative content of total chlorophylls. All treatments increased stomatal conductance, especially when the *Rhizobium* sp. C145 strain was inoculated, the treatment with the highest values of this variable (Table 4).

Inoculation of the formulation with the *Rhizobium* sp. C145 strain produced increases in nitrogen and phosphorus content in plant leaves. A similar effect was observed with the use of formulations based on *Rhizobium* sp. PL and Pectimorf® on phosphorus content (Table 3).

Treatment effects on the growth of peanut plants were also observed. The results showed that the application of the two formulations caused significant increases in height. A similar effect was found with the use of the *Rhizobium* sp. C145 strain formulation on root length (Figure 1A). None of the treatments affected the number of leaflets (Figure 1B). However, the application of the formulated mixture of *Rhizobium* sp. C145 and Pectimorf® caused an increase in the number of flowers (Figure 1B).

Finally, the use of the *Rhizobium* sp. PL strain and the corresponding formulation produced peanut plants with a smaller leaf area than those of the control treatment. None of treatments affected the dry mass of the aerial and root parts of peanut plants (Table 5).

DISCUSSION

According to the results of the typical Ferrallitic Red Leached soil analysis (Table 2) it presented slightly acid pH,

Table 3. Effect of inoculants and formulations based on *Rhizobium* and Pectimorf® strains on nodulation of peanut plants

Treatments	Main root		Secondary root	
	Number of nodules	Dry mass of nodules (g)	Number of nodules	Dry mass of nodules (g)
Control[a]	18.5+1.7	0.011+0.001	22.6+2.4	0.007+0.001 b
Rhizobium sp. C145	18.4+2.0	0.012+0.002	33.4+5.7	0.013+0.002 a
Rhizobium sp. PL	20.6+2.3	0.013+0.002	23.9+5.0	0.008+0.002 b
F1[b]	21.2+2.4	0.009+0.001	34.3+6.5	0.004+0.001 b
F2[c]	16.5+0.8	0.010+0.001	24.9+4.5	0.007+0.001 b
ESx	1.9 ns	0.001 ns	5.0 ns	0.002*

[a]Plants inoculated with Manitol medium - sterile yeast extract; [b]Plants treated with Formulate 1 (*Rhizobium* sp. C145+Pectimorf® 8 mg L^{-1}; [c]Plants treated with Formula 2 (*Rhizobium* sp. PL+ Pectimorf® 8 mg L^{-1}); Means + standard error of the mean are shown. Means with equal letters in the same column do not differ significantly (Tukey HSD p<0.05, n=10)

Table 4. Effect of inoculants and formulations based on *Rhizobium* and Pectimorf® strains on biochemical variables of peanut plants

Treatments	Relative total chlorophyll content (SPAD)	Stomatal conductance (mmol m² s-1)	Macronutrient content (%)	
			N	P
Control[a]	39.9+1.3bc	42.6+2.6 c	3.18+0.02 c	0.38+0.03 b
Rhizobium sp. C145	45.3+1.5 a	168.9+9.1 a	3.16+0.02 c	0.48+0.04 ab
Rhizobium sp. PL	42.9+1.1 ab	132.9+4.0 b	3.17+0.01 c	0.47+0.04 ab
F1[b]	37.3+1.1 c	129.9+4.1 b	3.39+0.02 a	0.54+0.06 a
F2[c]	36.2+0.9 c	145.6+8.9 ab	3.30+0.02 b	0.43+0.03 ab
SEx	1.23*	6.3***	0.019*	0.040*

[a]Plants inoculated with Manitol medium - sterile yeast extract; [b]Plants treated with Formulation 1 (*Rhizobium* sp. C145+Pectimorf® 8 mg L-1); [c]Plants treated with Formulation 2 (*Rhizobium* sp. PL+Pectimorf® 8 mg L-1); Means + standard error of the mean are shown. Means with equal letters in the same column do not differ significantly (Tukey HSD p<0.05. n=10)

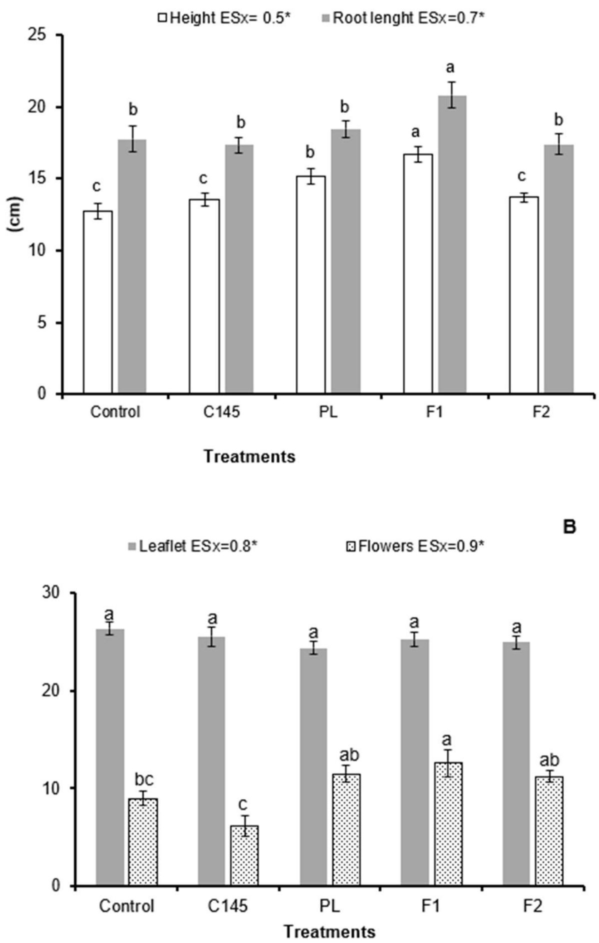
high content of organic matter and medium assimilable phosphorus, as well as low levels of potassium, calcium and magnesium (15).

Although the inoculation of *Rhizobium* sp. C145 did not cause an increase in the number of nodules on peanut plants, it produced nodules with a higher mass than the rest of the treatments. Previous research confirms that native rhizobial strains hinder inoculation success due to competition from native strains (17). However, this does not seem to be the case with *Rhizobium* sp. strain C145.

Another factor to be taken into account to explain the above is the specificity of the plant and the bacterium to establish symbiosis (18). The fact that inoculants based on *Rhizobium* sp. C145 strain, with lower concentration (4.2 x 10^7 CFU mL-1) causes greater nodular mass than the application of *Rhizobium* sp. PL strain, with higher concentration (1.9 x 10^9 CFU mL-1); may contribute to explain a greater specificity of the first of these strains with peanut plants. Nodules with greater mass would imply a greater content of bacteroids in their interior and with it the potentiation of the BNF (19).

The increase in the relative content of total chlorophylls, nitrogen-rich molecules, in peanut plants treated with inoculants of *Rhizobium* sp. strain C145 indicates a positive effect of these products on BNF. Chlorophyll synthesis is known to be closely related to the availability of nitrogen to the plant and the plant's ability to assimilate it. BNF allows a higher nitrogen supply, which would lead to an increase in photosynthetic pigment synthesis (20.

Chlorophyll content and stomatal conductance are some of the determinants that govern the photosynthesis process (21). Both variables are enhanced in peanut plants with the use of *Rhizobium* sp. C145 strain and its corresponding formulate. With this evidence, plants with a higher content of chlorophylls, a pigment that would allow a greater radiation harvest; and on the other hand, plants with greater stomatal conductance. which would allow a greater incorporation of CO_2 into the Calvin cycle, regardless of sharing a similar leaf area (Table 5), would enhance the photosynthesis process in them (22, 23). This would lead to a greater gain of carbon skeletons that can be used in protein synthesis, a process that would benefit from the activity of this bacterium in the nodules of peanut plants.



Control treatment: plants inoculated with Manitol medium - sterile yeast extract. F1: Formulated 1 (*Rhizobium* sp strain. PL+Pectimorf® 8 mg L-1. F2: Formulated 2 (*Rhizobium* sp strain. PL+Pectimorf® 8 mg L-1). C145+Pectimorf® 8 mg L-1). F2: Formulated 2 (*Rhizobium* sp strain. PL+Pectimorf® 8 mg L-1). Bars represent means + standard error. Equal letters. Not statistically different (Tukey HSD p<0.05n=10)

Figure 1. Effect of inoculants and formulations based on Rhizobium and Pectimorf® strains on height, root length (A) and on the number of leaflets and flowers (B) of plants of peanut

Table 5. Effect of inoculants and formulations based on *Rhizobium* and Pectimorf® strains on the growth of peanut plants

Treatments	Leaf area (cm^2)	Dry mass (%)	
		Aerial part	Root
Control[a]	601.4+15.0 a	1.8+1.7	0.20+0.01
Rhizobium sp. C145	543.1+22.6 ab	1.8+2.0	0.18+0.01
Rhizobium sp. PL	510.8+22.8 b	2.1+2.3	0.20+0.01
F1[b]	560.4+15.8 ab	2.1+2.4	0.17+0.01
F2[c]	525.8+14.6 b	1.6+0.8	0.17+0.01
SE x	18.6*	1.9 ns	0.0014 ns

[a]Plants inoculated with Manitol medium - sterile yeast extract; [b]Plants treated with Formulation 1 (*Rhizobium* sp. C145+Pectimorf® 8 mg L^{-1}); [c]Plants treated with Formulation 2 (*Rhizobium* sp. PL+Pectimorf® 8 mg L^{-1}); Means + standard error of the mean are shown. Means with equal letters in the same column do not differ significantly (Tukey HSD $p<0.05$. n=10)

Recent research shows such effects with formulations based on rhizobia strains and OGAs in the cultivation of beans (24).

In Cuba, there is only one published study on the benefits of microbial biostimulants in peanut cultivation. This study showed that the co-inoculation of the arbuscular mycorrhizal fungus *Glomus cubense* and the commercial product Azofert® significantly increased aerial dry mass and crop yield (4). However, the results of this research are the first in Cuba to demonstrate such effects in peanut crops, with the application of formulations based on combined microbial and non-microbial biostimulants. Other studies show that the combined application of Chitosan and Efficient Microorganisms potentiate the development of bean and peanut plants (25).

On the other hand, in the present investigation, an effect of formulations with Pectimorf® on the nitrogen and phosphorus content of peanut plants was proven. Previous studies showed that the foliar application of 344 mg ha^{-1} of the biostimulant caused significant increases in foliar nitrogen content in lettuce plants (*Lactuca sativa* L.). 12 and 30 days after sowing, which enhanced crop yield (26).

On the other hand, in the present investigation, an effect of formulations with Pectimorf® on the nitrogen and phosphorus content of peanut plants was verified. Previous studies showed that the foliar application of 344 mg ha^{-1} of the biostimulant caused significant increases in foliar nitrogen content in lettuce plants (*Lactuca sativa* L.). 12 and 30 days after sowing, which favored crop yield (26).

The application of Pectimorf® affects some physiological processes and stimulates the growth of beans and potatoes (11, 27). The stimulation of this product is attributed to its auxinic activity and positive effect on photosynthesis (28). Longer roots, as a result of Pectimorf® effect would explore a larger soil area, which would lead to a greater absorption of nutrients such as nitrogen and phosphorus, especially in plants treated with the *Rhizobium* sp. strain C145.

Previous research shows that as a result of root development, plant leaf growth is enhanced (11, 29). In the present investigation it is shown that none of treatments exceeds the control plants in the dry mass of the aerial and root part and the number of leaflets. However, the positive activity of Pectimorf® on root height and length was observed, an effect that was not observed with the use of rhizobium-based inoculants alone.

Peanut flowering covers 80 % of its development cycle and overlaps with fruiting (30). Enhancing this phase of the crop with the use of biological products could provide physiological benefits that would subsequently translate into higher yields. The application of formulations based on the *Rhizobium* sp. C145 strain and Pectimorf® can contribute to this end. This is the first evidence in Cuba that proves the effect of the combination of bacterial inoculants with Pectimorf®, in the flowering phase of peanut.

The results presented here constitute an approach to the possibility of increasing the growth and yield of a crop little studied in Cuba. The use of the combination of nationally produced, environmentally friendly and relatively cheap biostimulants is an attractive opportunity that contributes to the country's food security.

CONCLUSIONS

Peanut is a crop that has not been extensively studied in Cuba, compared to other legumes such as beans and soybeans. The application of formulations based on the *Rhizobium* sp. C145 strain and the commercial product Pectimorf® proved to be effective in increasing nodulation and growth of peanut. The application of these products to the legume may be a desirable and even necessary management option to improve its nutrition and productivity.

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