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# Effects of a New *Rhizobium*-Based Biofertilizer (Fertiriz) on Growth and the Yield of Cowpea (*Vigna unguiculata (L.) Walp*)

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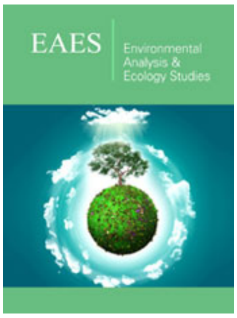


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## Abstract

Biofertilizers are an agroecological alternative to improve plants' development by increasing nutrient availability and acquisition. A technical evaluation trial was carried out to estimate the effect of a new *Rhizobium*-based biofertilizer (Fertiriz) on the development of cowpea bean (*Vigna unguiculata (L.) Walp*). The study included two assays in cowpea according to complete randomized designs with three replications. The first experiment was in chambers under semi-controlled conditions and comprised nine treatments: an absolute control (without any fertilizer), 50 and 100% mineral fertilizer (NPK), three lots of the bioproduct, and their combination with 50% NPK. The second assay was in an open field experiment and just involved the three lots and an absolute control; no mineral fertilizer was used. The first *Rhizobium* inoculation was before planting (10.8ml.kg$^{-1}$ of seeds); two re-inoculations were carried out at 21 and 45 days after sowing. Treatments confirmed the best results with the total recommended chemical fertilizer without statistical differences when 50% of mineral fertilizer was combined with the new biofertilizer inoculations. The general development (stem height and thickness), the number of pods per plant and grains yield were significantly higher in both experiments of cowpea when the new biofertilizer was used, compared to the non-inoculated control. The evaluation of nodulation showed a good number of nodules on roots, and most of them were active. Results validate technically the effectiveness of the new biofertilizer Fertiriz and demonstrated that it can save 50% of mineral fertilizer without affect the development or yield in cowpea.

**Keywords:** Biofertilizer; Biological nitrogen fixation; Plant growth-promoting *rhizobacteria*; Sustainable agriculture

## Introduction

Conventional agricultural systems during the Green Revolution increased considerably the food production and boost the crop yields up to unimaginable values. That kind of agriculture was very productive, but it has been high economic-environmental costs: loss of biological diversity, reduction of forest resources, soil erosion, greenhouse gas emissions, climatic changes, among others [1,2]. Under the difficult conditions imposed by climate change and the complexities of the global economy, agriculture faces the challenge of being a sustainable and efficient way to produce food. Due to the exorbitant cost of nitrogen fertilizers and the environmental damages on use of chemical fertilizers, biofertilizers could be the best alternative to chemical inputs and will help in reducing the rate of ecological disturbance to a great extent [3]. There are many definitions about 'biofertilizers', but in essence they are products that contain beneficial microorganisms and enhance soil fertility and crop productivity [4]; they are considered fundamental components of the agricultural sustainability [5]. For that reason, incorporating microorganisms, mainly as biofertilizers, into plant culture systems is a good strategy to increase yields and to decrease the use and environmental impact of chemical fertilization. A wide group of plant growth-promoting *rhizobacteria* has shown an important role in sustainable agriculture [6]. Among the bacterial genera most used in the production of biofertilizers, *Rhizobium*, *Bacillus* and *Pseudomonas* stand out [7].

Especially *Rhizobium* plays an essential biological function that helps in fixing nitrogen especially in leguminous plants like cowpea or common beans because these soil bacteria have the potential to form specific structures (called nodules) in legume roots. In effective nodules, they fix the atmospheric Nitrogen (N2) as ammonia and like this, plants take it for its growth. The Biological Nitrogen Fixation (BNF) contributes to 69% of the global nitrogen fixation and legume-rhizobia symbiosis supplies about 80-90% of the total N requirement of legume [3]. Recently, Kebede [8] published a wide review about the contribution, utilization, and improvement of legumes based BNF in agricultural systems. Symbiotic nitrogenous *rhizobia* can also solubilize phosphorus [9]. Several reviews deal with the complex biological interactions leading to infection and nodulation by *rhizobia* [10,11], especially in cowpea (*Vigna unguiculata (L.) Walp*) an important nutritional crop, with 23-32% protein in its dry grains, essential amino acids and other nutrients [12].

In Cuba, some researchers had studied *Rhizobium* or *Bradyrhizobium* strains, including *Rhizobium* or *Bradyrhizobium-based* biofertilizers (Biofert® or Azofert F®) [13,14]. Delgado et al. [15] showed some benefits of co-inoculation of arbuscular *mycorrhiza*l fungi and *rhizobia* in bean crops. Due to its positive effects on the crop's growth and yield, to develop *Rhyzobium*-based biofertlizers for multiple leguminous crops is a priority in latest research [16] around the world. Nevertheless, most of them, like in Cuba, are not commercial biofertilizers or they are not currently produced for different reasons; because of that, it needs further studies on these issues. In this sense, the Business Group of Biological and Pharmaceutical Laboratories (LABIOFAM) has obtained a new biofertilizer (registered as Fertiriz) from a good *Rhizobium* strain previously isolated and studied. As part of the new biofertilizer' technical evaluation, the objective of this work was to determine the effect of the new *Rhizobium*-based biofertilizer Fertiriz on the development of cowpea crop var. 'Cubanita 666'.

## Materials and Methods

Experiments were developed according to randomized complete designs with three replications: one assay in chambers and the second on open field, all of them in areas from the Research Institute of Tropical Roots and Tubers Crops, in Santo Domingo, Cuba.

### Plant material and the experimental design

The Cuban commercial variety 'Cubanita 666' of cowpea was used; seeds were obtained from the national service of certified seed. Two experiments were developed: the first one, in chambers with a conventional mixed substrate (70% organic matter (filter cake) and 30% soil). Two or three seeds previously inoculated or not, according with each treatment, were planted at 0.70mx0.25m.

Treatments included various combinations as well as two additional un-inoculated control treatments: an absolute control (without any fertilization) and a control with 100% of the complete formula (NPK) 9-13-17) at a dose of 70kg ha-1. Although in Cuba, for different reasons, the mineral fertilization is not very common in cowpea [17], globally it's wide recognized the importance of a well nutritional balance in this crop, consequently, different rates of NPK (from 30 to 150kg ha$^{-1}$) has been used [18,19]. The 'lots' were the result of three different fermentation processes of the biofertilizer Fertiriz under the same conditions. Total treatments were:

T1- Absolute Control (without any fertilization).

T2- Control (100% of the recommended NPK mineral fertilizer (9-13-17).

T3- Lot 1 *Rhizobium* (Lot 1 from the new Rhizobium-based biofertilizer).

T4- Lot 2 *Rhizobium*.

T5- Lot 3 *Rhizobium*.

T6- Lot 1 *Rhizobium*+50% NPK.

T7- Lot 2 *Rhizobium*+50% NPK.

T8- Lot 3 *Rhizobium*+50% NPK.

T9- 50% NPK (half of the recommended NPK mineral fertilizer).

Due that in Cuba rarely the cowpea crop receives mineral fertilizer [17], the second experiment was conducted in the field without mineral fertilizer and only included four treatments:

T1- Absolute Control (without any fertilization).

T2- Lot 1 *Rhizobium* (Lot 1 from the new *Rhizobium*-based biofertilizer).

T3- Lot 2 *Rhizobium* (Lot 2 from the new *Rhizobium*-based biofertilizer)

T4- Lot 3 *Rhizobium* (Lot 3 from the new *Rhizobium*-based biofertilizer)

In all cases, the inoculation was carried out by coating the seed (only with *Rhizobium* at a dosage of 10.8 ml.kg$^{-1}$ of seeds) prior to sowing, and immediately, they were planting in chambers or in field according to each experiment. The *Rhizobium* used was the new biofertilizer (Fertiriz) produced as a liquid bioproduct by the Business Group of Biological and Pharmaceutical Laboratories (LABIOFAM) (Cuba). Applications at 21 and 45 days after planting (in both assays) were made using a manual sprayer directly on the soil around the roots zone with a dosage of 500ml ha$^{-1}$ (2,5ml L$^{-1}$); re-inoculations can improve the colonization process. The farm trial was only irrigated three times: after planting and before each biofertilizer application. All the recommended cultivation practices were followed, including plant protection measures.

 

## Measurements and statistical analysis

At 15, 30 and 45 days after planting (dap), measurements of plant height (cm) (with a tape, measured from the basis of the stem to the terminal bud) and stem diameter/stem thickness (mm) (with a caliper), were recorded on ten randomly selected plants from each treatment during the first assay. Same variables were measured similarly in the field assay. The presence and quality of nodules were sampled at 50 and 75 days after sowing (das) in both experiments. Nodules per sampled plants (10) were detached from the roots, counted and annotated. After being gently washed, each selected nodule (10 per plant, per treatments) was cut transversally to observe the internal color. Nodules' nitrogenase activity were verified by a macroscopic study: identified as positive those nodules with reddish coloration inside, which indicates the production of leghemoglobin [20]. In both experiments, at harvest (75das), the following variables were evaluated in ten randomized plants per treatment: number of pods per plant, number of seeds per pod and weight of 100 seeds (g). For each selected plant, the seeds were separated from their pods and counted; 100 seed weight (g) per plot was determined. Individual net plots per treatments were harvested at maturity to estimate the yield (t $ha^{-1}$). The collected data by each assay were subjected to the Analysis of Variance (ANOVA) appropriate to the completely randomized design. All statistical procedure was according to the experimental design and using the tools from the IBM.SPSS/PC+statistical package version 23.0 for Windows® [21]. Whenever differences existed among means values, the comparison of them was carried out with the test of Tukey for P≤0.05.

## Results

The survival percentage of plants after a week behaved above 98% in both assays. All plants, regardless of the treatment received, maintained a good appearance and their typical morphological characteristics for each variety in both developed assays. Nevertheless, a substantial increment on plants height and stem thickness were observed, which were significantly different at 0.05 level of significance to the absolute control (without fertilizer) (Figure 1), as much in chambers as in the field. In the chambers' assay, during the first 15 days there were no differences between the treatments for both variables, however, after a month difference began to be appreciated between them, especially for the stem thickness with the highest value for treatments with 100% of mineral fertilizer (NPK) or with 50 % NPK plus *Rhizobium* inoculations (Fertiriz biofertilizer) without significant differences between them.

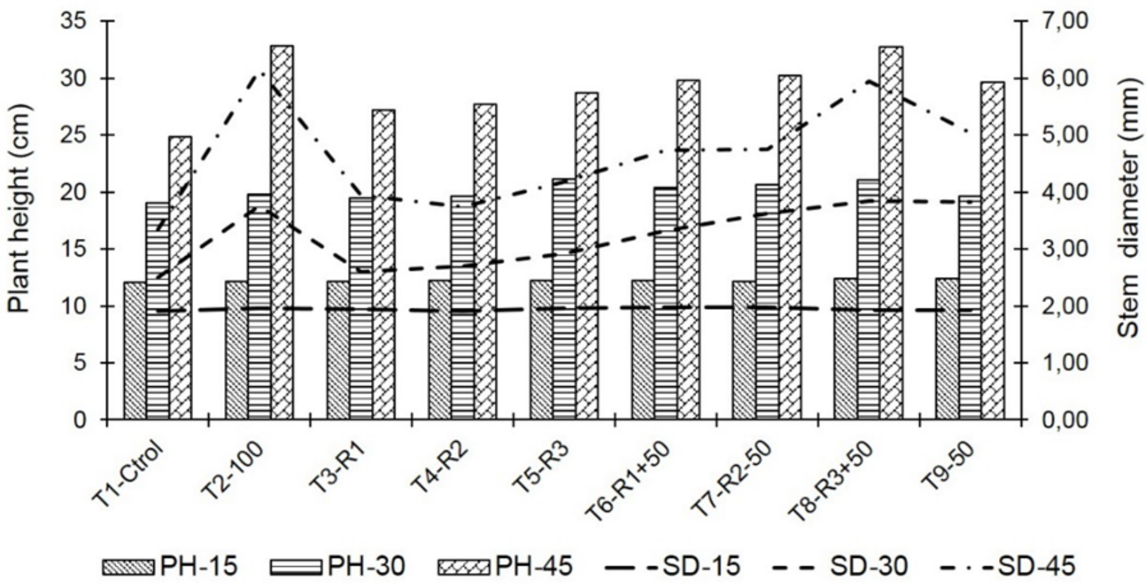


**Figure 1:** Vegetative development of the cowpea var. 'Cubanita 666' at 15, 30 and 45 days after sowing, with the use of Fertiriz, a *Rhizobium*-based biofertilizer (in chambers).
Legend: PH-plant height (cm) at 15, 30 and 45 days after sowing; SD- stem diameter/thickness (mm) at 15, 30 and 45 days after sowing.
T1-Ctrol: Absolute control (without fertilizer); T2-100: 100% mineral fertilizer (NPK, 9-13-17); T3-R1, T4-R2, T5-R3: three lots of the new *Rhizobium*-based biofertilizer; T3-R1+50, T4-R2+50, T5-R3+50: three lots of the new biofertilizer with the addition of 50% of the mineral fertilizer; T9-50: 50% of the mineral fertilizer.

Based on the data collected, it appeared that different combinations (*Rhizobium*+mineral fertilizer) may influence positively the quality of plants, especially for plant height and stem thickness. After 30 days, the plants continued their differential development, increasing the value of both variables but the most evident results were detected after 45 days of planting. The higher values were measured for treatments T2-100 (100% NPK) and T8-R3+50 (*Rhizobium* Lot 3+50% NPK), without statistical differences between them neither with remain combinations that included the chemical fertilizer. An increment of Control treatment had the lowest plants with 24.83cm for plant height and 3.33cm for stem diameter. The best treatments showed an increment of around

8.0cm (24.4%) and more than 2.6mm (43.9%) in plant height and stem thickness, respectively, from the absolute control. In general, inflorescences in the cowpea's assays appeared after 25 days of planting and the first flowers opened at 30-35 days. Plants from the control treatment (without fertilization), flowered less homogeneously (flowers of the same inflorescence with different development or with only one opened flower per inflorescence); while those inoculated showed at least 3-4 well developed flowers per inflorescence. The presence of nodules per plant was sampled at 50 and 75 days (harvest), observing a greater number of them in the inoculated plots (Figure 2) and, in general, a decrease in their number over time. Most of nodules were effective, according to the internal reddish color revealed when they were transversally cut. Nodules were mainly spherical but many of them showed an irregular surface, especially during their advanced development stage (more old).

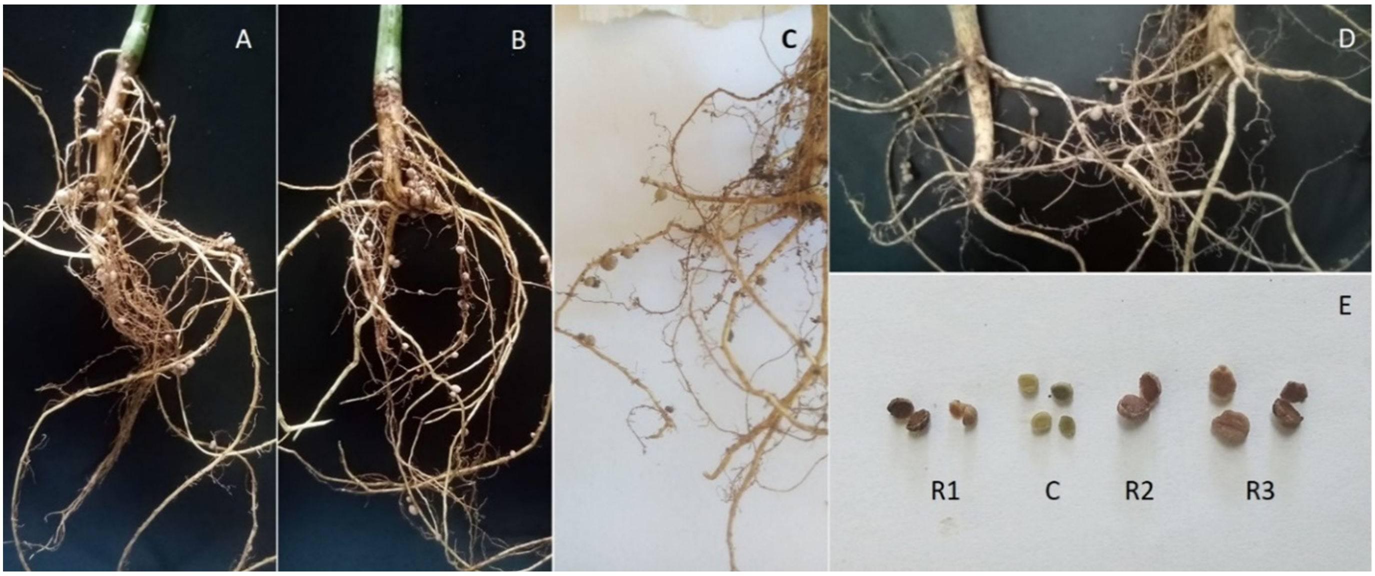


**Figure 2:** Nodulation in cowpea var. 'Cubanita 666' inoculated with three lots of a new *Rhizobium*-based biofertilizer (in chambers).
Legend: A and B- nodulation 50 days after sowing. C and D- nodulation at 75 days after sowing. E- nodules collected at harvest (75 days) cut transversally; R1, R2 and R3- nodules of plants inoculated with the three lots of the biofertilizer; C-control (without inoculation).

During the harvest, the observed number of pods varied from eight to seventeen with the worse results for the absolute control (9.9 pods per plant) (Table 1). The best values were determined when *Rhizobium* was combined with 50% NPK fertilizer (12.8 pods) 100% NPK mineral fertilizer (12.5 pods). Interestingly, no significant differences were detected at 0.05 level of significance between all treatments with Fertiriz+50% NPK and 100% NPK. Statistical differences were found for the average number of nodules per plant, number of seeds per pod and the weight of 100 seeds; in all cases, the absolute control showed the lower values (15.8, 7.7, 19.39g, respectively). Interestingly, the higher nodulation was observed in treatments with *Rhizobium* and 50% of mineral fertilizer (>65%), with significant differences over the control with 100% of NPK fertilizer. Even, in those variants where only Fertiriz was added, the number of nods was superior to the 100% NPK treatment. The weight of 100 seeds (g) is one of the most important variables for the yield and the best results were observed in those treatments where the new biofertilizer Fertiriz was used together with the 50% of mineral fertilizer, without statistical differences with the control with 100% of NPK fertilizer. Nevertheless, it is of noticing that values were superior numerically for the combination Lot 3 of Fertiriz plus 50% of NPK fertilizer (22.76g).

**Table 1:** Effects of the new *Rhizobium*-based biofertilizer (Fertiriz) with a mineral fertilizer (NPK) combination in the cowpea var. 'Cubanita 666' (in chambers).

*Means followed by the same letter in a same column are not significantly different according to Tukey's HSD test (P≤0.05).

Legend: PH-45, plant height (cm) at 45 days after sowing; SD-45, stem diameter/thickness (mm) at 45 days after sowing.

T1-Ctrol: Absolute control (without fertilizer); T2-100: 100% mineral fertilizer (NPK, 9-13-17); T3-R1, T4-R2, T5-R3: three lots of the new *Rhizobium*-based biofertilizer; T3-R1+50, T4-R2+50, T5-R3+50: three lots of the new biofertilizer (R1, R2, R3) with the addition of 50% mineral fertilizer; T9-50: 50% mineral fertilizer

| Treatments | No. Nodules | No. Pods/Plant | No. Seeds/Pod | Weight of 100 Seeds (g) |
|---|---|---|---|---|
| T1-Ctrol | 15.8 e | 9.4 b | 7.7 b | 19.39 d |
| T2-100 | 21.9 cd | 13.4 a | 11.1 a | 22.22 ab |
| T3-R1 | 28.5 bcd | 11.2 ab | 9.8 ab | 20.82 c |
| T4-R2 | 28.6 bcd | 11.3 ab | 9.3 ab | 20.95 c |
| T5-R3 | 29.4 abc | 11.4 ab | 9.4 ab | 20.91 c |
| T6-R1+50 | 35.3 ab | 13.2 a | 11.2 a | 22.26 ab |
| T7-R2+50 | 35.6 ab | 13.0 a | 11.2 a | 22.57 ab |
| T8-R3+50 | 36.2 a | 13.4 a | 11.9 a | 22.76 a |
| T9-50 | 26.8 cd | 11.8 ab | 10.1 ab | 21.40 bc |
| $S\bar{x}$ | 1.65* | 0.80* | 0.62* | 0.28* |

Consequently, statistically significant difference at P≤0.05 was also recorded in grain yield due to different treatments under field conditions (Figure 3), but again, the worse results were observed for the absolute control (0.81t $ha^{-1}$) which was statistically inferior to all treatments. Treatment with 100% of mineral fertilizer NPK was the best (1.38t ha-1) without significant differences with treatments that combined the inoculation of three lots of Fertiriz and 50 % NPK, with important increments compared to the uninoculated control (at least, more than 0,5t $ha^{-1}$). In the field experiment, results were similar: in all cases, when the new *Rhizobium*-based biofertilizer was used, it was detected a favorable growth and superior to the control treatment, where any fertilization source was applied, from the first stages of the plantation. A satisfactory colonization with high number of effective nodules (Figure 4); excellent plant development and a good number of pods per plant were observed when the biofertilizer was applied (results not showed). No significant differences were observed for lots of treatments at P≤0.05.

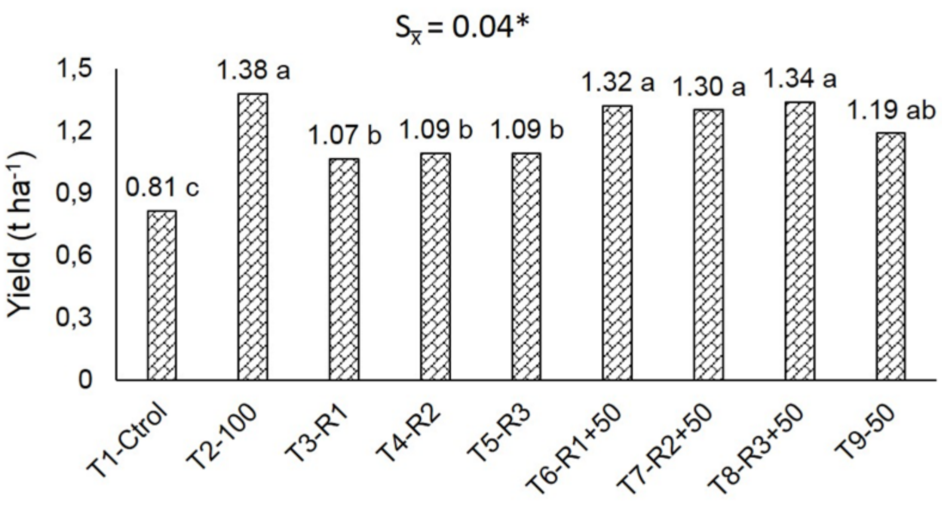


**Figure 3:** Effect of *rhizobia* inoculation with three lots of a new Rhizobium-based biofertilizer (Fertiriz) and a mineral fertilizer (NPK) combination in cowpea var. 'Cubanita 666' (in chambers).
Legend: T1-Ctrol: Absolute control (without fertilizer); T2-100: 100% mineral fertilizer (NPK, 9-13-17); T3-R1, T4-R2, T5-R3: three lots of the new *Rhizobium*-based biofertilizer; T3-R1+50, T4-R2+50, T5-R3+50: three lots of the new biofertilizer (R1, R2, R3) with the addition of 50% mineral fertilizer; T9-50: 50% mineral fertilizer.
*Means followed by the same letter in a same column are not significantly different according to Tukey's HSD test (P≤0.05).

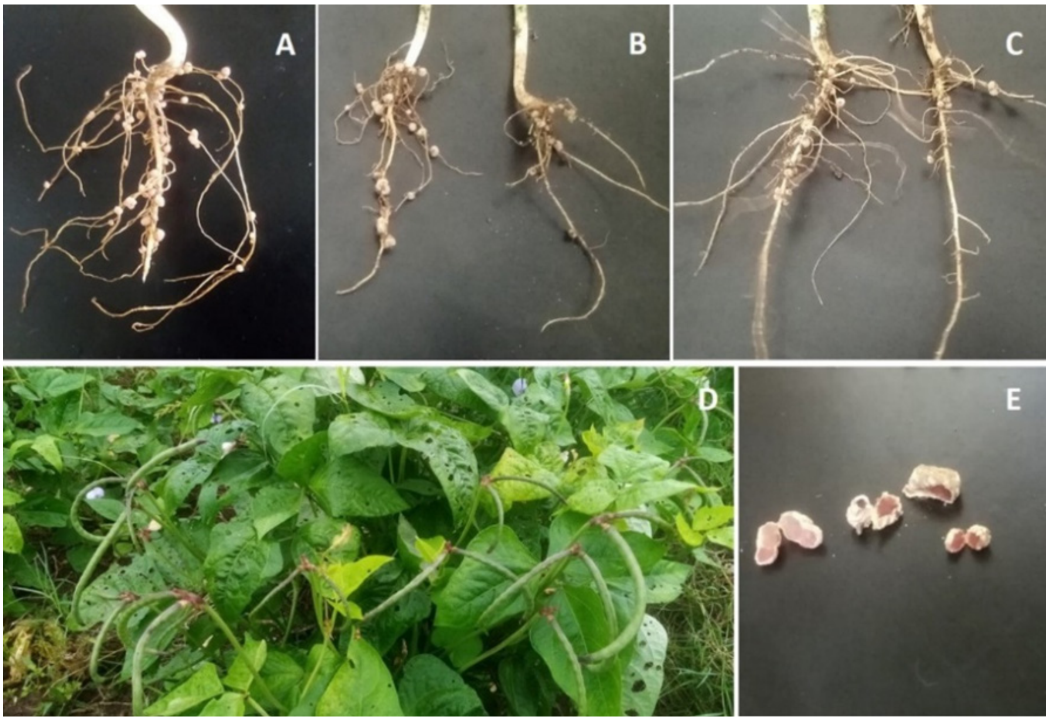


**Figure 4:** Evidence of nodulation and plant development in cowpea var. 'Cubanita 666' inoculated with three lots of a new *Rhizobium*-based biofertilizer (Fertiriz) in the field.
Legend: A- roots from plant inoculated with *Rhizobium* lot 3 (50 days after sowing); B, C- roots from plants inoculated with three lots of *Rhizobium* (75 days after sowing), D- sheathed cowpea plants (lot 1); E- effective nodules (lot 1).

With abundant nodules and the internal reddish color as indicator of its effectiveness, their presence validates the positive effect of the new biofertilizer Fertiriz already observed during the previous assay in chambers. Nodules mainly exhibited a globose/ spherical shape, but cylindrical and irregular too. Some nodules were found in roots from non-inoculated plants (absolute control) but in most of them, the cross section showed nodules with a very faint pink or green color, as in chambers was detected too. Nodules measured from less than 2mm to more than 4mm, but many of them had between two and 4mm. As in chambers, at harvest time not only good development was observed in terms of the quantity and quality of the produced pods, but also interesting nodulation both in the proximal area to the stem and in the distal area of roots, well away. Interestingly, large nodules (3-4mm or more) and mostly active according to cross section, were found in plants previously inoculated.

The obtained yield under farm conditions confirmed the technical effectiveness of the Fertiriz as new biofertilizer (Figure 5). No statistical differences were detected between the three lots inoculation. The lowest yield value was obtained from the control treatment (0.87t $ha^{-1}$), while the highest yield values were obtained from the seed inoculation treatments which increased by 45.9 % or more. The use of Fertiriz alone incremented the cowpea yield in 0.4t ha-1, demonstrated the effectiveness of the new biofertilizer, already detected in chambers with the same variety, and probed that it can be incorporated to this crop production system. No significant differences were observed between the development of cowpea plants in chambers or in the field, except for the presence of some pests, such as: chrysomelids (*Diabrotica sp*) and sucking insects (*Empoasca spp.*) with an incidence rate of 20% or less, and also quite a lot of whitefly (*Bemisia tabaci*) (40%) in the chambers' assay. For its control, a biological product Nicosave was applied at a dose of 800 ml per liter of water. In the field, only appeared some chrysomelids (*Diabrotica sp*) and sucking insects (*Empoasca spp.*) with a low incidence rate (<10%); the same bioplaguicide was apply three times each seven days.

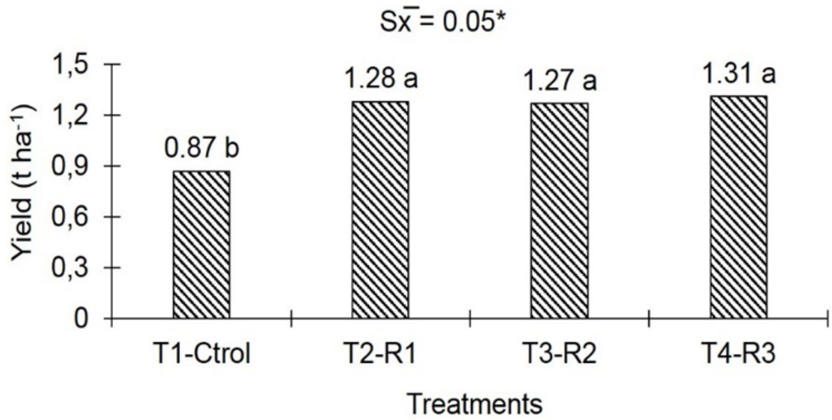


**Figure 5:** Effect of *rhizobia* inoculation with three lots of a new Rhizobium-based biofertilizer (Fertiriz) on the yield in cowpea var. 'Cubanita 666' (in the field).
Legend: T1-Ctrol: Absolute control (without inoculation); T2-R1, T3-R2, T4-R3: three lots of the new *Rhizobium*-based biofertilizer (Fertiriz).
*Means followed by the same letter in a same column are not significantly different according to Tukey's HSD test (P≤0.05).

In general, the Fertiriz biofertilizer showed a superior performance in all the tested parameters or variables when compared to the uninoculated control, in both experiments (under chambers or field conditions). Results were related to an effective symbiotic association between *Rhizobium* and cowpea plants of 'Cubanita 666' variety. Interestingly, the study has demonstrated the great potential of Fertiriz to improve cowpea growth and yield under the Cuban agricultural conditions in order to reduce synthetic fertilizers in agriculture for promoting legumes production by a sustainable technique.

## Discussion

The study assessed the effect of *Rhizobium* inoculation by the new biofertilizer Fertiriz on the cowpea crop var. 'Cubanita 666'. Experiments showed the best general performance when the bacteria were included, with positive effects on plant growth parameters due to the significant increase in most of the evaluated variables: plant height, stem diameter/thickness, number of pods/plant, number of seeds/pod, weight of 100 seeds and yield, compared with un-inoculated treatment (absolute control). In absence of others possible reasons associated to the observed differences, the new *Rhizobium*-based biofertilizer was responsible for these results. In both assays, the un-inoculated control exhibited relatively poor growth in all growth parameters evaluated. In absence of others possible reasons associated to the observed differential positive results in the inoculated treatments, might be due to the symbiotic relationship between the Rhizobium inoculated and root nodules of legume. Several authors have reported similar findings in cowpea and common beans [13,22,23]. Indeed, the use of *rhizobial* inoculants is a common agronomic practice to ensure adequate Nitrogen (N) availability for crops. Around 80 % of biologically fixed N comes from this symbiosis in different legume species [24]. Previous research have demonstrated an increase of fixed N in inoculated legume plants with *Rhizobium* relative to un-inoculated ones [25,26].

The significant increase of plant height and stem diameter after the inoculation of seeds with Fertiriz, coincides with previous similar research. The *Rhizobium* application increased crop growth by improving plant height, seed germination, leaf chlorophyll, and N content [23,27]. Studies made by Iqbal et al. [16], determined the existence of a linear correlation between the height of the plant and the circumference of the cowpea stem. Presumably, a good initial colonization of seeds during the inoculation process had an important effect on the plant growth, as was already reported by Noumavo et al. [28]. In addition, *Rhizobium* also produce significant amount of phytohormones such as Indole Acetic Acid (IAA) that promote plant growth [3] and can also solubilize phosphorus [11,29]. The appearance and characteristics of flowers coincided in general terms with the information recorded in the crop technical guide [17]. The proper nutrient management is one of the main factor for increasing the percentage of nutrients availability in the soil which influences better growth and development of the crop, and its yield. In the present study, a general good nodulation was observed, but, nodulative aspects of the cowpea var. 'Cubanita 666' were especially benefited by the Fertiriz applications in chambers. Treatments with Fertiriz+50% NPK showed higher number of nodules per plant (35.6) than the alternative with 100% NPK (21.9), expressed in an increment of 62,5% of nods; referred to the absolute control (15.8) differences were more than 125%.

During the field experiment, treatments with Fertiriz showed similar tendency. All fertilized variants, independently of what kind of source (lot) was used, statistically overpassed the absolute control. It coincides with results informed by Jiménez et al. [14] and dos Santos et al. [30] in common beans, or by Issoufa et al. [31] in cowpea, showing the efficiency of the inoculant introduced in the crop compared with chemical fertilizer. In any typical process to produce a biofertilizer, strains selected as inoculants are typically grown at a fermenter scale and used to coat seeds of compatible legume hosts to introduce them into the soil [32]. Some reinoculation actions improve the process and both used alternatives were effective according to the obtained results. Because of that, two applications of Fertiriz at 21 and 45 days after sowing represented an approach that could enhance the rhizobial inoculum and to reinforce its biological activity. Consequently, they improved the bacteria colonization, increased nodule occupancy and maximized the rhizobial inoculant efficacy in the cowpea 'Cubanita 666'.

In both experiments, it was detected a high number of nods (and big ones) in the proximal region to the stem (Figure 2 & 4). These findings are in agreement with the findings of Vlassak & Vanderleyden [33] and it is related with the Fertiriz's application scheme. Mentioned authors coincide that the inoculation method of coated seeds (here followed by two re-inoculations in the soil around the roots area), frequently results in a high density of bacteria near the seed, with a good nodulation in the upper tap root but wake in more distal parts and in lateral roots due to the low density of the inoculant strain in the bulk soil. However, it must be considered that some bacteria die after seed-coating owing to osmotic and desiccation stress under the field conditions [4]. The well plant growth and better production indicators (pods, seeds and yield) observed in both assays agree with previous studies that demonstrated positive effects associate to the important symbiotic relationship *Rhizobium*-legume [10,11]. There are direct and indirect effects of *Rhizobium* inoculation on the nodule formation in cowpea, which help to fix nitrogen in soil as well as increases the fertility of the soil [34]. In the case of this research, numerous nods appeared before 50 days after planting. It is the result of an efficient symbiosis, due to inoculate the appropriate *rhizobia* which resulted in the early formation of effective nodules for efficient nitrogen fixation [8].

The principal shape and size of root nodules counted in cowpea var. 'Cubanita 666' coincides with description made by Singh et al. [35]; they are usually smooth and spherical, about 5mm in diameter. These nodules provide an advantage for N2-fixation in which nitrogenases are protected in bacteroids from atmospheric oxygen [36]. During this research, after a transversal cut of nods, it was detected a reddish color in most of them, a typical characteristic of effective nodules that according to other authors is associate to the positive nitrogenase activity and the production of leghaemoglobin [20]. The nodules turn green during ageing due to breakdown of leghaemoglobin to green bile pigments. When the nodules die, stationary phase *rhizobia* are released and can multiply by using degradation products of nodules as substrate [3]. The presence of nodules in non-inoculated plants (absolute control), probed the promiscuity of cowpeas by this *rhizobacteria* and the presence of pre-existing native rhizobia in the soil where this experiment was conducted. It can affect the connection between *rhizobia* applied as inoculant and a competition for nodule occupancy with resident *rhizobia*, which according to previous reports, limiting its composition and effectiveness [37].

Some observed differences between the two assays (chambers or field), for example in height, number of pods or nods quantity, agree with previous considerations that microbial interactions in the soil depend of many factors [38]. The nitrogen-fixing *rhizobia* activity could be specific for plant genotype and can change during the life cycle of the plant, the root zone, the *rhizosphere* microbial community, among others [39,40].

The efficacy of inoculation usually be different because inoculant rhizobia must compete with edaphic conditions, with resident rhizobia for resources and nodule occupancy [41], or according to the phosphorus supply in the soil [8]; they can reduce the process of infection and nodule development and becomes a barrier to the symbiotic effectiveness. The soil nutrient deficiency can also contribute to less nodulation by local strains [42]. High level of synthetic fertilization to the crop inhibits or decreases the effectiveness of bioproducts [43]. All those findings explain the observed results in terms of nodulation and that is particular consistent with the present study results for nodulation variables when the 100% on NPK fertilizer was used in cowpea. The presence of abundant nodules in cowpea plants inoculated with the biofertilizer, even at harvest time, was an interesting finding, since many of them attached to fine roots remained in the soil during the plant extraction process in the field. This fact constitutes a potential inoculum of the bacteria that contributes to a better microbiological composition of the soil, an important aspect towards the sustainability of agriculture. It has been recognized previously by other authors as a significant contribution in the transfer of nitrogen as a consequence of its decomposition in the soil [44]. The use of rhizobia strains that nodulate well as part of the Fertiriz biofertilizer and the fact that they are persistent in the soils when their host legumes were not cropped will be beneficial for farmers. In this case, new studies will be necessary to test whether the good nodulation observed in cowpea roots would imply good future colonization of the soil after harvest. Consequently, other studies and in-depth knowledge of factors responsible for rhizobial survival and persistence will be essential.

As for the yield, best results were reached in treatments where the Fertiriz was used. It indicated its positive contribution to the well development of cowpea under the experimental conditions. These results coincide with those informed before by Acuña et al. [45] in common bean, who demonstrated in a wide study of efficient strains of *Rhizobium* in several countries of Central America, a significant increment in the yield only with the use of the microorganisms. Like in the present research, the use of half of recommended mineral fertilization plus inoculation, was better than inoculation only. Our results for number of pods/plant, number of seeds/pod and weight of 100 seeds (g) were comparable with those obtained by Estrada et al. [13], when they studied the effect of Azofert®, a Cuban inoculant based on bacteria of the genus *Rhizobium*, in two common bean varieties. The yield in their research was superior with 2.15t ha-1, but under normal irrigation conditions throughout the crop cycle, compared with the current results without irrigation (>1.27t ha$^{-}$1). Different researchers reported that rhizobia inoculation increased the yield in some studies on different species [23,26]. In an experiment conducted in Namibia under different climatic conditions in cowpea yields improved on average by 47% [46]. In the same way, in our study, cowpea var 'Cubanita 666' yields increased around 62% when

*Rhizobium* (Fertiriz) was combined with 50% of mineral fertilizer NPK (in chambers assay), and 47% using only the biofertilizer (in the field), compared with the non-inoculated treatment.

In general, the positive efficiency of the *Rhizobium* present in the Fertiriz is consistent with previous studies. Some efficient N-fixing strains of rhizobia have successfully been formulated into commercial biofertilizers and are widely applicable to legume crops [47]. Their inoculations in farm can guarantee the required nitrogen levels by plants and substantially reduce the application of chemical fertilizers [48]. An important finding of this research was high values in plant growth and remain variables, included the yield, for treatments with Fertiriz+50% of the NPK recommended mineral fertilizer, without statistical differences with the use of 100% NPK. This result agree with same findings when researchers from Iraq used 150-200kg of chemical fertilizer per hectare as a reference for cowpea fertilization and combined it with different organic and biofertilizer sources [49]. There are numerous studies focused on knowing the economic, environmental and functional balance for the use of biofertilizers in combination with reduced levels of synthetic fertilization in agricultural crops [50,51]. The interactions among cowpea and the biofertilizer Fertiriz will require further studies and new approaches, but it confirmed that the nitrogen fixation carried out by *Rhizobium* permits the legume to be less dependent on chemical fertilizers as compared to the non-leguminous plants [52]. Considering the growing interest and practical results of inoculation techniques with *Rhizobium* as a tool to mitigate the harmful effects of mineral fertilization on the environment, future researches with Fertiriz should additionally include new assays in open field conditions for cowpea and common beans crops using more combination with mineral fertilizers (complete formula -NPK, and Nitrogen fertilizer). This study highlights the possibility of improving cowpea development and productivity through combined use the new biofertilizer Fertiriz with the half of the recommended rate of synthetic fertilizer. This combination could reduce the environmental pollution and moderate the investment in chemical fertilizers by smallholder farmers.

## Conclusion

Applications of the new biofertilizer Fertiriz together with 50% of NPK mineral fertilizer resulted in positive interactions for most of the plant growth parameters and yield in the cowpea crop var. ‘Cubanita 666’. The only inoculation of the *Rhizobium*-based biofertilizer can improved plant development and yield too, probing its contribution to the sustainability of the Cuban cowpea production systems.

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