



# Interaction Effects between Organic Fertilizers and Biofertilizers on the Growth of *Stevia rebaudiana* Bertoni

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## Abstract

In the last decade *Stevia rebaudiana*, has become one of the most demanded crops in the market dedicated to health care and food. However, the main consuming countries, from Asia and Europe, are constantly demanding organic products. Therefore, the purpose of this study was to evaluate the effect of organic fertilizers in combination with biofertilizers in the growth of *Stevia*. A randomized block design with divided plots (large and small) and three replications were used. In the large plots, the cuttings of *stevia* were inoculated with 1) *Rhizophagus intraradices* fungus (**Rhiz**); 2) A mixture of bacteria *Bacillus* spp. (**Bacl**) and *Azospirillum brasilense* (**Azo**); 3) *Trichoderma* spp. fungus (**Trch**); 4) A treatment with no inoculant (**c**). These inoculation treatments were combined, in the smaller plots with three sources of organic fertilizers named: 1) Sheep manure (**Shim**); 2) Bocashi (**Bcsh**); 3) chicken manure (**Chm**); 4) A treatment with any organic fertilizer. The plot with no inoculants nor organic fertilizers was the Absolute Control (**Ac**). There was a positive relationship between organic fertilizers and biofertilizers in the growth of *Stevia*. The highest yield corresponded to the **Rhiz + Bcsh** with 450.00 kg·ha<sup>-1</sup> followed by **Rhiz + Shim**, and **Trch + Chm** both with 410.00 kg·ha<sup>-1</sup>. The treatment with the lowest yield was the **Ac** with 120.00 kg·ha<sup>-1</sup>.

## Subject Areas

Agricultural Engineering

## Keywords

Natural Sweetener, Inoculation, Biofertilizers

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### 1. Introduction

The management of organic fertilizers has traditionally been used by farmers in small areas of land, incorporating organic materials (manure, household fruit and vegetable waste) into the agrosystems [1]. The application of organic fertilizers dates back to the times of the Aztecs and Mayans, who used fish as a source of phosphorus [2]. The Aztecs intensely used the so-called *chinampas*; a kind of rafts covered with underground mud to grow flowers and vegetables in swampy soils with high humidity and abundant organic fertilizers [3]. The soft mud, as silt, was extracted at a maximum depth of three meters from the bottom of the lakes and placed on the upper part of the platforms. Water lilies and other floating organic materials were also used [3].

While time was passed by the population growth in an accelerated way and in consequence there was a need to intensively produce agricultural crops so the ancient production systems fell into disuse. However, intensive agricultural activities to the detriment of organic matter reserves and reduce soil fertility [4]. In recent years, at the global level, the incorporation of fertilizers and organic manures (manure and compost) for soil bioremediation in agricultural soils has regained importance [5] [6] [7]. Organic fertilizers can satisfy the nutrient demand of crops while significantly reducing the use of chemical fertilizers and improving the characteristics of the vegetables to be consumed [8]. Furthermore, organic fertilizers are able to improve the soils attribute that have been deteriorated by the excessive use of agrochemical and over-exploitation [1].

On the other hand, biofertilizers are other components that can contribute to improving soil deterioration and stimulating crop growth and productivity [9]. They are products that contain microorganisms, which when inoculated can live associated or in symbiosis with plants to improve nutrition [10]. These micro-organisms are found naturally in the soil and comprise various groups [11]. The bacterias commonly used in agriculture are of the genus *Rhizobium* and *Azospirillum* [12]. The fungus *arbuscular mycorrhiza* has been reported as a microorganism capable to stimulate vegetative growth when soil nutrients such as N, P and K are absorbed and introduced into the plants [13]. The growing interest in developing agriculture with low use of agrochemicals is based on the awareness of taking care the environment and the high cost and low efficiency of inorganic fertilizers so farmers are seeking new alternatives such as the use of biological and organic fertilizers [14]. In the particular case of *Stevia rebaudiana* (Bertoni), it is important to achieve a safe natural production due to the fact that leaves are the directly consume product. The *steviosides* and *rebaudiosides* (*steviol glycosides*), found in the leaves, are the most important chemical compo-

nents of stevia and are 200 to 300 times sweeter than common cane sugar (containing sucrose) [15] [16] [17]. They comprise nine glycosides (1) rebaudioside A, (2) steviolboside, (3) stevioside, (4) rubusoside, (5) rebaudioside B, (6) rebaudioside C, (7) rebaudioside D, (8) rebaudioside F and (9) dulcoside A, which can be determined by high-performance liquid chromatography (HPLC) [18]. Due to the foregoing, the production and management of stevia must be carried out by applying technologies that are more friendly to the environment and to human health.

A possible way to cope with the problem is by using biofertilizers such as *arbuscular mycorrhizal* fungi [19] and organic fertilizers. The organic products can supply micro and macronutrients to the crops and have the advantage of improving the soil structure for good aeration and moisture retention. In this way, soil erosion can be diminished and soil temperature is regulated [20]. Therefore, the purpose of this study was to evaluate the effect of organic fertilizers, in combination with biofertilizers, on the growth of *S. rebaudiana*.

## 2. Materials and Methods

This work was carried out under field and irrigation conditions in a red soil (*Luvoso*) at the Uxmal Experimental Station belonging to the National Institute of Forestry, Agricultural and Livestock Research (INIFAP). It is located in the municipality of Muna, Yucatán, Mexico at 20°29'08.1 North latitude and 89°24'39 West longitude with an altitude of 50 meters above sea level. The climate is of the Aw type, which is the driest of the warm subhumid ones with summer rains, average annual precipitation and temperature of 900 mm 25°C respectively [21].

## 3. Establishment of the Experiment

The experiment was established on a loamy-silty texture soil with pH of 7.8, electrical conductivity (EC) of 1.1 dS·m<sup>-1</sup>, organic matter (OM) of 4.8%, with 0.13% nitrogen, 43.4 mg·kg<sup>-1</sup> of phosphorus, 229 mg·kg<sup>-1</sup> of available potassium. A complete random block design, with three replications, was used in splitted large and small plots. In the large plots, biofertilizers were applied to high quality selected cuttings of *Stevia rebaudiana* Bertoni (Creole variety) coming from six months old plants.

The cuttings were inoculated with: 1) *Rhizophagus intraradices* (**Rhiz**) at 1 spore mL<sup>-1</sup> (INIFAP<sup>MR</sup> Mycorrhiza brand); 2) Mixture of *Bacillus spp.* (**Bacl**) and *Azospirillum brasilense* (**Azo**) (1 × 10<sup>8</sup> cfu·mL<sup>-1</sup>) (BactoCROP<sup>MR</sup>); 3) *Trichoderma spp.* (**Trch**) at 1 × 10<sup>8</sup> cfu·mL<sup>-1</sup> (INI04-INIFAP brand); 4) a treatment control with just the corresponding biofertilizer was considered to see the effect of the inoculants alone. The amount of 70 g·L<sup>-1</sup> of inoculants, in solid forms, were diluted in tap water and stirred them until having a homogeneous solution. The inoculation was carried out according to the methodology proposed by Lozano and Ramírez [22], consisting of soaking the cuttings of stevia for one minute in the solution before sowing.

In the smaller plots, three types of organic fertilizers were applied: 1) Sheep manure (**Shim**) (15 t·ha<sup>-1</sup>); 2) Bocashi (**Bcsh**) (15 t·ha<sup>-1</sup>); 3) Chicken manure (**Chim**) (15 t·ha<sup>-1</sup>); 4) Unfertilized treatment was considered. In **Table 1** the chemical analysis of each organic fertilizers are noted.

All inoculation treatments combined with the organic fertilizers, including their corresponding controls, made up 16 treatments. The Absolute Control (**Ac**) was the treatment with no organic fertilizer nor inoculant.

Prior to the transplant, the organic fertilizers were spread manually in the small plots. The transplant was carried out on June 2016 at a population density of one hundred thousand plants per hectare. The plants were pruned (formation pruning) 15 days after transplantation. The first and unique harvest was done two months after transplanting.

Drip irrigation was used with 1500 tape gauge, emitters every 30 cm with a water flow rate of 1.5 L·h<sup>-1</sup>. The hoses were covered with 100 µ thick silver/black pad plastic.

### Measured Variables for Stevia under Field Conditions

The growth of 10 representative stevia plants, from each plot, were evaluated measuring the next parameters: 1) Height of the plant measured from the base of the stem to the last foliar apex; 2) Production and distribution of dry biomass (stem and leaves) dried in a forced air oven at 65°C until constant weight; 3) Total dry leaf yield obtained at 65 days after transplantation. All parameters were submitted to an Analysis of Variance (ANOVA) and to a mean comparison test

**Table 1.** Chemical analysis of organic fertilizers used in the experiment.

| Soil attributes  | Sheep manure | Bocashi | Chicken manure |
|--|--------------|---------|----------------|
| pH   | 7.64         | 7.36    | 7.50           |
| EC (mS/cm <sup>-1</sup> )                              | 38.50        | 11.93   | 20.40          |
| OM (%)   | 32.16        | 9.78    | 30.15          |
| N-NO <sub>3</sub> (mg·Kg <sup>-1</sup> )               | 1,728        | 408     | 1,620          |
| PO <sub>4</sub> -P (mg·Kg <sup>-1</sup> )              | 390          | 115     | 870            |
| S-SO <sub>4</sub> <sup>2-</sup> (mg·Kg <sup>-1</sup> ) | 68.33        | 20.00   | 436.67         |
| Na <sup>+</sup> (Meq/100 g)                            | 10.43        | 3.13    | 8.13           |
| K <sup>+</sup> (Meq/100 g)                             | 28.13        | 9.95    | 15.60          |
| Ca <sup>2+</sup> (Meq/100 g)                           | 30.00        | 33.50   | 23.00          |
| Mg <sup>2+</sup> (Meq/100 g)                           | 18.63        | 9.00    | 16.08          |
| CEC (meq/100g)   | 87.20        | 55.58   | 62.81          |

**Note:** pH = Hydrogen ions activity measuring grade of acidity or alkalinity, C.E. = electrical conductivity, OM = organic matter, N-NO<sub>3</sub><sup>-</sup> = nitric nitrogen (Brusini), PO<sub>4</sub>-P = phosphorus from phosphates (Bray method), S-SO<sub>4</sub><sup>2-</sup> = sulfur from sulfates, Na<sup>+</sup> = sodium, K<sup>+</sup> = potassium, Ca<sup>2+</sup> = calcium, Mg<sup>2+</sup> = magnesium, CEC = Cation Exchange Capacity (by Ammonium Acetate 1N pH 7).

(Tukey,  $p \leq 0.05$ ) using the SAS statistical package version 9.2 [23].

## 4. Results and Discussion

### 4.1. Plant Height as Related to Organic Fertilizers and Biofertilizers

Statistically significant differences ( $p \leq 0.05$ ) were observed between treatments inoculated with **Rhiz** plus organic manures. The combination of **Shim** plus **Rhiz** showed the maximum height (15.14 cm). **Shim** with no inoculants had the lowest one (11.38 cm) as it is shown in **Table 2**. Biofertilizers showed a stimulating

**Table 2.** Hight (cm), and weight (g/plant) of dry leaves and stems of *Stevia* with biofertilizers and/or organic fertilizers. 65 days after transplantation.

| Treatment   | Leaf              |                      | Stem                 |       |
|---|-------------------|----------------------|----------------------|-------|
|   | Height (cm)       | Dry weight (g/plant) | Dry weight (g/plant) |       |
| <i>R. intraradices</i><br>( <b>Rhiz</b> )   | <b>Shim</b>       | 15.51                | 4.1                  | 2.2   |
|   | <b>Rhiz</b>       | 16.61                | 2.8                  | 1.2   |
|   | <b>Bcsh</b>       | 15.41                | 4.5                  | 2.6   |
|   | <b>Chim</b>       | 15.79                | 3.0                  | 1.1   |
|   | DMS               | NS                   | NS                   | NS    |
|   | C.V. (%)          | 18.06                | 66.16                | 68.99 |
| <i>Azospirillum brasiliense</i> ( <b>Azo</b> ) +<br><i>Bacillus</i> sp. ( <b>Bacl</b> ) | <b>Shim</b>       | 12.28b               | 2.5                  | 1.5   |
|   | <b>Azo + Bacl</b> | 14.24ab              | 1.8                  | 1.0   |
|   | <b>Bcsh</b>       | 14.54a               | 2.7                  | 1.2   |
|   | <b>Chim</b>       | 15.96a               | 2.4                  | 1.1   |
|   | DMS               | 2.045                | NS                   | NS    |
|   | C.V. (%)          | 12.78                | 53.21                | 67.02 |
| <i>Trichoderma</i> spp.<br>( <b>Trch</b> )  | <b>Shim</b>       | 15.36                | 2.5                  | 1.6   |
|   | <b>Trch</b>       | 14.64                | 1.9                  | 1.0   |
|   | <b>Bcsh</b>       | 16.10                | 2.1                  | 0.8   |
|   | <b>Chim</b>       | 15.46                | 4.1                  | 2.5   |
|   | DMS               | NS                   | NS                   | NS    |
|   | C.V. (%)          | 25.52                | 68.10                | 83.71 |
| No biofertilizers   | <b>Shim</b>       | 15.14a               | 2.4                  | 1.1   |
|   | <b>Ac</b>         | 13.00ab              | 1.2                  | 1.0   |
|   | <b>Bcsh</b>       | 14.78a               | 2.5                  | 1.5   |
|   | <b>Chim</b>       | 11.38b               | 2.5                  | 2.0   |
|   | DMS               | 2.47                 | NS                   | NS    |
|   | C.V. (%)          | 16.23                | 60.02                | 82.33 |

Note: Values with the same letters are statistically equals according to Tukey Test ( $p < 0.05$ ). C.V. = Coefficient of Variation.

effect on plant growth.

The **Rhiz** alone, obtained a higher height compared to non-inoculated plants. This was to be expected since *Arbuscular mycorrhizal* fungi (AMF) form abundant external hyphae as an extension of roots. In this way, water absorption and nutrient translocation (mainly phosphorus) is enhanced, stimulating the plant growth [24]; this may also be related to a stimulating effect of phytohormones released by the AMF [25].

When organic fertilizers were inoculated with the mixture of **Azo** and **Bacl** significant statistical differences ( $p \leq 0.05$ ) were observed. The interactions with **Chim** registered the maximum height with 15.96 cm (**Table 2**), followed by **Bcsh** with 14.54 cm. When **Azo** plus **Bacl** were applied with no organic fertilizers showed a height of 14.24 cm. The lowest height was with **Shim** alone with 12.28 cm high.

Certain species of bacteria such as *Pseudomonas* or *Bacillus* can exert a direct action by stimulating plant growth and, at the same time, antagonizing pathogens and/or stimulating plant defenses [26]. Once into the roots, the *Azospirillum* bacterium is capable to convert gaseous nitrogen into available nutritional forms in order to stimulate better plant growth [27]. It is highly possible that bacterium stopped to produce inorganic nitrogen (from the air nitrogen) as long as organic fertilizers was applied. This effect was mainly appreciated when the height (14.24 cm) with **Azo + Bacl + Shim** was compared to the **Shim** alone (12.28 cm).

The plants inoculated with **Trch** and mixed with **Bcsh** (16.10 cm), **Chim** (15.46 cm) and **Shim** (15.36 cm), had the highest height (**Table 2**) as compared to **Trch** alone (14.64 cm). The effect of *Trichoderma* in promoting plant growth is widely known; this is related to the production of indoleacetic acid (IAA), the ability to solubilize phosphates and produce compounds called *siderophores* capable to improve nutrients availability [28]. However, the beneficial effect of *Trichoderma* on stevia growth has been studied very little [29].

Studies such as those of Garcés [30], mentioned that both height and green matter production of alfalfa increased at the addition of solid organic fertilizer enriched with *Trichoderma*. Organic fertilizers have high content of growth stimulators such as free amino acids.

In relation to the effect of organic fertilizers alone, with no biofertilizers, significant statistical differences ( $p \leq 0.05$ ) were observed between treatments. The **Shim** and **Bcsh** treatments showed the highest plant height, with 15.14 and 14.78 cm respectively, followed by the **Ac** with 13.0 cm and **Chim** with 11.38 cm (**Table 2**).

Cegarra *et al.* [31] reported that organic fertilizers have high mineral nitrogen content and significant amounts of other nutritive elements for plants. Depending on the applied level, they can increase the content of soil organic matter, improve moisture retention and regulate the pH [32] [33]. They can increase potassium [34], calcium and magnesium [35] [36] availability in the soil.

#### 4.2. Weight of Dry Leaves (g/plant) as Related to Treatments

No statistical differences ( $p \leq 0.05$ ) were found between yields of dry leaves when stevia was treated with bioinoculants and organic fertilizers (**Table 2**). The highest weight of dry leaves was obtained with **Rhiz** combined with **Bcsh** (4.5 g/plant) followed by **Rhiz** plus **Shim**, and **Trch** plus **Chim** with 4.1 g/plant each one. The lowest weight (1.2 g/plant) was found when plants were not inoculated and organic manures not applied (**Ac**). Regardless of the manures treatments, the best plant response was observed when **Rhiz** was inoculated. This favorable effect can be explained, in part, to better absorption of unmobile nutrients from the soil such as phosphorus, zinc and copper when roots, due to **Rhiz** colonization can explore more soil volume (length and depth) [37].

#### 4.3. Weight of Dry Stems (g/plant) According to Treatments

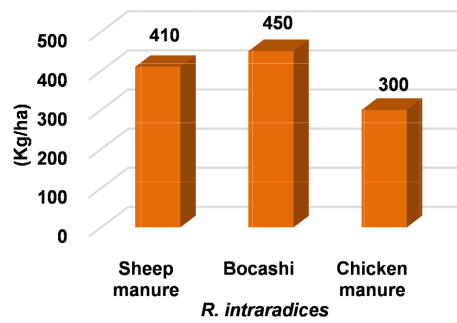
The stem is of great importance for the survival and growth of stevia. The cuttings are produced from the stems, so plants with great potential for homogeneous production can be selected and preserved. The results showed that the treatments: **Rhiz** + **Bcsh** (2.6 g/plant), **Trch** + **Chim** (2.5 g/plant), **Rhiz** + **Shim** (2.2 g/plant) and **Chim** alone had the highest stem weight (**Table 2**). The other treatments, with the lowest stem weight, ranged from 0.8 to 1.5 g/plant. Therefore, the use of different organic fertilizers and biofertilizers, used in this work, has an unequal impact on the final weight of the stem. The previous findings are in disagreement with the normal expectation that both endo-mycorrhizal fungi and N-fixing bacteria are capable, per se, of stimulating plant growth and reproduction of annual [38] and perennial crops [39] under nursery conditions.

#### 4.4. Yield of Dried Leaves of Stevia ( $\text{kg}\cdot\text{ha}^{-1}$ )

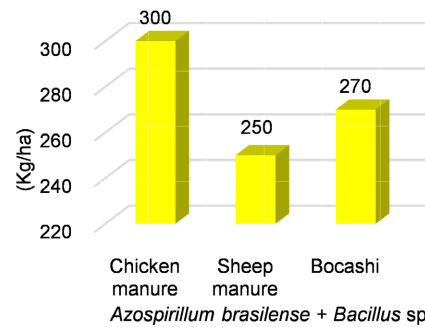
The ANOVA of dry leaves ( $\text{Kg}\cdot\text{ha}^{-1}$ ) at the first cut (65 days after transplanting) showed statistical differences between treatments. **Figures 1(a)~(d)** are shown the yields obtained with biofertilizers and/or organic biofertilizers. Statistical analysis detected significant differences between treatments at first cut. The highest yield corresponded to **Rhiz** + **Bcsh** with  $450.00 \text{ kg}\cdot\text{ha}^{-1}$ , followed by **Rhiz** + **Shim** and **Trch** + **Chim** with  $410.00 \text{ kg}\cdot\text{ha}^{-1}$  both. The treatment with the lowest yield was the Absolute Control (**Ac**) with  $120.00 \text{ kg}\cdot\text{ha}^{-1}$ .

The combination of **Trch** + **Shim** and **Bcsh** showed low yields with  $250.00$  and  $210.00 \text{ kg}\cdot\text{ha}^{-1}$  respectively (**Figure 1(c)**). This could be related to some factors such as: 1) The natural mechanism of the biological product, with a chemical substance which needs suitable environment to work properly [40], Native strains extracted from local soils would be more functional due to its biological adaptation to the environment [41]; 2) The low yield of **Trch**, is also related to the principle of competitive exclusion, also known as Gause's law; suggesting that two species competing for the same resources cannot coexist if other ecological factors are constant [42].

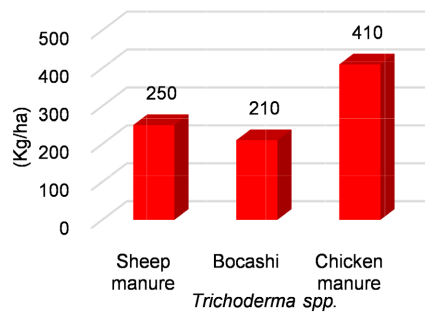
On the other hand, the yields with organic fertilizers (**Shim**, **Bcsh**, **Chim**) did



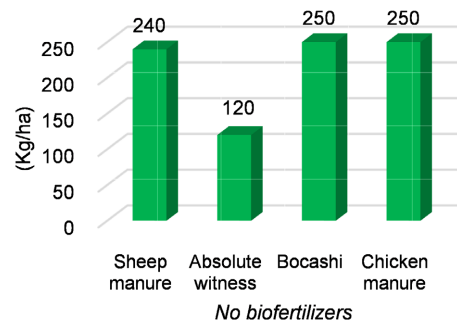
(a)



(b)



(c)



(d)

**Figures 1.** (a) *R. intraradices* (**Rhiz**); (b) *Azospirillum brasilense* (**Azo**) + *Bacillus* sp. (**Bacl**); (c) *Trichoderma* spp. (**Trch**); (d) *No biofertilizers*. Interaction effects between Organic Fertilizers and Biofertilizers on the yield of stevia dry leaves (kg·ha<sup>-1</sup>) 65 days after transplanting under field conditions.



not show important differences when inoculated with the mixture of **Rhiz** + **Bacl** (**Figure 1(a)**); and the yields, ranging from 250.00 to 300.00 kg·ha<sup>-1</sup> is similar to those observed without inoculation (240.00 to 250.00 kg·ha<sup>-1</sup>). Although in this work the advantage of using Plant Growth Promoting Rhizobacteria (**PGPR**) was not reflected, there are reports suggesting the potentiality they have to enhance the contents of lycopene, total sugars and ascorbic acid in fruits [43]. The **PGPR** are able to increase the number of fruits per plant and their quality when bacteria are capable to synthesize phytohormones such as cytokinins and Indole Acetic Acid (IAA). They have the advantage of fixing gaseous nitrogen, solubilizing phosphorus and are capable to inhibit phytopathogenic microorganisms [44].

## 5. Conclusion

The use of organic fertilizers combined with biofertilizers improved the growth of *Stevia rebaudiana*. The use of *Rhizophagus intraradices* (**Rhiz**) being the best biofertilizer to be combined with the organic fertilizers (**Shim**, **Bcsh**, **Chim**) since the yields of dry leaves ranged from 300.00 to 450.00 kg·ha<sup>-1</sup> while the Absolute Control (**Ac**) had just 120.00 kg·ha<sup>-1</sup>. It seems that both biofertilizers and organic fertilizers, either alone or in combination can induce better production of *Stevia rebaudiana*.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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