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**Original article**

# Response of *Stevia rebaudiana* Bertoni to the strains inoculation of arbuscular mycorrhizal fungi

## Respuesta de la especie *Stevia rebaudiana* Bertoni a la inoculación con cepas de hongos micorrízicos arbusculares

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**ABSTRACT:** *Stevia rebaudiana* Bertoni is a perennial species of high commercial value for its medicinal properties. In order to determine the effect of the inoculation of efficient strains of arbuscular mycorrhizal fungi in stevia cuttings, this research was carried out under semi-controlled conditions at the National Institute of Agricultural Sciences. Disease-free cuttings were used, with a length of 10 cm and with five pairs of leaves for each one. Three AMF strains were studied: *Rhizoglomus irregulare*, *Funneliformis mosseae* and *Glomus cubense*, in addition to a control without inoculation, in the first experiment. For the second experiment, the strain with the best performance from the previous test was used, in addition to two combinations of substrate (soil+organic fertilizer). Treatments were distributed in a completely randomized design with three repetitions and 20 plants for each one. Evaluations were carried out at the end of the nursery stage of the crop and variables related to the fungal function and the growth and development of plants were analyzed. Results demonstrated the AMF inoculation effectiveness on the variables related to the growth and development of stevia cuttings at 40 days after planting. *Rhizoglomus irregulare* strain showed a better performance under the experimental conditions evaluated.

**Keywords:** mycorrhizae, effectiveness, inoculum, growth, development, medicinal plants.

**RESUMEN :** La *Stevia rebaudiana* Bertoni es una especie perenne de alto valor comercial por sus propiedades medicinales. Con el objetivo de determinar el efecto de la inoculación de cepas eficientes de hongos micorrízicos arbusculares (HMA) en esquejes de estevia, se desarrolló esta investigación en condiciones semicontroladas en el Instituto Nacional de Ciencias Agrícolas. Se utilizaron esquejes libres de enfermedades, con una longitud de 10 cm y con cinco pares de hojas por cada uno. En el experimento 1 se estudiaron tres cepas de HMA: *Rhizoglomus irregulare*, *Funneliformis mosseae* y *Glomus cubense*, además de un control sin inoculación. Para el segundo experimento se empleó la cepa de mejor comportamiento del ensayo anterior, además de dos combinaciones de sustrato (suelo + abono orgánico). Los tratamientos se distribuyeron en un diseño completamente aleatorizado con tres repeticiones y 20 plantas por cada uno. Las evaluaciones se realizaron al finalizar la etapa de vivero del cultivo y se analizaron variables relacionadas con el funcionamiento fúngico y con el crecimiento y desarrollo de las plantas. Los resultados demostraron la efectividad de la inoculación de los HMA en las variables relacionadas con el crecimiento y desarrollo de los esquejes de estevia a los 40 días después de su plantación. La cepa *Rhizoglomus irregulare* mostró un mejor comportamiento en las condiciones experimentales evaluadas.

**Palabras clave:** micorrizas, crecimiento, desarrollo, plantas medicinales.

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## INTRODUCTION

*Stevia rebaudiana* Bertoni is a perennial species native to Paraguay of high commercial value for its medicinal properties (1). Although botanical seeds can be used in the reproduction process of this species, this method is not the most efficient to guarantee plants of excellent quality because high heterogeneity is obtained in the offspring in terms of germination percentage, plant growth, as well as stevioside production; therefore, agamic propagation constitutes the most effective method that guarantees the characteristics of the mother plant (2).

However, the growth habit and limited size of cuttings make this propagation method slow and in some cases it is considered inefficient to produce the volumes of plants demanded by growers (3).

In this context, arbuscular mycorrhizal fungi (AMF) represent a group of soil microorganisms that establish symbiosis with numerous plant species of agricultural interest (4). Among the main benefits of this interaction are: the direct effects on mineral nutrition, especially on the absorption of macro and micronutrients (5); the induction of tolerance to biotic (e.g. pathogens) (6) and abiotic (e.g. drought and salinity) stress conditions (7); their participation in phytoremediation processes (8); and their contribution to the stability of soil aggregates (9).

Some studies refer to the mycorrhizal symbiosis effectiveness in different plant production technologies (10), specifically in nursery systems, demonstrating significant increases in the growth and quality of plants, as well as their subsequent transplanting to the field (11).

The objective of this research was to determine the inoculation effect with efficient strains of arbuscular mycorrhizal fungi on cuttings of *Stevia rebaudiana* Bertoni under semi-controlled conditions.

## MATERIALS AND METHODS

The research was conducted under semi-controlled conditions, in the department of Sustainable Agroecosystems Management (MAS, according ist acronyms in Spanish) belonging to the National Institute of Agricultural Sciences (INCA), located at kilometer 3½ of Tapaste road, San José de las Lajas municipality, Mayabeque province. This area was located at 23º north latitude and 82º 12'west longitude, at an altitude of 138 m a.s.l. (12).

Disease-free cuttings of *Stevia rebaudiana* Bertoni, collected from the flower beds of the garden area of the department's own garden of medicinal plant varieties, were used. For their collection, the uniformity of the cuttings with a stem length of 10 cm and five pairs of leaves per cutting was taken into account.

**Experiment 1**. Inoculation effect of three AMF strains on the growth and development of stevia under nursery conditions.

For the substrate, a Ferrallitic Red Leached soil was used, according to the Cuban Soil Classification (13), which was correlated with ferrallitic, lixic, (distric, rodic, arcillic) Nitisol (14) and mixed with organic fertilizer (cow manure) in a 1:1 ratio, some of its chemical characteristics are shown in Table 1.

Three AMF strains were studied: *Rhizoglomus irregulare* (Błaszk., Wubet, Renker & Buscot) (INCAM-11), *Funneliformis mosseae* (T.H. Nicolson & Gerd.) (INCAM-2), *Glomus cubense* (Y. Rodr. & Dalpé) (INCAM-4) (15), belonging to the INCA Laboratory of Arbuscular Mycorrhizae strain collection. The inoculum concentration was certified by the Mycorrhizal Quality Control Laboratory of INCA (35 average spores $g^{-1}$ of inoculant) and 1 g of inoculant was applied per plant, making a hole located under the stevia cuttings at the time of planting.

Treatments were distributed in a completely randomized design with three replicates and 20 plants per replicate and consisted of the three AMF strains and a control without inoculation. From the results of this experiment, the best performing AMF strain was selected for the conduct of subsequent studies.

**Experiment 2.** Evaluation of the effect of inoculation of efficient AMF strains and different substrate combinations on stevia cultivation under semi-controlled conditions.

The same soil described in experiment 1 was used and two substrate combinations [soil+organic manure (cow dung)] were formulated (1:1 and 3:1). Some of the chemical characteristics are shown in Table 2.

The methodologies used for chemical determinations coincided with those described in experiment 1. Four treatments were evaluated and distributed in a completely randomized design with three replications and 20 plants per treatment (Table 3).

The cuttings were planted in polyethylene trays, expanded with 70 alveoli containing the substrate mixtures described in each experiment. The plants were grown at an average temperature between 26.5 and 29.6 °C, with a relative humidity of 68.25 and 87.05 % (Tapaste Meteorological Station). The cultural attentions were carried out according to the technology of the crop (16).

**Table 1.** Chemical characteristics of the substrate used

| pH $H_2O$ | OM (%) | $P_2O_5$ (mg $kg^{-1}$) | Exchangeable cations | | | | |
|---|---|---|---|---|---|---|---|
| | | | $Ca^{2+}$ | $Mg^{2+}$ | $K^+$ | $Na^+$ | CIB |
| | | | (cmol$_c$kg$^{-1}$) | | | | |
| 6.9 | 29.3 | 35.23 | 24.2 | 3.61 | 2.21 | 0.92 | 30.94 |

Chemical determinations: pH, potentiometry; organic matter (OM), Walkley Black (1934); phosphorus ($P_2O_5$), extraction with $H_2SO_4$ 0.025 M and determination by spectrometer; exchangeable cations, $Ca^{2+}$ and $Mg^{2+}$ (extraction with NH4Ac 1 mol $L^{-1}$ at pH 7 and determination by complexometry), Na+ and K+ (extraction with boiling $HNO_3$ and determination by flame photometry)

The evaluations were carried out at the end of the nursery stage of the crop (40 days after planting). Fifteen plants were randomly selected for each treatment (n=15) and the following evaluations were performed:

*Fungal indicators:* 250 mg of secondary roots were taken, carefully washed with abundant water, dried in an oven at 70 °C until constant mass was obtained, and clarified and stained (17). The intercept method was used to determine the frequency of mycorrhizal colonization and the intensity of colonization or visual density (DV) (18).

*Plant growth indicators:* for the determination of aerial and root dry mass (g), the samples were placed in an oven at 70 ºC (BrBOXUN) and weighed on a digital technical balance (Acom JW-1, precision level 0.1 g) until a constant mass was obtained. In addition, the length (cm) and stem diameter (mm) were determined.

Statistical processing of the data was performed by simple rank analysis of variance. Means were compared according to Tukey's multiple range test ($p \leq 0.05$) when significant differences were observed between treatments. In all cases, the statistical package Statgraphics Centurion-2013 was used, under the *Windows 7* operating system.

## RESULTS AND DISCUSSION

### Experiment 1

Figure 1 shows the inoculation effect of the different AMF strains, for the variables related to fungal functioning (frequency and intensity of mycorrhizal colonization), on stevia cuttings at 40 days after planting. A positive response was observed for both variables when inoculating the different strains, with values that differed significantly ($p \leq 0.05$) and they were higher than those achieved in control plants. Similarly, a differential response was shown among the strains studied, with *R. irregulare* standing out with higher values, *F. mosseae* with average figures and finally, *G. cubense* obtained lower values, with respect to the rest of the inoculated strains.

In the case of mycorrhizal colonization frequency (Figure 1A), the highest values were obtained when inoculating *R. irregulare* (20 %), *F. mosseae* behaved with lower figures (17 %) and *G. cubense* reached the lowest values (15 %). Similar behavior among the different inoculated strains was observed for the mycorrhizal colonization intensity variable (Figure 1B).

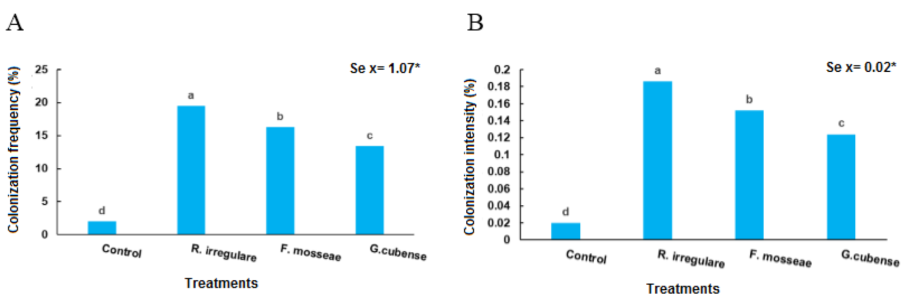
**Table 2.** Chemical characteristics of the final composition of the two substrate combinations used in experiment 2

| pH $H_2O$ | OM (%) | $P_2O_5$ (mg $kg^{-1}$) | Exchangeable cations | | | | |
|---|---|---|---|---|---|---|---|
| | | | $Ca^{2+}$ | $Mg^{2+}$ | $K^+$ | $Na^+$ | CIB |
| | | | (cmol$_c$kg$^{-1}$) | | | | |
| **1:1 (50 % soil: 50 % OM)** | | | | | | | |
| 7.2 | 21.5 | 30.85 | 24.3 | 3.41 | 1.38 | 0.89 | 29.98 |
| **3:1 (75 % soil: 25 % OM)** | | | | | | | |
| 7.0 | 14.7 | 35.23 | 26.4 | 4.21 | 2.03 | 1.01 | 33.65 |

**Table 3.** Description of treatments evaluated in experiment 2

| Treatments | Description |
|---|---|
| 1 | Control (50 % S + 50 % OM) |
| 2 | AMF + 50 % S + 50 % OM |
| 3 | Control (75 % S + 25 % OM) |
| 4 | AMF + 75 % S + 25 % OM |

AMF: selected species; S: soil MO: organic matter




A: colonization frequency (%); B: colonization intensity (%)

Bars with equal letters in each graph do not differ significantly ($p \leq 0.05$) according to Tukey's Multiple Range Test

**Figure 1.** Inoculation effect of AMF strains in the fungal functioning at 40 days after stevia planting

One of the benefits related to mycorrhizal symbiosis is its ability to stimulate plant growth and development, so Figure 2 shows the effect of inoculation of the different AMF strains on the variables aerial and root dry mass, as well as on the length and stem diameter of stevia plants 40 days after planting.

Significant differences (p≤0.05) were observed between the plants of the inoculated treatments and the control, with higher values in the mycorrhized plants. For these variables related to plant growth and development, a differential response was observed among the different strains under study, with higher values when inoculating *R. irregulare*, with intermediate figures behaved *F. mosseae* and *G. cubense* showed the lowest values.

Arbuscular mycorrhizal fungi have been widely studied for their role in mineral nutrition, an aspect of great importance due to the direct effects of this symbiosis on the growth, development and yield of mycorrhizal plants, in relation to controls (19). This effect is explained by the fact that mycorrhized plants require more photosynthates to satisfy their demand and that of the fungus, in such a way that a harmonic development between both is guaranteed (20). Therefore, in the presence of an effective mycorrhizal symbiosis, the synthesis and translocation of carbon substances from photosynthesis necessary to maintain the symbiotic process increases, which leads to a better relationship between the host and the AMF, stimulating the variables related to plant growth and development (21). In the same sense, it is suggested that the functioning of the mycorrhizal symbiosis is subject to the interaction of several factors, with special emphasis on the edaphic environment and soil pH (22).

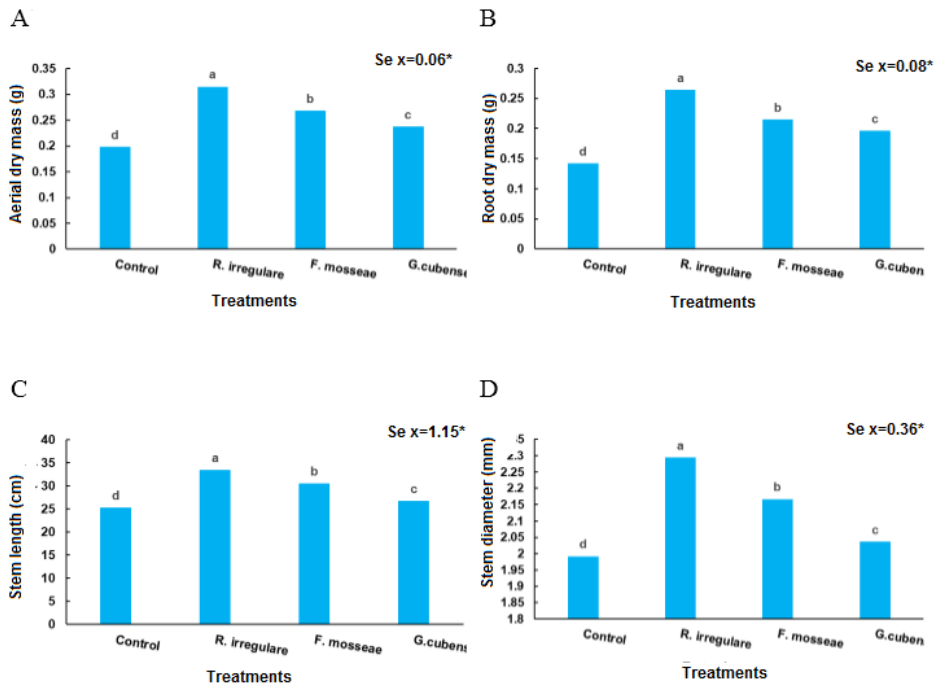
Therefore, the response found in the indicators of mycorrhizal functioning (Figure 1) and its consequent effect on the growth indicators of stevia plants (Figure 2), was determined by the characteristics of the substrate used in this study (Table 1). The chemical analysis indicated the existence of medium fertility, represented by average available P content and adequate levels of organic matter and calcium, which enhanced the activity of *R. irregulare*, compared to the rest of strains under study.

In this sense, the behavior described by *R. irregulare*, as the strain with the best response, coincided with studies carried out during the acclimatization phase of banana plants (*Musa* spp.), where significant increases were obtained in the survival of plants inoculated with an efficient AMF strain, in correspondence with their edaphic environment (23), although some studies refer to the non-specific character of the mycorrhizal symbiosis due to the diversity of families of the plant kingdom that are susceptible to be colonized, each plant species has a different degree of mycorrhizal dependence (24).

## Experiment 2

Figure 3 shows the effect of the HMA-substrate combination on variables related to fungal functioning (frequency and intensity of mycorrhizal colonization), on stevia cuttings 40 days after planting.

In a general sense, a positive response was observed when applying *R. irregulare* species in both substrate combinations, as all inoculated variants differed significantly (p≤0.05) from the control treatments, which showed lower values. It was observed that, in both mycorrhizal functioning




A: aerial dry mass (g); B: root dry mass (g); C: stem length (cm); D: stem diameter (mm).

Bars with equal letters in each graph do not differ significantly (p≤0.05) according to Tukey's Multiple Range Test

**Figure 2.** Effect of inoculation of different AMF strains on variables related to stevia growth and development 40 days after planting

variables [frequency (Figure 3A) and intensity (Figure 3B) of colonization], the higher values were reached in the treatments where *R. irregulare* was inoculated on the substrate composed of 75 % soil + 25 % MO.

The responses found in the fungal variables demonstrate that inoculation with an efficient AMF strain, from early stages of the crop and the presence of adequate soil and OM combinations, favors optimal mycorrhization, which is very convenient for the management of inoculants and their integration in crop technology (25).

In this study, the superior effect of mycorrhization was found in the combination 75 % Soil+25 % OM, so this ratio guaranteed an adequate availability of nutrients for the functioning of the AMF strain *R. irregulare*, in correspondence with the pH value reflected in the chemical analysis of the substrate (Table 2). Studies conducted referred to the differential response between various AMF strains in different soil environments and the results showed high correlations ($R^2$=0.97) between the effectiveness of the inoculated fungus strains and soil pH (25).

On the other hand, Table 4 shows the effect of the AMF-substrate combination on the aerial and root dry mass variables, as well as on the length and stem diameter of stevia plants 40 days after planting.

In the variables related to plant growth and development, higher values were reached in the treatments where *R. irregulare* was applied in the substrate composed of 75 % soil+25 % OM, differing significantly (p≤0.05) from the rest of the variants analyzed. On the other hand, the control treatments showed lower values, regardless of the type of substrate combination evaluated.

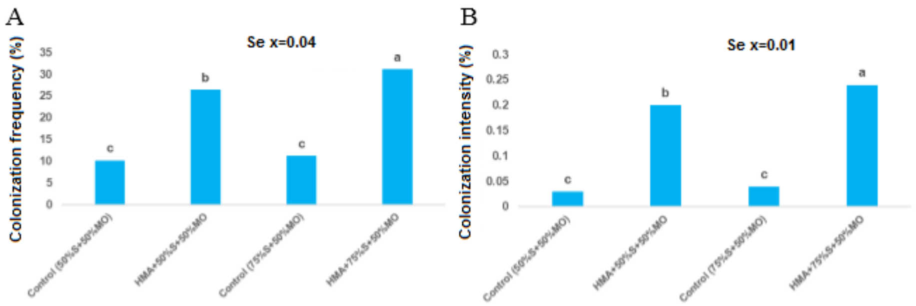
The increase in variables related to plant growth and development in treatments where *R. irregulare* was applied in the presence of the substrate combination 75 % soil+25 % OM (Table 4), indicates the effectiveness in the inoculation of this strain, which corresponded with the chemical analysis of the soil (Table 2).

The direct effect of AMF on plant growth and development has been an aspect widely addressed in the literature, since the presence of these obligate symbionts allows the plant to explore different sites or niches and transport water and nutrients of low mobility, from distances that exceed the depletion zones surrounding the roots and stimulate plant growth in mycorrhized plants compared to controls (21).

The response found in growth and development variables when inoculating *R. intraradices* coincided with other results where the same AMF strain was used in the same crop, finding a significant increase in the number of leaves, stem length and aerial dry mass.

## CONCLUSIONS

The effectiveness of AMF inoculation on variables related to the growth and development of stevia cuttings 40 days after planting is tested; being the strain *R. irregulare* the one that showed a superior response in the combination of substrate 75 % soil+25 % OM, under the experimental conditions evaluated.




A: colonization frequency (%); B: colonization intensity (%).

Bars with equal letters in each graph do not differ significantly (p≤0.05) according to Tukey's Multiple Range Test

**Figure 3.** Effect of AMF-substrate combination on mycorrhizal performance indicators 40 days after stevia planting

**Table 4.** Effect of the AMF-substrate combination on growth and development variables of stevia plants 40 days after planting

| Treatments | Stem length (cm) | Stem diameter (mm) | Aerial dry mass (g) | Root dry mass (g) |
|---|---|---|---|---|
| Control (50 % S + 50 % OM) | 23.15 d | 2.32 b | 1.24 d | 0.19 d |
| AMF + 50 % S + 50 % OM | 31.74 c | 2.51 b | 1.38 c | 0.24 c |
| Control (75 % S + 25 % OM) | 24.87 b | 2.39 b | 1.45 b | 0.30 b |
| AMF + 75 % S + 25 % OM | 38.10 a | 2.90 a | 1.57 a | 0.38 a |
| Se x | 0.45 | 0.06 | 0.03 | 0.01 |

Means with equal letters in each column do not differ significantly (p≤0.05) according to Tukey's Multiple Range Test. AMF: *R. irregulare*; S: soil MO: organic matter

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