Agrociencia

ARBUSCULAR MYCORRHIZAL FUNGI AND INDOLE-3-BUTYRIC ACID ON ROOTING AND GROWTH OF *Stevia rebaudiana* Bertoni STEM CUTTINGS

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Citation: Quiñones-Aguilar EE, Ríos-Sandoval M, Valerio-Landa SD, Hernández-Montiel LG, Rincón-Enríquez G. 2026. Arbuscular mycorrhizal fungi and indole-3-butyric acid on rooting and growth of *Stevia rebaudiana* Bertoni stem cuttings. **Agrociencia.** https://doi.org/ 10.47163/agrociencia.v60i3.3392

Editor in Chief:
Dr. Fernando C. Gómez Merino

Received: September 27, 2025.
Approved: April 14, 2026.
Published in Agrociencia:
April 27, 2026.

ABSTRACT

Stevia is an herb used as a raw material to manufacture a low-calorie natural sweetener. Stevia plants are generally propagated by stem cuttings. Nonetheless, this method is not sufficient to meet global demand. The aim of this study was to determine the effect of applying an arbuscular mycorrhizal fungus (AMF) consortium and indole-3-butyric acid (IBA), in both powder and solution, on the greenhouse propagation of stevia stem cuttings. Applying AMF and IBA promoted greater growth across the different parameters assessed in stevia stem cuttings, increasing mycorrhizal colonization, particularly arbuscule content in roots. Therefore, the use of AMF and IBA should allow the production of stevia stem cuttings with greater vigor in a shorter period of time, reducing crop production costs by optimizing the dosage and methods of plant hormone application.

Keywords: mycorrhizal colonization, vegetative propagation, plant hormone.

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a perennial herb that belongs to the family Asteraceae and has gained global interest as a natural low-calorie sweetener (Kumar *et al*., 2019; Dyduch-Siemińska *et al*., 2020; Muñoz-Labrador *et al*., 2023). Stevia leaves are a source of diterpene glucosides (mainly steviosides and rebaudiosides), compounds that are 300 times sweeter than saccharose (Adari *et al*., 2016; Ameer *et al*., 2017; de Andrade *et al*., 2024). Additionally, steviosides exhibit several health-promoting properties, such as blood pressure-lowering effects in individuals with hypertension, increased insulin levels in blood, and the elimination of reactive oxygen species, among others (Ciriminna *et al*., 2019; Shahu *et al*., 2023; Munir *et al*., 2024).

Currently, stevia extracts have been approved for use in food and beverages in most countries worldwide, including Japan, Singapore, Switzerland, the United States, the

United Kingdom, Russia, China, India, Canada, and Brazil (Ashwell, 2015; Siddique *et al*., 2016; Tey *et al*., 2016; Farhat *et al*., 2019). Worldwide demand has increased the cultivation area of this crop. However, low germination and seed viability have necessitated the use of vegetative propagation strategies, such as stem cutting cultivation (applying indole-3-butyric acid, IBA). Nevertheless, this propagation method is not sufficient to meet the seedling demand required by the global market for stevia commercial production, in addition to the fact that IBA must be applied constantly to plants (Sharma *et al*., 2015; Galo, 2019). Moreover, these hormones have been reported as weak growth promoters in stevia (Kassahun and Mekonnen, 2011).

An alternative to improve the propagation of plant cuttings is the use of beneficial microorganisms, which improve root and shoot growth (Bezerra *et al*., 2019; Vicente-Hernández *et al*., 2018). Arbuscular mycorrhizal fungi (AMF) are microorganisms from the phylum Glomeromycota and are obligate symbionts of nearly 90 % of terrestrial plant species (Ganugi *et al*., 2019; Ferreira *et al*., 2021; Szentpéteri *et al*., 2023). The symbiosis, denominated mycorrhizal, involves the transfer of nutrients from the fungus to the plant (mainly phosphorus and nitrogen) and carbon sources from the plant to the fungus (mainly sugars and lipids) (Luginbuehl *et al*., 2017).

The inoculation of different AMF species promotes plant growth and provides protection against abiotic and biotic stress (Trinidad-Cruz *et al*., 2017a; Chen *et al*., 2018; Wahab *et al*., 2023; Bhupenchandra *et al*., 2024). The interaction of mycorrhizae with hormones such as auxins has been previously reported, suggesting a positive correlation between auxin content and the level of mycorrhizal colonization (Chen *et al*., 2023; Abd-Alla *et al*., 2024).

In *S. rebaudiana*, AMF application has increased yield and improved the nutritional, physiological, and quality attributes of the harvested crop. In particular, an increase in stevioside and rebaudioside content due to inoculation with AMF has been reported (Hoseini *et al*., 2016; Tavarini *et al*., 2018). Nonetheless, no information exists on the effect of AMF during the initial rooting and propagation stages in stevia plants. Therefore, the objective of this research was to quantify the effect of AMF inoculation and IBA application on the propagation of stevia stem cuttings in a greenhouse, hypothesizing that AMF and its interaction with IBA positively affect cutting quality.

MATERIALS AND METHODS

Arbuscular mycorrhizal fungi inoculums

The study was performed in the Plant Biotechnology greenhouse at the Research and Assistance Center in Technology and Design of the State of Jalisco, A.C. (CIATEJ), located at 20°42′ N, 103° 28′ W, and an altitude of 1675 m. *Funneliformis mosseae*, *Rhizophagus intraradices*, and two mycorrhizal consortia, “Las Campesinas” (consisting of *Acaulospora* spp., *Claroideoglomus* sp., *Entrophospora* sp., *Funneliformis* sp., *Glomus* spp., and *Septoglomus* sp.) and “Cerro del Metate” (*Acaulospora* spp., *Glomus* spp., *Septoglomus* sp., and *Dentiscutata* sp.) (Trinidad-Cruz *et al*., 2017b), were used in the inoculation experiment. These belonged to the microorganism collection of the Phytopathology Laboratory at CIATEJ.

Indole-3-butyric acid treatments and seedbeds

Three concentrations of indole-3-butyric acid (IBA: 0, 0.075, and 0.15 %) in two application forms (powder and liquid solution) were prepared. Radix 1500 (Intercontinental Import Export, Celaya, Mexico), formulated as an impregnated powder containing 0.15 % indole-3-butyric acid, was used as the IBA source for the powder treatments, and reagent-grade IBA at ≥98.0 % (Sigma-Aldrich, St. Louis, MO, USA) was used to prepare the liquid treatments.

For powder application, IBA powder was added to perlite powder (Agrolita ground, 0.01–0.001 mm in diameter, ACCIMIN, Mexico City, Mexico) until it reached each of the evaluated concentrations. For liquid solution application, IBA was diluted in sterile distilled water at 0, 0.075, and 0.15 %. For stevia stem cutting rooting, a seeder with sterile substrate (120 °C, 1.05 kg cm^{-2}, 6 h), composed of a mixture of perlite, peat moss, and coconut fiber in a volume ratio of 2:6:2, was used.

Treatment applications, mycorrhizal fungi inoculation, and growing conditions

Stevia stem cuttings of 10 cm in height, with two leaves at the main apex, were used. The stems were provided by Agrostevia SAPI de C.V. (Tepic, Mexico). IBA powder impregnation was performed 1 cm from the base of each cutting before transplant, whereas treatments with IBA solution were carried out by immersing the cuttings for 1 h.

AMF inoculation was performed using 20 g of inoculum (equivalent to 40 spores) deposited on the substrate during transplant. The seeders were maintained in the greenhouse at a relative humidity of 80–90 % using a fogger system with tap water and a photoperiod of 16 h of light for 45 d. At this point, the experiment ended, and all measurements of the response variables were taken.

Microbiological response and plant growth

Root staining was performed using the method of Phillips and Hayman (1970) 45 d after the experiment was established, and mycorrhizal activity in the roots was evaluated as the percentage of total, hyphal, vesicular, and arbuscular colonization (McGonigle *et al*., 1990) using an optical microscope (Velab VE-BC3 Plus, Mexico City, Mexico). The plant growth variables of height (cm), root length (cm), stem diameter (mm), number of leaves, and number of lateral roots (NLR) were measured, and root and shoot dry weight (g) were determined after oven-drying the plant tissue at 65 °C for 120 h.

Experimental design and statistical methods

A completely randomized experiment was performed with a bifactorial treatment arrangement: (1) AMF factor with five levels: (a) *Funneliformis mosseae*; (b) *Rhizophagus intraradices*; (c) “Las Campesinas” consortium; (d) “Cerro del Metate” consortium; and

(e) without AMF; and (2) IBA factor with six levels: (a) IBA mixed with perlite powder at 0 , (b) 0.075 , and (c) 0.15 %; and (d) IBA dissolved in water (solution) at 0, (e) 0.075, and (f) 0.15 %. The combination of bifactorial levels resulted in a total of 30 treatments. Four replicates were performed per treatment.

Data for microbiological and plant growth variables were analyzed using bifactorial and unifactorial analysis of variance (ANOVA) after assessing the assumptions of normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett test). When significant differences were found, a comparison of means (Tukey's test) was performed; both analyses were conducted at a significance level of 5 % ($p \leq 0.05$) using the statistical program Statgraphics Centurion XVII (StatPoint, 2005).

RESULTS AND DISCUSSION

Microbiological response

Differences in mycorrhizal colonization were quantified in stevia stem cuttings treated with AMF and IBA (Table 1). The highest total AMF colonization was observed in cuttings inoculated with *Rhizophagus intraradices*; however, this value was not statistically significant compared to the other AMF treatments and the treatment with a 0.15 % IBA solution.

The highest value of arbuscular content was observed in cuttings colonized by *Funneliformis mosseae*, "Las Campesinas," and "Cerro del Metate" consortia; vesicle content was highest in cuttings inoculated with "Cerro del Metate," and hyphal content in cuttings inoculated with *R. intraradices* (Figure 1). No differences were observed in mycorrhizal colonization (%) for the AMF × IBA interaction. Stem cuttings treated with 0.15 % IBA solution showed an increase in total, arbuscular, and hyphal colonization, whereas vesicular colonization increased with 0.075 % IBA solution.

Differences in total arbuscular colonization were observed in the analysis of the effect of IBA application on stevia roots with each AMF (Figure 2). Both variables for *F. mosseae* increased with the application of 0.15 % IBA solution, whereas for *R. intraradices*, only total colonization increased with the application of IBA at 0.075 and 0.15 % in solution and 0.075 % in powder form. No differences were observed in the remaining treatments.

Table 1. Effect of arbuscular mycorrhizal fungi (AMF) and indole-3-butyric acid (IBA) on mycorrhizal colonization of stevia stem cuttings, 45 d after the experiment was established.

Factor	Mycorrhizal colonization (%)			
	Total	Arbuscular	Vesicular	Hyphal
		AMF		
"Cerro del Metate"	17.3 ± 3.6 a*	1.8 ± 0.6 ab	4.5 ± 1.5 a	11.0 ± 1.9 bc
"Las Campesinas"	19.8 ± 3.2 a	2.8 ± 0.7 a	3.0 ± 0.7 ab	13.9 ± 2.3 ab
Funneliformis mosseae	20.5 ± 3.3 a	3.0 ± 1.1 a	0.8 ± 0.3 b	16.7 ± 2.4 ab
Rhizophagus intraradices	21.2 ± 3.6 a	0.2 ± 0.1 b	0.3 ± 0.3 b	20.7 ± 3.5 a
Non-AMF	3.0 ± 1.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	3.0 ± 1.0 c
		IBA		
IBA powder 0 %	9.4 ± 3.1 b	1.0 ± 0.4 ab	1.7 ± 1.0 a	6.7 ± 2.1 c
IBA powder 0.075 %	11.8 ± 3.0 b	1.1 ± 0.4 ab	1.1 ± 0.5 a	9.7 ± 2.6 bc
IBA powder 0.15 %	15.0 ± 2.9 b	0.7 ± 0.3 b	1.3 ± 0.7 a	13.0 ± 2.6 abc
IBA solution 0 %	13.3 ± 3.0 b	2.0 ± 0.9 ab	1.2 ± 0.5 a	10.2 ± 2.0 bc
IBA solution 0.075 %	20.5 ± 3.9 ab	1.1 ± 0.4 ab	2.7 ± 1.2 a	16.7 ± 3.4 ab
IBA solution 0.15 %	28.1 ± 4.5 a	3.6 ± 1.4 a	2.5 ± 1.4 a	22.0 ± 3.3 a
		Interaction AMF × IBA		
F-distribution	1.10	1.57	0.77	1.60
p-value	0.3652	0.0795	0.7412	0.0698

*The values are the means ± standard error (SE) of four replicates. The same letter indicates no significant differences between treatments according to Tukey's test ($p \leq 0.05$).

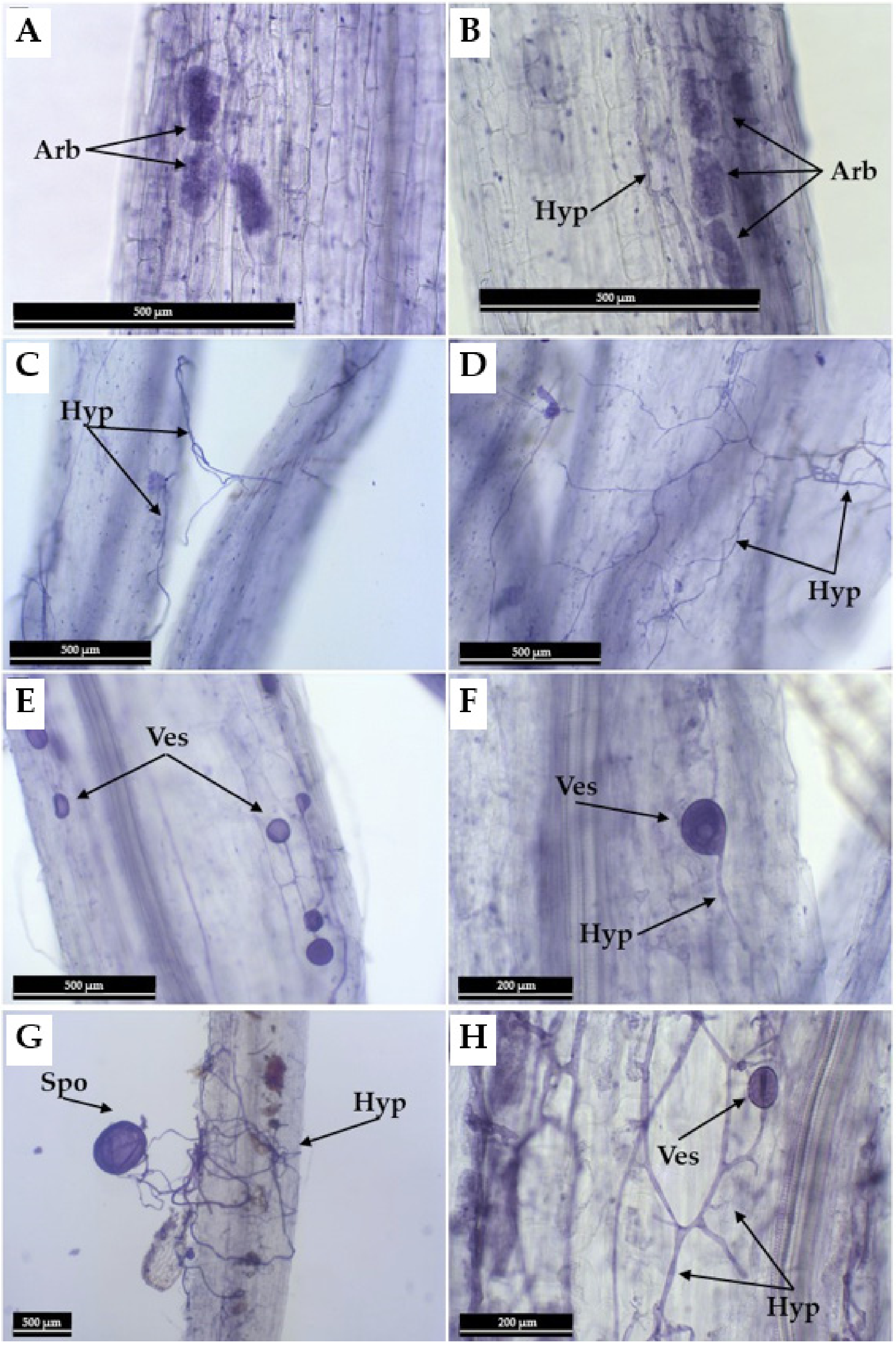

Figure 1. Arbuscular mycorrhizal fungi (AMF) structures in *Stevia rebaudiana* Bertoni roots. *Funneliformis mosseae* (A, B); *Rhizophagus intraradices* (C, D); "Cerro del Metate" (E, F); "Las Campesinas" (G, H). Arb: arbuscule; Hyp: hypha; Ves: vesicle; Spo: spore.

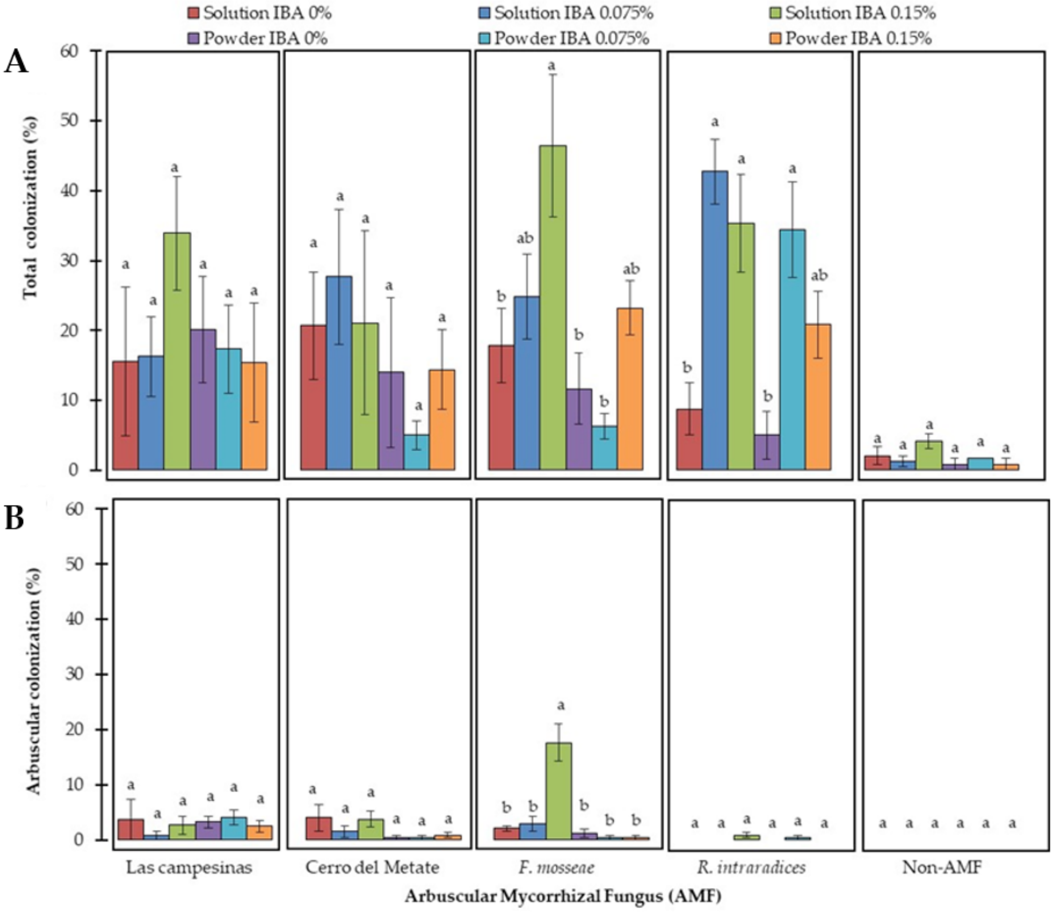

Figure 2. Effect of indole-3-butyric acid (IBA) on mycorrhizal colonization of stevia stem cuttings, 45 d after the experiment was established. A: total colonization; B: arbuscular colonization. The results are shown as means (n = 4) and the bars above the columns indicate the standard error (SE). The same letter indicates no significant differences between treatments according to Tukey's test ($p \leq 0.05$).

Plant growth response

Differences were observed in stevia stem cutting growth variables with AMF and IBA (Table 2). For root dry weight, the highest values were observed in stevia stem cuttings without AMF; for root length, greater values were observed in the "Cerro del Metate" consortium, *F. mosseae*, and *R. intraradices*, as well as in cuttings treated with the recommended IBA dose (0.15 %). In the other morphological variables, no differences were observed in the response of stevia stem cuttings to AMF inoculation. In the analysis of the AMF effect and that of each IBA application on stevia stem cuttings, differences in root length and leaf dry weight were observed (Figures 3 and 4). The highest root length values were observed in cuttings treated with IBA 0.075 %

Table 2. Effect of arbuscular mycorrhizal fungi (AMF) and indole-3-butyric acid (IBA) on the morphological parameters of stevia stem cuttings, 45 d after the experiment was established.

Factor	Height (cm)	Root length (cm)	Stem diameter (mm)	Number		Dry weight (g)	
				Leaves	Lateral roots	Shoot	Root
AMF							
“Cerro del Metate”	12.8 ± 0.3 a*	7.3 ± 0.1 a	2.1 ± 0.1 a	11.2 ± 0.4 a	20.7 ± 1.0 a	0.22 ± 0.01 a	0.08 ± 0.00ab
“Las Campesinas”	11.7 ± 0.2 a	6.4 ± 0.2 b	2.0 ± 0.0 a	10.8 ± 0.4 a	19.0 ± 1.2 a	0.20 ± 0.01 a	0.06 ± 0.00 b
Funneliformis mosseae	12.2 ± 0.3 a	7.1 ± 0.2 a	2.0 ± 0.0 a	10.7 ± 0.3 a	17.9 ± 1.1 a	0.21 ± 0.01 a	0.06 ± 0.00 b
Rhizophagus intraradices	12.1 ± 0.4 a	7.1 ± 0.1 a	2.0 ± 0.0 a	11.8 ± 0.4 a	17.9 ± 1.2 a	0.21 ± 0.01 a	0.08 ± 0.01 ab
non-AMF	12.4 ± 0.3 a	7.2 ± 0.2 a	2.1 ± 0.0 a	11.9 ± 0.5 a	17.9 ± 1.2 a	0.23 ± 0.01 a	0.09 ± 0.01 a
IBA							
IBA powder 0 %	11.0 ± 0.3 b	6.6 ± 0.1 b	2.0 ± 0.0 a	10.5 ± 0.5 a	14.7 ± 1.1 b	0.18 ± 0.01 b	0.06 ± 0.00 b
IBA powder 0.075 %	12.5 ± 0.4 a	7.1 ± 0.2 ab	2.1 ± 0.1 a	11.8 ± 0.5 a	22.9 ± 1.0 a	0.23 ± 0.01 a	0.09 ± 0.01 a
IBA powder 0.15 %	13.0 ± 0.3 a	7.6 ± 0.1 a	2.0 ± 0.0 a	11.1 ± 0.4 a	18.9 ± 1.3 ab	0.21 ± 0.01 ab	0.07 ± 0.01 b
IBA solution 0 %	12.0 ± 0.4 ab	6.8 ± 0.2 b	2.1 ± 0.1 a	11.3 ± 0.5 a	18.5 ± 1.1 ab	0.22 ± 0.01 ab	0.07 ± 0.01 ab
IBA solution 0.075 %	12.5 ± 0.3 a	7.0 ± 0.2 ab	2.1 ± 0.0 a	11.6 ± 0.5 a	17.8 ± 1.3 b	0.22 ± 0.01 ab	0.07 ± 0.01 b
IBA solution 0.15 %	12.4 ± 0.3 ab	6.9 ± 0.2 b	2.1 ± 0.0 a	11.4 ± 0.5 a	19.0 ± 1.3 ab	0.23 ± 0.01 a	0.07 ± 0.01 ab
Interaction AMF × IBA							
F-distribution	1.42	3.45	1.24	1.11	0.74	0.75	1.98
p-value	0.1362	0.0000	0.2451	0.3566	0.7759	0.7593	0.0159

*The values are the means ± standard error (SE) of four replicates. The same letter indicates no significant differences between treatments according to Tukey’s test ($p \leq 0.05$).

+ *F. mosseae* (powder and solution) and IBA 0.15 % + *F. mosseae* (powder and solution). No differences in root length were observed in cuttings treated with IBA powder at 0 and 0.15 %, and IBA solution at 0 % + AMF. In shoot dry weight, differences were observed only with the application of IBA solution 0.15 % + AMF. The interactions found between AMF and IBA were observed in root variables (length and dry weight) (Table 2 and Figure 3). In particular, the “Las Campesinas” consortium and *F. mosseae* were influenced by the IBA application form and concentration, with a significantly higher effect for both root variables in stevia stem cuttings treated with *F. mosseae* + IBA solution 0.15 % (Figures 3 and 4).

The traditional propagation method in *S. rebaudiana* is the cultivation of cuttings using IBA as a root promoter (Rakibuzzaman *et al*., 2018; Pigatto *et al*., 2018; Galo, 2019; Muslihatin *et al*., 2023; Neisiani *et al*., 2024); however, the use of beneficial microorganisms, such as AMF, may represent an alternative in vegetative propagation (Abdel-Rahman *et al*., 2019; Tchiechoua *et al*., 2019; Dewir *et al*., 2023; Taghizadeh *et al*., 2023).

In this study, AMF did not promote plant growth in stevia stem cuttings; however, mycorrhizal symbiosis was successfully established, as evidenced by the presence of mycelium, arbuscules, and vesicles. These structures actively participate in the

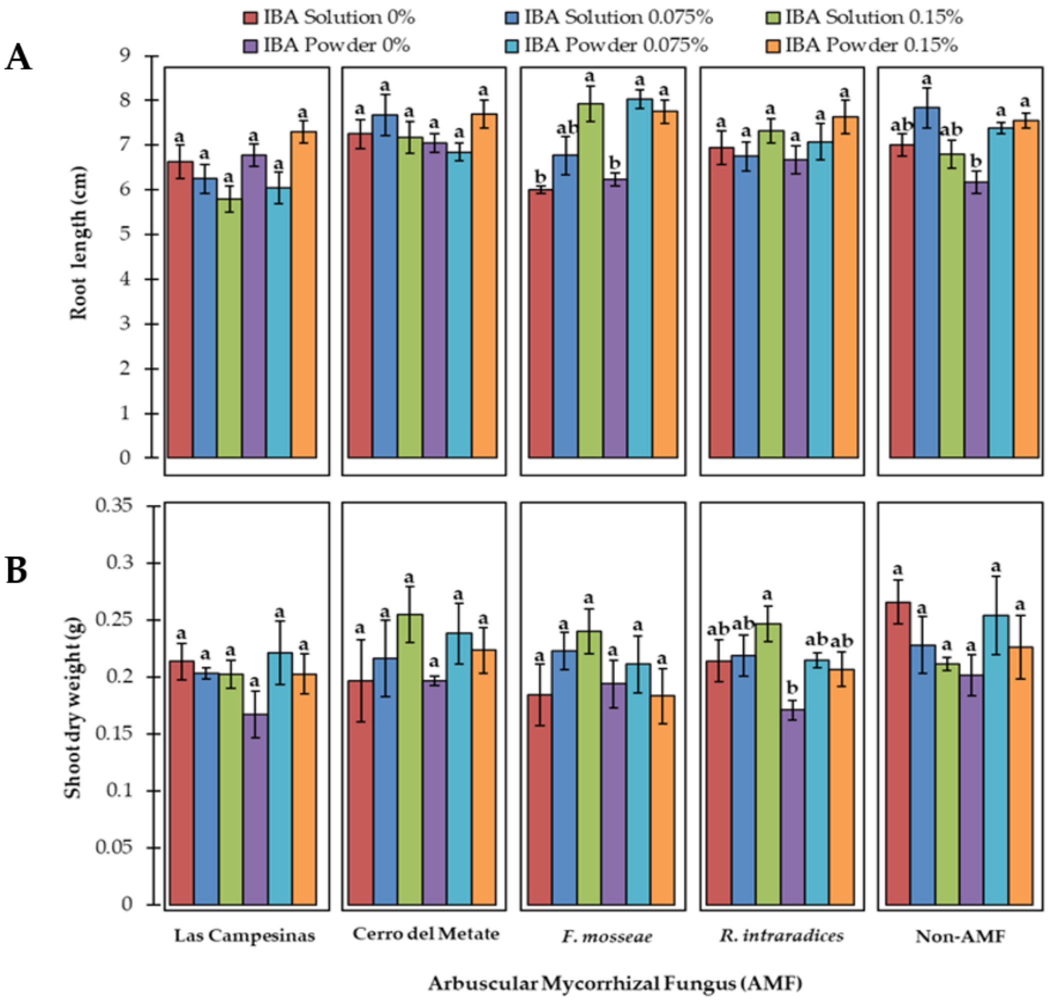

Figure 3. Effect of arbuscular mycorrhizal fungi (AMF) and indole-3-butyric acid (IBA) on growth of stevia stem cuttings, 45 d after the experiment was established. A. root length; B: shoot dry weight. The results are shown as means (n = 4) and the bars above the columns indicate the standard error (SE). The same letter indicates no significant differences between treatments according to Tukey's test ($p \leq 0.05$).

storage, exchange, and transport of soil nutrients to the host (Fernandes *et al*., 2019; Chenchouni *et al*., 2020; Tchiechoua *et al*., 2019;). It is necessary to evaluate plant growth over a longer period to observe the effects of AMF on stevia plants. Moreover, since the substrate used was nutrient-poor, the stevia cuttings grew mainly at the expense of their accumulated reserves, making the benefits related to nutrient uptake by AMF barely detectable, which could explain why no marked differences in growth were observed among AMF treatments. On the other hand, the presence of hyphae in the non-mycorrhizal treatment is attributed to microorganisms that colonize after

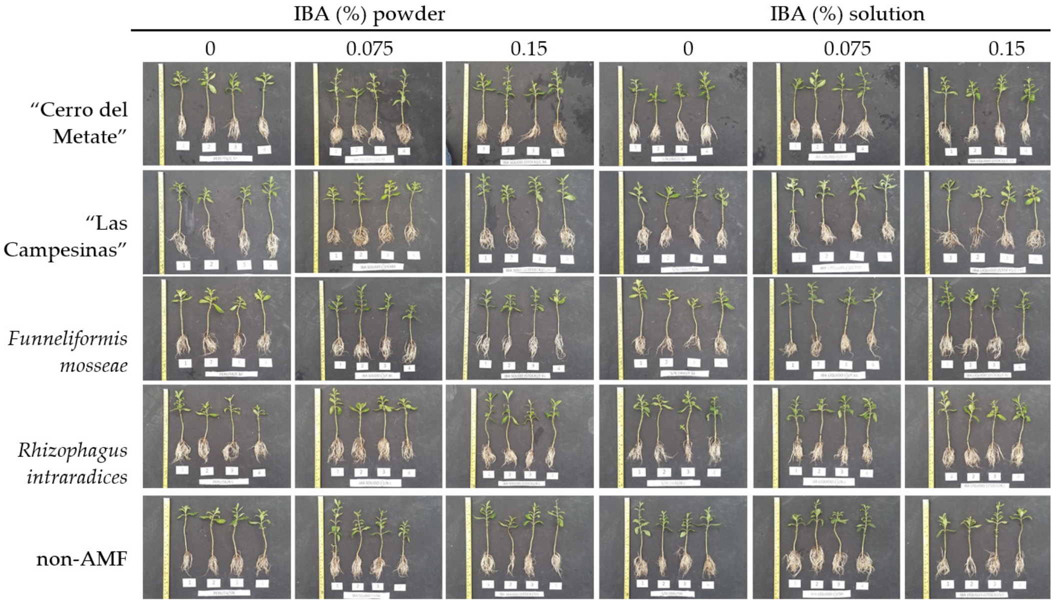

Figure 4. General appearance of stevia plant inoculated with arbuscular mycorrhizal fungi (AMF), 45 d after the experiment was established, across indole-3-butyric acid (IBA) treatments (powder and solution). The yellow bar indicates a scale of 30 cm.

the experiment is established, through spores carried by the wind; however, their presence is very low, and they correspond to non-mycorrhizal fungi, as no arbuscules or vesicles were observed.

The colonization of AMF in stevia plants has been previously reported (Bonfante and Genre, 2010; Tedone *et al*., 2020); however, the results of this study show for the first time that different AMF species and consortia can colonize stevia stem cuttings during the rooting stage under greenhouse conditions. In particular, the presence of arbuscules in plant roots indicates functional symbiotic activity between AMF and the host, as nutrient exchange occurs in this fungal structure between the endophyte and the plant (Bao *et al*., 2019; Thirkell *et al*., 2020). Furthermore, the presence of mycelium and vesicles in roots indicates a differential response of stevia to colonization by the different AMF species inoculated. These results agree with those reported by Quiñones-Aguilar *et al*. (2016), where the same plant species may exhibit differential growth responses depending on colonization levels by different AMF consortia and species. According to Chen *et al*. (2018), the plant response to AMF inoculation may result from functional specificity between endophytes and the host, which could explain the differences in endophyte proliferation levels observed among the AMF consortia and species inoculated.

Moreover, stevia stem cuttings grew when inoculated with AMF, but root length and dry weight values were lower compared to those without AMF inoculation. Li *et al*.

(2015) reported that plants modify their root architecture during symbiotic association with AMF as a strategy to improve water and nutrient absorption; that is, plants reduce the energy expenditure required for root production and instead take advantage of the external AMF mycelium to absorb water and soil nutrients (Wang *et al*., 2011). Additionally, plants have been reported to reduce growth parameters (mainly fresh and dry biomass) because mycorrhizal symbiosis requires a high carbon (C) allocation to maintain the association between endophyte and root (Tavarini *et al*., 2018).

In relation to plant hormones such as auxins (IBA), these are defined as promoters of plant growth and development, intervening in plant responses to both biotic and abiotic factors and promoting lateral root development (Sukumar *et al*., 2012; El-Banna *et al*., 2024; Song *et al*., 2024). Several studies have indicated that auxins act as signaling molecules in regulating interactions between AMF and the host, mainly in root architecture, modulation processes, endophyte spore germination, and arbuscule formation (Etemadi *et al*., 2014; Chen *et al*., 2017).

In this study, the application of IBA in solution increased mycorrhizal colonization in stevia stem cuttings compared to IBA in powder, which showed no effect on colonization levels. IBA powder can be denatured as a phytohormone by several factors, particularly chemical or biological oxidation by microorganisms, whereas IBA in solution does not present these potentially negative effects, as reported in studies on rooting of peach cuttings (*Prunus persica* (L.) Batsch) (Lesmes-Vesga *et al*., 2021). On the other hand, in nasturtium plants (*Tropaeolum majus* L.) inoculated with *R. intraradices*, IBA levels (free and total) are significantly increased compared to plants without AMF (Jentschel *et al*., 2007). In this context, it is hypothesized that when IBA powder is denatured, the available amount that promotes mycorrhizal colonization is reduced, which could explain the low colonization levels observed in stevia cuttings in this study.

Moreover, the IBA application dose played an important role in plant rooting response (Faisal *et al*., 2018; Fan *et al*., 2020), with reports indicating that low concentrations of this hormone improve root length and number in plants (Hoseini *et al*., 2016). Similarly, better mycorrhizal colonization is expected at low IBA doses (Chen *et al*., 2022), possibly because excess auxins affect root architecture as well as the hormonal balance required for proper plant function. Thus, the use of low IBA doses combined with AMF in stevia stem cuttings may represent an effective strategy for obtaining vigorous plants while reducing crop production costs by optimizing plant hormone dosage and application method.

CONCLUSIONS

This study showed the capability of arbuscular mycorrhizal fungus (AMF) to colonize stevia stem cutting during their rooting stage. Furthermore, the application of indole-3-butyric (IBA) influenced on endophyte colonization levels in the host root. Particularly, stimulating cutting by low IBA dose could be important to improve AMF-

Root interaction, mycorrhizal colonization, and obtaining plants with greater vigor. Although no significant differences were observed in the growth of cuttings inoculated with AMF compared to those not inoculated, colonization was successfully established. Therefore, longer term studies evaluating response parameters are needed to determine these differences. Furthermore, studies are needed to determine the influence of IBA application on the establishment of symbiosis between the plant and the endophyte. Integrating knowledge of the effects of applying different AMF species and consortia is crucial for the development and application of sustainable agricultural production of stevia.

ACKNOWLEDGEMENTS

We thank Diana Fischer for translation/editorial services.

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