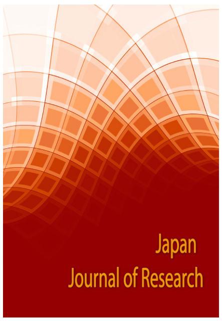
Japan Journal of Research

Original Article



Growth and sweetener content in Stevia rebaudiana Bert. biofertilized with Rhizophagus intraradices (Schenck & Sm.) Walker ted & Schüßler, and Azospirillum brasilense Tarrand, Krieg & Döbereiner in a substrate with bovine manure added

Juan Francisco Aguirre-Medina[1], Flor R Bartolón-Morales[1], Mayra Martínez-Solis[1], Jorge Cadena-Iñiguez[2*], Valeria A Martínez-Sias[2]

[1]*Facultad de Ciencias Agrícolas, Campus IV, Universidad Autónoma de Chiapas, Mexico.*

[2]*Campus San Luis Potosí, Colegio de Posgraduados (COLPOS), Mexico.*

Correspondence

Jorge Cadena-Iñiguez

Campus San Luis Potosí, Colegio de Posgraduados (COLPOS), Calle Iturbide No. 73, Salinas de Hidalgo, S.L.P., C.P. 78600 México.

Tel: 4969634000

E-mail: jocadena@colpos.mx

- Received Date: 30 May 2020;
- Accepted Date: 10 June 2020;
- Publication Date: 12 Oct 2020.

Abstract

Growth and sweetener content were evaluated in *Stevia rebaudiana* Bert. cv. Morita 2, biofertilized with *Rhizophagus intraradices* and *Azospirillum brasilense* in substrates (soil + sand (1: 1 v / v), and soil + bovine manure (0.70: 0.30 v / v) in nursery. 4 g of each biofertilizer were applied to the transplant in the bottom of the hole *A. brasilense* in a concentration of 9 x 106 bacteria g-1 and *R. intraradices* 40 spores g-1 of soil and 95% colonization in a completely randomized design and six replications, microorganisms and substrates were combined, with Morpho-physiological variables of yield, final content of N, P, sweeteners and colonization of roots at 60, 90 and 120 days. The largest initial growth was in soil plus bovine manure, while *R. intraradices* and *A. brasilense* induced a greater increase. in the morpho-physiological yields at 120 days, also registering a higher proportion of sweetener, N and P.

Introduction

Stevia rebaudiana Bert. is a perennial [1] plant belonging to the *Asteracecae* family. It is endemic to southeastern Brazil and northern Paraguay where it is used traditionally for nutritional and medicinal purposes [1]. Leaves contain terpene glucosides [2] which are synthetized within the gibberellic acid route parting from mevalonate [3]. The active constituents of the leaves, especially estevioside and A rebaudioside have a sweetening effect, which is greater than glucose [4]. Their concentration is influenced by genotype, environmental conditions and agronomic practices such as nutrition [5].

In Mexico, stevia production acreage has increased and application of synthetic fertilizers is recommended at rates of 105 kg of N, 23 kg of P_2O_5 and 180 kg of K_2O. ha-1 [6]. However, synthetic fertilizer application can harm the environment and increase production costs [7]. In this context, special attention has been given to feed plants with organic manures and/or soil microorganisms which establish a beneficial symbiosis in the host plant root system and significantly affect its function, especially under biotic or abiotic stress conditions. Hence, these beneficial microorganisms are considered agents of biological fertilization or bio fertilizers which do not contaminate the environment.

As of 2002 in Mexico, there was an increase in agricultural crops bio fertilized with endomycorrhizal fungi and nitrogen-fixing bacteria such as *Rhizobium* and *Azospirillum*, especially in black beans and maize [8]. Today, the Mexican ministry of Agriculture [9] reports an increase in bio-fertilizer use in farms from 2014-2015. This allowed for the non-application of 69 589 mt of synthetic fertilizers and the resulting decrease of 22.7 thousand tons of CO_2 produced as well as an increase in crop yield in 15% of the crops produced.

It is also evident that some organically produced vegetables present high contents of secondary phenolic metabolites[10], such as stevoides, [11], these increase when microorganisms are inoculated into substrates having high organic matter contents [12]. In *stevia* biomass increase is enhanced [5].

The objective of the present experiment was to determine the effect of biofertilization with two microorganisms alone and combined, on the vegetative development of *Stevia rebaudiana* Bert. when grown in substrates with and without bovine manure added and to identify sweetener, N and P content in leaves.

Citation: Aguirre-Medina JA, et al. Growth and sweetener content in Stevia rebaudiana Bert. biofertilized with Rhizophagus intraradices (Schenck & Sm.) Walker ted & Schüßler, and Azospirillum brasilense Tarrand, Krieg & Döbereiner in a substrate with bovine manure added. Japan J Res. 2020;1(2):1-5.

Material and Methods

The experiment was carried out under nursery conditions at the experiment farm of the Agricultural Sciences Faculty of the University of Chiapas (UNACH) located in Huehuetan, Chiapas Mexico (15° 00´ y 15° 30´ N, 94° 30´ y 95° 00´ O) at an altitude of 44 meters above sea level. Climate is classified as Am (w") i g according to García [13]. This defines it as warm humid tropical with summer rains, an average rainfall of 2500 mm and high low temperatures of 38º and 15º C. Substrates used were composed of soil (classified as a eutric flavisole), bovine manure and previously washed and graded river sand. Manure was ground in a hammer mill and sterilized (15 PSI at 250 °C for 20 min). 30% v/v of manure was added to substrate bags in the corresponding treatments. Substrate Analyses were carried out at the laboratory of the Agricultural Sciences Faculty (UNACH). Physical and Chemical characteristics of the soil/sand substrate were as follows: Loamy sand texture (Bouyucos hydrometer) with a 1:2 aqueous solution pH of 5.78, 2.63% O.M. content, (Walkley-Black), 0.13 % N (Micro Kjelddhall), 14.2 ppm P (colorimetry), 64.2 ppm K++ (atomic spectrophotometry), 474 ppm Ca++, 58.0 ppm Mg++, 102.5 ppm Na++, and 5 Meq.100 g-1 cation exchange capacity(CEC). 0.05 ds.m-1 electrical conductivity (conductimeter). For the soil/manure substrate: Loamy sand texture (Bouyucos hydrometer) with a 1:2 aqueous solution pH of 6.64, 4.13% OM (Walkley-Black), 0.20 % N (Micro Kjelddhall), 206.52 ppm P (colorimetry), 1610.00 ppm K++ (atomic spectrophotometry), 1425.00 ppm K++ (atomic spectrophotometry), 385.00 ppm Mg++, 141.00 ppm Na++, 0.87 ds.m-1 electrical conductivity (conductimeter). Black plastic perforated bags (25 x 35 cm 6 k capacity) were filled with the two substrates and placed on iron structures.

Rhizophagus intraradices propagated on a sterile soil with *Brachiaria decumbens* Stapf as a host with 95% root system colonization was used. Product contained 40 spores g^{-1} plus propagules. *Azospirillum brasilense* was prepared with 9x106 cells g^{-1} and impregnated on a peat substrate donated by the microbiological Soil Science laboratory of the Microbiologic Sciences Center of the University of Puebla (BUAP).

Stevia rebaudiana Bert. cv Morita II cuttings were obtained from a farm located 10 km from the experimental site. Vigorous plants without insect or disease damage were selected.

Approximate length of the cuttings was 10 ± 2 cm taken from the upper 1/3 of the plant, and deposited in sterile water for transport, and planting.

4.0g of each inoculum were applied to the corresponding treatments placing them at the bottom of the planting holes in the pot at transplant.

The five treatments were: 1) Soil+ sand, 2) Soil + bovine manure, 3) Soil + bovine manure + *Rhizophagus intraradices*, 4) Soil + bovine manure + *Azospirillum brasilense* 5) Soil + bovine manure + *Azospirillum brasilense* + *Rhizophagus intraradices*. All treatments with six repetitions each in a completely randomized design within the nursery.

Four repetitions were considered in the nitrogen, phosphorus, stevioside, A rebaudioside and steviol analyses. The experimental unit was one container with one plant. Plants were irrigated with distilled water. Destructive samplings were taken at 60, 90 and 120 DAS.

Variables evaluated

Morphological (Number of leaves and branches) and physiological (dry weight of aerial and root components) variables were registered with three destructive samplings. Yield components of the root and aerial parts were weighed in a semi analytic balance after drying in a forced air muffle oven at 75-80ºC to constant weight.

Leaf area was registered in cm^2 with a leaf area integrator (LI-COR, LI 3100). N and P content was obtained by foliar analysis. N was determined by microkjeldahl and phosphorus content with an Olsen/spectrophotometer (Thermo Fisher Scientific Model 400 ¼) at the soil and water laboratory of the Agricultural Sciences Faculty of the UNACH in Huehuetan, Chiapas, Mexico.

For the stevioside, A-rebaudisoid and steviol analyses, fresh leaves were oven dried at 60 ºC up to constant weight and then ground in an electric mill until a fine powder was obtained (1 mm mesh). Compounds were determined according to Hashimoto and Morigasu [14], using an HPLC chromatograph at the Plant Chemistry Laboratory of the College of Postgraduate studies in Montecillo, Mexico. Colonization percentage was quantified only for Rhizophagus intraradices Schenk y Smith using the Phillips and Hayman [15] technique. One hundred root segments 1.5 – 1.6 cm long were observed with an optical microscope with an oil immersion lens (100X).

Experimental design

Experimental design was completely randomized with six repetitions and data was analysed with the SAS 8.1[16] version GLM procedure. Media were compared with the Tukey ($P \le 0.05$) test. Percentage values of N and P nutrients were converted to arch sines for statistical analyses.

Results and Discussion

Morphological components

Number of leaves and branches was statistically different ($P \le 0.05$) between treatments during the three samplings (Table I). At 50 DAS highest increase in both plant was observed when the substrate received added bovine manure and lowest leaf number was observed in treatments receiving both microorganisms together. Initial development of biofertilized plants during symbiotic initiation, seems to be related to photosynthate demand of the root system [17], as occurs with endomycorrhizal fungi (Wright et al. 2005). In the case of *Rhizophagus intraradices* at 60 DAS it coincides with 35% root colonization. At 90 DAS the number of leaves increased in this treatment and root colonization increased to 65%. At 120 DAS highest leaf number occurred in the biofertilized with two microorganisms and endomycorrhizal fungus colonization reached 72%.

Lack of response in the first two evaluations coincides in time with response found with response observed in other plants with endomycorhysic fungi with different colonization percentages. In *Tabebuia donnell-smithii* this slow phase occurred at 84 DAS [18] and in *Coffea canephora* (perre ex Froehner, 54 DAS [19].

Biomass increase of *Stevia* through time in biofertilized treatments seems to be influenced by the establishment of symbiosis in the host plant roots and by photosynthate demand. Consequently, plant growth response depends on its capacity make them available especially when energy demand is increased in order to favor plant-bacteria recognition mechanisms during symbiosis establishment [17].

During symbiosis establishment, nutrient transport by endomycorhysic fungi, or nitrogen fixation by bacteria seems to be minimal and results are expressed by differential induction of plant development of *S. rambaidiana* Bert. in time.

Time (days)	Treatment	Number.plant^{-1}		Dry weight (g.plant^{-1})			Leaf area (cm^2.plant^{-1})
		Leaves	Branches	Root	Stem and branches	Leaf	
60	Soil: Sand	172 b*	5.6 bc	0.76 a	0.701 c	1.75 b	231.67 d
	Soil:Bovine manure	197 a	6.1 ab	0.80 a	1.583 a	2.29 a	409.75 a
	Soil:Bovine manure + *Azospirillum brasilense*	173 b	6.6 a	0.63 b	1.003 b	2.31 a	385.20 b
	Soil:Bovine manure + *Rhizophagus intraradices*	160 b	4.8 cd	0.40 c	0.476 d	0.84 c	252.75 c
	Soil:Bovine manure + *Rhizophagus intraradices* + *Azospirillum brasilense*	111 C	4.0 d	0.29 c	0.371 e	0.80 c	174.75 e
	CV	5.2	9.2	11.1	6.1	7.7	3.5
90	Soil: Sand	247 b	6.0 b	1.51 a	5.43 a	4.74 b	1170.1 b
	Soil:Bovine manure	301 a	7.5 a	1.48 a	5.60 a	5.31 a	1411.5 a
	Soil:Bovine manure + *Azospirillum brasilense*	253 b	5.3 bc	0.92 b	3.86 b	3.47 d	1262.4 b
	Soil:Bovine manure + *Rhizophagus intraradices*	324 a	5.3 bc	0.80 c	4.01 b	3.96 c	1470.5 a
	Soil:Bovine manure + *Rhizophagus intraradices* + *Azospirillum brasilense*	209 c	4.3 c	0.97 b	3.90 b	3.48 d	911.7 c
	CV	6.6	14.6	5.8	3.3	4.5	6.1
120	Soil: Sand	499 b	14.1 b	2.17 a	9.77 b	6.97 c	1791.4 b
	Soil:Bovine manure	484 cd	10.0 c	1.06 d	9.07 c	7.30 c	1710.2 b
	Soil:Bovine manure + *Azospirillum brasilense*	527 c	13.8 b	2.05 a	10.92 a	9.78 a	2676.9 a
	Soil:Bovine manure + *Rhizophagus intraradices*	452 d	9.1 c	1.34 c	8.58 d	8.19 b	1801.6 b
	Soil:Bovine manure +*Rhizophagus intraradices* + *Azospirillum brasilense*	730 a	16.5 a	1.69 b	11.09 a	8.06 b	2444.9 a
	CV	6.3	9.2	8.1	2.7	5.6	4.7

CV = Coefficient of variation (%). *Values with different letter within each factor and column are statistically different (P ≤ 0.05)

Table 1. *Growth of Stevia rebaudiana Bert. biofertilized with microorganisms and additioned with bovine manure*

Increase in leaf number when different crops are biofertilized with mycorrhysic fungi has been reported in *C canephora* (Pierre ex Froehrner [19], and in *Theobroma cacao* [20] and can be related to increases in nutrient and water absorption capacity of the root system, induced by mycelium ramification of endomycorrhizal fungi acting as an extension of the root absorption surface [21].

In *C. canephora* (Pierre) ex Froehner 15 leaves were increased with biofertilization with two microorganisms [19]. Number of branches per plant increased as well. Increase in vegetative and reproductive development has been reported in different annual crops biofertilized with endomycorrhizal fungi and *Azospirillum brasilense* [8].

Physiological components

Initial dry weight of the root system in *Estevia* also showed increased biomass in treatments without microorganisms at 60 and 90 DAS, especially in the soil plus manure substrate in which N, P and Mg content increased notably as did organic matter. In biofertilized treatments, at 60 DAS root biomass was lowest by 21%, with *Azospirillum brasilense,* 50% with *Rhizophagus intraradices* and 63% with the two microorganism symbiosis in comparison with the bovine manure substrate (P ≤ 0.05). At this time, with the same treatment relation, lowest dry matter assigned to the root system was with *Rhizophagus intraradices*. It seems likely that fungus hypha substitute root hairs of the radical system and the plant transports more photosynthates to the aerial parts. Similar results have been reported by Aguirre-Medina and Kohashi-Shibata [22] in black beans with *Glomus intraradices.*

On the other hand, with *Azospirillum brasilense* it increased at 120 DAS. Root growth is generated by phytohormone production by bacteria [23; 24], as indolacetic acid [23; 24; 25], cytokines, gibberellins, that induce more root hairs and consequently favor nutrient intake [26]. In annual crops, *Azospirillum brasilense*. Also induces increase in root biomass when applied in coinoculation with *Azospirillum* and *Glomus* in *Phaseolus vulgaris L.* and *Zea mays L.* [27].

Dry weight of stems and branches also showed less biomass with biofertilized treatments during the first two samplings and highest values were found in the soil plus bovine manure substrate.

In the third sampling at 120 DAS biofertilization of *Azospirillum brasilense* and coinoculation with *Rhizophagus intraradices* and *Azospirillum brasilense* showed greater biomass assignment to branches and stems and this was statistically significant (P ≤ 0.05 %). Different studies show that inoculation with fungi and bacteria induce a synergistic effect in their interaction [28,29]. However, carbohydrate demand increases with coinoculation of more than one microorganism and it is estimated that plants in symbiosis with endomycorrhizal fungi, transfer about 20% of total assimilated carbon [30]. In our particular case, increase in accumulated biomass in the treatment with both microorganisms together indicates functional compatibility of these with the plant and suggests that the host plant was able to supply sufficient carbon to the microorganisms.

Results shown above explain why buildup of dry matter within *Stevia rebaudiana* plant organs varies according to the microorganism applied and its effect is differential in time. Leaf blade weight of *Stevia rebaudiana* was similar to the dry matter assigned to the main stem and branches. During the first two samplings, highest biomass buildup in the leaf blade occurred in treatments which were not inoculated with microorganisms. Statistical differences in favor of treatment with *Azospirillum brasilense* occurred at 60 and 120 DAS. Dry biomass in this treatment was 43% higher than the soil-sand control. Similar results have been reported by Portugal [11] when evaluating different endomycorrhysic fungi on vegetative development of *Stevia rebaudiana* with increased plant biomass as of 60 DAS. Aguirre-Medina [31] in *Cedrella odorata* found higher biomass at 112 DAS with *Azospirillum brasilense* and in the manure substrate control in comparison with *Rhizophagus intraradices* alone and in coinoculation with *Azospirillum brasilense*.

Leaf area of *Stevia rebaudiana* showed significant statistical differences ($P \leq 0.05$ %) between treatments at the first sampling date in favor of the soil plus bovine manure treatment. At 90 DAS statistical differences favored *Rhizophagus intraradices* ($P \leq 0.05$ %) and at 120 DAS highest leaf area occurred when the two microorganisms were coinoculated. Although studies have shown that endomycorrhizal symbiosis lacks taxonomic specificity [32], evidence suggests that there might be functional compatibility between the plant, the substrate and the introduced microorganisms.

Similar results have been obtained when both microorganisms are coinoculated in other perennial crops such as *T. cacao* L. [20] under nursery conditions. In our experience, double symbiosis (*Rhizophagus* + *Azospirillum*) was the best combination increasing biomass in stevia.

With regard to the steviosid, A-rebausteviosid and steviole contents, statistically significant

($P \leq 0.05$ %), differences were observed between treatments (Figure 1).

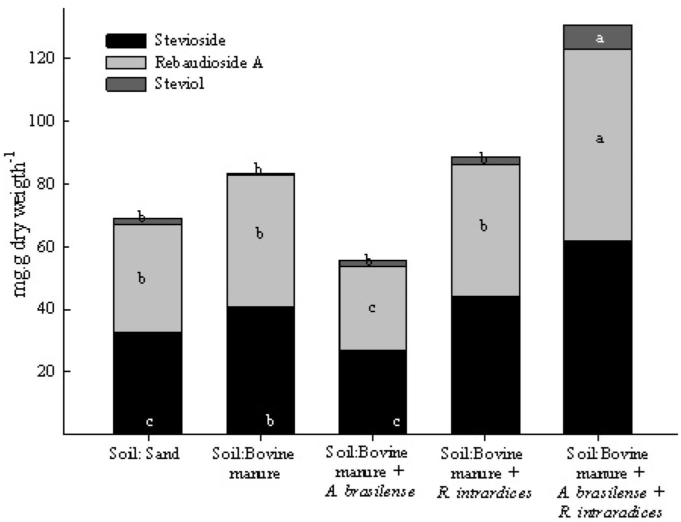


Figure 1. Sweetener content in *Stevia rebaudiana* growing with two microorganisms in two substrates in the nursery. The vertical line indicates ± the standard error of four repetitions. The different letters in each column are statistically different ($P \leq 0.05$). Coefficient of variation (%): Stevioside 8.4, Rebaudioside A 8.3 and Steviol 48.7.

Lowest sweetener content was observed in the treatment biofertilized with *Azospirillum brasilense* and the highest, when biofertilized with both microorganisms. *Rhizophagus intraradices* induced an important increase in sweetener content of leaves and similarly in leaves in the bovine manure treatment. In this case, P content increased upon addition of bovine manure and this could probably have influenced sweetener concentration in the leaves. This nutrient forms an essential part of many glucophosphates such as uridin diphosphate glucose, UDP-glc, a glucose donator in the diterpene glucoside synthesis. Shibata [30] Biofertilization with *Rhizophagus intraradices*, increased P content (0.43 %) in leaves and represented a 19.4 % increase with regard to control.

On the other hand, the lowest P value in leaves was registered with *Azospirillum brasilense* showing values similar to those of substrates without microorganisms (0.38%) as well as the lowest sweetener content value in plant tissue. In mycorrhized plants P content in plant tissue is favored. Numerous studies have shown that mycorrhized plants absorb soil P more efficiently than non colonized plants [34] through fungal hypha affinity to phosphate ions [35] and especially in low-input production systems.

When stevia is grown in nutrient-deficient soils, Jarma [36] did not find variations in total glucoside content but there is a decrease of A-rebaudiside withdeficiencies in P, rabica and copper.

Leaf nitrogen content in biofertilized plants with added bovine manure was highest as compared with the control. In other studies, inoculation with R*hizophagus intraradices* + *Azospirillum brasilense* increased N content in plant tissue of *C rabica* [36].

Biofertilization of *Stevia rebaudiana* Bertoni with *Rhizophagus intraradices* and/or *Azospirillum brasilense* microorganisms induced differential development in different morphological and physiological plant yield components. Leaves, branches and dry biomass of main stems plus branches, of leaf blades and foliar area were increased with double symbiosis. *Stevia rebaudiaana* Bertoni plants biofertilized with two microorganisms maintain a higher proportion of sweeteners, N and P.

Authors' Contributions

The preparation of the material, data collection and statistical analysis were performed by Flor Rocío Bartolon-Morales, Mayra Martínez-Solís and Valeria Abigail Martínez-Sias with the supervision of Juan Francisco Aguirre-Medina and Jorge Cadena-Iñiguez. The first version of the manuscript were made by Rocío Bartolon-Morales, Mayra Martínez-Solís and Valeria Abigail Martínez-Sias and was reviewed and corrected by Juan Francisco Aguirre-Medina and Jorge Cadena-Iñiguez. All authors read and approved the final manuscript.

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