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Original Article

Biomass production and steviolglycoside content of *Stevia rebaudiana* Bertoni var. Morita II, under field conditions

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ABSTRACT

Dry leaf production of *Stevia rebaudiana* Bertoni under Cuban climatic conditions requires the identification of the agro-technical variables with the greatest impact on yield. Therefore, the aim of this work was to evaluate the effect of planting distance on biomass production and steviolglycoside content. Plants were planted in the field at three planting distances: 15x20 cm; 20x20 cm and 25x20 cm. Variables plant height, number of branches per plant, fresh and dry mass of both leaves and stems, and flowering percentage were evaluated after 180 days. In addition, the yield in dry mass per hectare was estimated and the steviolglycoside content was determined. The best results for the agronomic variables evaluated were obtained with the longest planting distance (25x20 cm), except for plant height and flowering percentage. However, these two variables did not influence the behavior of leaf yield (1.48 t ha^{-1}) or steviolglycoside content (450.89 mg g^{-1} dry mass) in the plants of this treatment, indicating a direct relationship of these latter variables with the planting distance of *Stevia rebaudiana* var. Morita II grown under these conditions.

Key words: yield, planting distance, sweet grass

INTRODUCTION

Stevia rebaudiana Bertoni is an herbaceous plant of the Asteraceae family widely produced in the world and its main component is steviolglycosides (stevioside and rebaudioside). The sweetener obtained from this plant has a high natural non-caloric sweetening power and is approximately 300 times sweeter than sucrose [(1-3)]. Its importance is especially relevant in current conditions, where healthier foods are demanded to counteract nutritional disorders such as *Diabetes mellitus* type II [(4)].

The presence in this plant leaves of natural compounds, called steviolglycosides, is what provides the sweetening properties. Steviolglycosides are tetracyclic diterpenes derived from the same kaurenoid precursor as gibberellic acid. Their intense sweetness and use as a sweetener made them a subject of scientific and commercial interest since they first came to the attention of Europeans in 1899 [(5)].

This plant is naturally propagated by seed. However, the low level of seed germination and loss of viability are limiting factors for its large-scale use [(6-8)].

Micropropagation and propagation by cuttings are alternatives to counteract these disadvantages. They are widely used and have feasibility from the economic point of view, quality of propagules produced and satisfaction of the demand for this plant [(9,10)]. However, it is important to establish the adequate agro-technology of the crop and to evaluate the biomass yields and the quality of propagules under production conditions [(11,12)].

Crop planting distance can be a taxing factor in plant growth and development. For *Stevia rebaudiana* Bertoni it is very important to adjust this agro-technical variable if it is taken into account that the product to be harvested in this crop is the foliage. This plant has a pivot root, filiform, which does not go deep; that is, it is distributed close to the surface [(11)], in this sense, a greater planting distance can be favorable for plants, due to the space provided, both aerial and in the soil. This spacing facilitates the expansion of lateral branches and a better uniformity in terms of sunlight received by plant leaves. On the other hand, it also provides more space for root development and better nutrient absorption per plant. In addition, it limits excessive self-shading that could cause decreases in the average photosynthetic rate per unit leaf area, with detriments in the total biomass per plant [(13)]. However, a very large spacing can decrease yields per unit area [(14)].

In Cuba, *S. rebaudiana* cultivation is recent and has not been generalized. There is only one work that reported the study of its behavior in the field at the San Antonio de los Baños Experimental Station of Medicinal Plants, Artemisa province [(13)]. Therefore, it is recognized that there is little information on the agro-technical management necessary for the production of biomass on an industrial scale [(14)]. Therefore, it is necessary to carry out studies related to its crop in the field, under conditions specific to each region where the crop will be developed.

Therefore, the aim of this work was to evaluate the effect of planting distance on biomass production and steviolglycoside content of *Stevia rebaudiana* Bertoni var. Morita II, under field conditions.

MATERIALS AND METHODS

This research was carried out at the UEB Medicinal Plants of Ceballos Agroindustrial Enterprise, Ciego de Avila. The crop used was *Stevia rebaudiana* Bertoni var. Morita II, developed in Japan by Toyosigue Morita. The advantage of this variety is that it has higher dry leaf yields and better chemical content than other varieties. The research was conducted in the period from April to November 2018.

Plant material

As vegetal material were used cuttings (sprouts with a length of approximately 8-10 cm), coming from mother plants of *Stevia rebaudiana* Bertoni propagated *in vitro*, with 120 days after sowing, kept in the Bioplant Center Adaptation Area from Ciego de Avila and free of pests (Figure 1). They were previously cultivated in polyethylene bags of 1.2 L capacity, containing as substrate a mixture of Ferrallitic Red Compacted Ferrallitic soil and filter cake (1:1 v/v) [15].

Figure 1. Rooted cuttings of *Stevia rebaudiana* Bertoni planted in the nursery of Ceballos Agroindustrial Enterprise

Prior to planting, cuttings were put in contact at the base with rooting powder composed of Naftalene Acetic Acid (NAA) and Indole Butyric Acid (IBA) (2000 mg kg^{-1} each), individually and immediately buried in the substrate at a depth of approximately 1-2 cm, according to Instructions of the Bioplant Center Adaptation Area.

The rooted cuttings with 20 days of age were transferred and planted in field conditions, in May 2018, in the UEB of Medicinal Plants. The total area planted for the experiment was 0.025 ha (1374 plants) on Compacted Red Ferrallitic soil with Neutral pH and clay texture [15]. Soil preparation was done by animal traction. Planting was done manually. A 2 m border was left on each side of the plot. Treatments consisted of three planting distances: treatment (Tment) 1: 15x20 cm (333 333 plants ha^{-1}), Tment 2: 20x20 cm (250 000 plants ha^{-1}) and Tment 3: 25x20 cm (200 000 plants ha^{-1}). A randomized complete block design was used, with 185 plants for treatment 1, 153 plants in treatment 2 and 120 plants in treatment 3. Each of these blocks was repeated three times. Forty plants (n=40) were randomly selected and identified for each treatment, for a total of 120 plants. Three evaluations were made every 60 days for a period of 180 days.

The evaluations were conducted at each pruning and they are described below:

- Plant height was measured for lateral sprout formation. A tape measure was used and the result was expressed in centimeters (cm).
- The number of branches per plant was quantified.
- The presence or absence of flowers per plant per treatment was observed and the percentage of plants with flowers was calculated.
- Leaves were separated from plant branches. The fresh mass of leaves and stems was determined on a technical balance (SARTORIUS TE 412). It was expressed in grams per plant (g plant^{-1}).
- Total leaves of each plant and stems were dried in an oven (HS 62A) at 70 °C for 72 h. The dry mass was determined on a balance (SARTORIUS TE 412). Dry mass was determined on a SARTORIUS TE 412 balance and expressed in grams per plant (g plant^{-1}).
- Biomass yield per hectare was estimated from the variable dry mass of leaves per plant and the number of plants per hectare. It was expressed in tons per hectare (t ha^{-1}).

Extraction and quantification of steviolglycoside-rich extracts

Steviolglycosides were extracted and quantified from dried leaves of *Stevia rebaudiana* Bertoni collected from each pruning. Leaves were crushed in a mill (Mikro-Feinmuhle-Culatti model, MFC) with a particle size of 0.7 µm, maintaining the mass/volume ratio: 1:10 (m/v). A clarified aqueous extract was obtained for each treatment. For this, 10 mL of distilled water was heated to 100 °C and 1 g of dried leaves was added. The extraction was carried out for 3 h at rest. It was then centrifuged at 21891xg for 20 min (Heal Force model centrifuge). The precipitate was discarded and the supernatant was clarified with activated carbon (2 %) [(16)], to eliminate pigments that could interfere in the determination of steviolglycosides. It was shaken for 20 min in vortex shaker (IKA GENIUS3) and centrifuged at 21891xg for 20 min. This assay was performed three times. Three replicates were used for each pruning. Subsequently, the absorbance at 210 nm of the clarified aqueous crude extracts of *Stevia* was measured in spectrophotometer (Pharmacia LKB, Ultrospec II, HOLLAND) to determine the content of steviolglycosides. Three determinations were performed for each replicate. The result was expressed as mg steviolglycosides g^{-1} dry mass.

Statistical processing

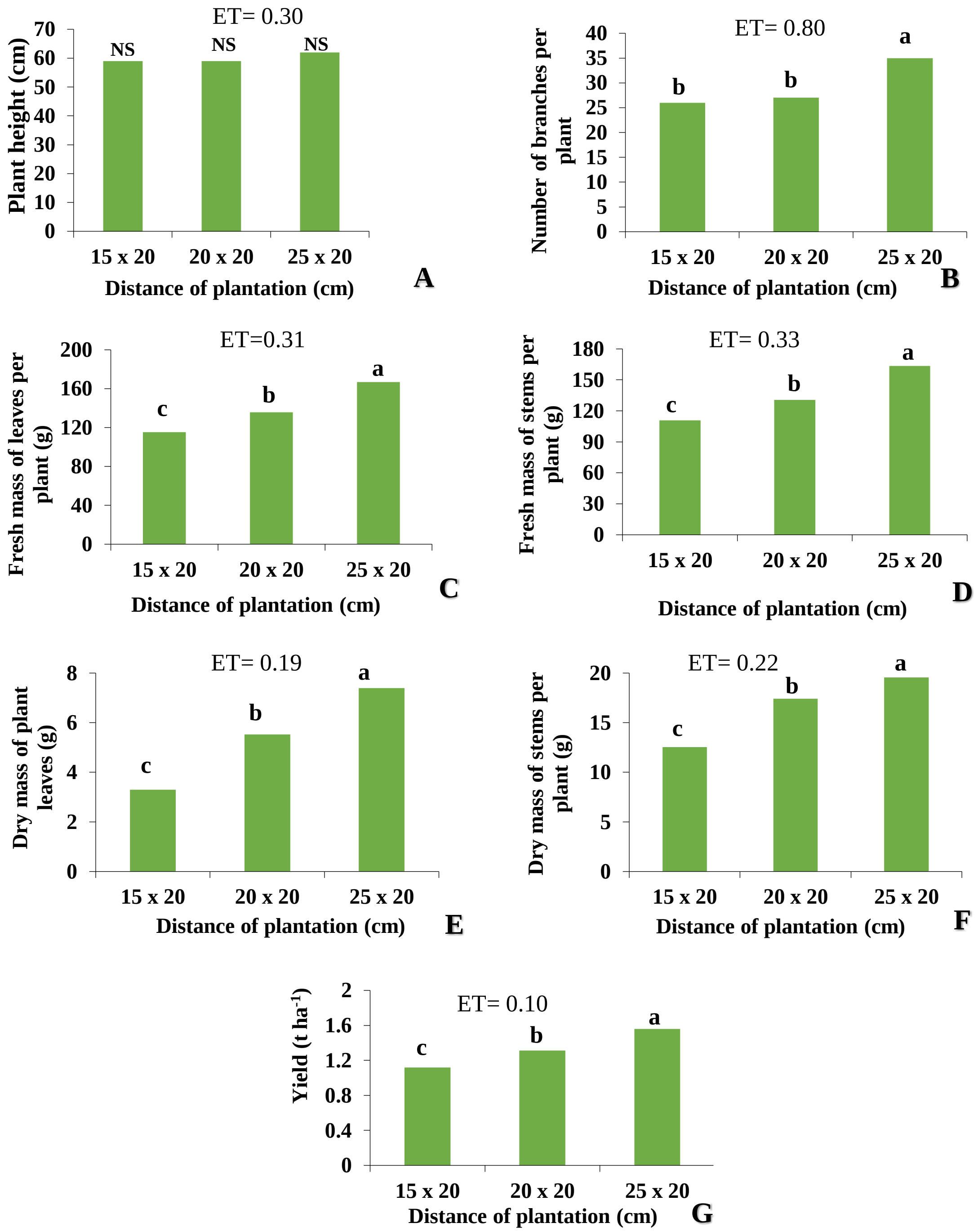
The Kolmogorov Smirnov test and Levene's test were used to check assumptions of normal distribution and homogeneity of variances, respectively. When these assumptions were met, the variance test analysis (simple ANOVA) for fixed effects was applied and when significant differences were found, Tukey's

DHS multiple comparisons test was applied with a statistical significance level of p≤0.05. In all cases, *Statistical Package for Social Sciences* (SPSS) version 21.0 was used as the statistical processor. No statistical test was performed for the flowering percentage variable.

RESULTS AND DISCUSSION

Effect of planting distance on plant biomass production

Plant height is shown in Figure 2A. No significant differences in this agronomic variable were observed in the three planting distances studied. Plant height was 58, 59 and 62 cm, at distances 15x20, 20x20, 25x20 cm, respectively. Lower results for this variable (45, 20 and 37.5 cm) were reported under field conditions [(17-19)], respectively.



A) plant height; B) number of branches per plant; C) fresh mass of leaves; D) dry mass of leaves; E) fresh mass of stems; F) dry mass of stems; G) biomass yield

Bars with different letters indicate significant differences according to ANOVA and Tukey tests ($P \leq 0.05$; n= 40)

Figure 2. Effect of planting distance on evaluated growth variables and biomass production of *Stevia rebaudiana* Bertoni var. Morita II plants under field conditions

It is considered that the distance of 25x20 cm could be feasible. Plants have more space for leaf development and competition for nutrients will be reduced. In addition, excessive self-shading is limited, which could cause decreases in the average photosynthetic rate per unit of leaf area with detriments in the total biomass per plant [20].

Branch number per plant of *Stevia rebaudiana* Bertoni under field conditions was significantly higher at the 25x20 cm planting distance (35 branches per plant) (Figure 2B). The distances of 15x20 and 20x20 cm presented lower values that did not show significant differences between them for this trait. The longer planting distance could be favorable for plant nutrition as well as for the development of photosynthesis.

The planting distance effect on the fresh mass of leaves per plant of *Stevia rebaudiana* Bertoni, under field conditions, is shown in Figure 2C. The highest values were reached with the 25x20 cm planting distance with 166.8 g per plant, followed by the other two planting distances 20x20 and 15x20 cm. Significant differences existed among all treatments.

The longer planting distance was more favorable, perhaps because of the space provided both above and below ground, which facilitates the expansion of lateral branches and better uniformity in terms of sunlight received by plant leaves. On the other hand, the distance also provides more space for root development and better nutrient absorption per plant [(21)].

The fresh mass of *Stevia rebaudiana* Bertoni stems under field conditions (Figure 2D) showed a similar behavior to the fresh mass of leaves. There were significant differences among all treatments. The highest values were reached with the planting distance of 25x20 cm with 163.60 g plant^{-1}, followed by the distances of 20x20 cm (130.51 g plant^{-1}) and 15x20 cm (110.73 g plant^{-1}).

Leaf dry mass per plant was significantly higher with the 25x20 cm planting distance (Figure 2E), with value of 7.4 g plant^{-1}. There were also significant differences between the 20x20 cm (5.53 g) and 15x20 cm (3.3 g) planting distances.

Similar results for this variable (4 to 7 g plant^{-1} of leaf dry mass per plant) were observed under field conditions [(22-24)]. However, other authors found higher values (6.25 to 10.57 g plant^{-1}) for this variable [(24)].

The dry mass of stems per plant of *Stevia rebaudiana* Bertoni under field conditions with different planting distances is shown in Figure 2F. There is significant difference in this agronomic variable at the three planting distances. The best results were observed with the 25x20 cm distance, coinciding with a previous study [(25)]. The distances of 20x20 and 15x20 cm continued to decrease, with 17.42 and 12.55 g plant^{-1}, respectively. Thus, in the present investigation, the increase in planting distance anhanced plant growth and development, which is reflected in most of the agronomic variables described.

Planting distance positively influenced the yield of *Stevia rebaudiana* Bertoni plants (Figure 2G). The highest yield was obtained with the 25x20 cm planting distance with 1.48 t ha^{-1}, followed by that observed at the 20x20 cm planting distance (1.38 t ha^{-1}) and the lowest corresponded to the 15x20 cm (1.10 t ha^{-1}).

Planting distance results on the flowering percentage of *Stevia rebaudiana* Bertoni plants showed that all plants flowered in November, with no significant differences. Flowering is independent of planting distance, being more dependent on photoperiod, time of year and low temperatures [(26)].

Effect of planting distance on steviolglycoside content

Table 1 shows the results when evaluating the planting distance effect on the steviolglycoside content of *Stevia rebaudiana* Bertoni. The best results were obtained when the planting distances 25x20 cm and 20x20 cm were used with 450.89 and 447.34 mg g^{-1} DM, respectively, with no statistical differences between them.

Table 1. Effect of planting distance on steviolglycoside content in leaves of *Stevia rebaudiana* Bertoni var. Morita II, grown under field conditions

Planting distance (cm)	Steviolglycosides (mg g^{-1} DM)
15x20	365.65 b
20x20	447.34 a
25x20	450.89 a
$\bar{x}$	421.29
ET	0.35

Means with different letters indicate significant differences according to Tukey DHS test for p≤0.05 and n= 40

DM: leaf dry mass

These results are superior to those reported in previous studies carried out under nursery and *in vitro* laboratory conditions [24,26], respectively. In addition to the study conditions, differences in sweetener content with respect to this work could be related to several factors such as the growth cycle of the plant [27], the plant age [28], the region where it is grown [29], and the planting density used [30].

Specifically, planting distance is a special factor in the growth and development of *Stevia* plants and their content of steviolglycosides [30], although other authors report an insignificant effect in their results [31]. The root of Stevia rebaudiana Bertoni, is pivoting, filiform and does not go deep, that is to say, it is distributed near the surface [32]. The possibility of competition among plants of this species cannot be ruled out. Competition with undesirable plants can be ruled out, since the furrows were kept clean of weeds during evaluation time. However, the planting distance of 25x20 cm may be beneficial for better nutrient uptake per plant. Likewise, it can favor an efficient illumination and consequently, the development of photosynthesis and therefore a high content of steviolglycosides.

In the evaluations carried out in the present study, all the results for variables analyzed were superior at the 25x20 cm planting distance, with the exception of the variables plant height, flowering percentage and steviolglycoside content, which did not show significant differences.

CONCLUSION

Planting distance influences biomass production and yield of *S. rebaudiana* var. Morita II, grown under field conditions, as well as steviolglycoside content in leaves; 25x20 cm being the recommended distance for study conditions.

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