



## Plant densities and harvesting times on productive and physiological aspects of *Stevia rebaudiana* Bertoni grown in southern Brazil

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### ABSTRACT

*Stevia* (*Stevia rebaudiana* Bertoni) is a species characterized by producing non-caloric substances with high sweetening potential. Among these substances, rebaudioside A and stevioside are produced in greater quantity. Plant density and harvesting time are factors that affects biomass and sweetening compounds yield in this species. The objective of this research was to evaluate the effect of plant densities and harvesting times on the productive and physiological characteristics of stevia in southern Brazil. The experimental design was in randomized blocks, in a split-plot scheme, with 9 treatments comparing the effect of three planting densities (166 667, 83 333 and 33 333 plants ha<sup>-1</sup>) in the plots and three harvesting periods (before, in the beginning and in full flowering) in the subplots. Harvesting at the beginning of flowering promoted higher dry leaf biomass yield and, when associated with the lowest planting density, promoted higher levels of rebaudioside A and stevioside. The lowest planting density resulted in greater leaves biomass accumulation, whereas the highest density promoted higher yields per area. Interaction between density of 166 667 plants ha<sup>-1</sup> and the harvest at the onset of flowering promoted higher yields of rebaudioside A (43.22 kg ha<sup>-1</sup>) as well as higher rebaudioside A/stevioside ratio (0.60).

**Key words:** diterpene glycosides, natural sweetener, rebaudioside A, stevioside.

### INTRODUCTION

*Stevia rebaudiana* Bertoni, popularly known as stevia, sweet grass or honeyleaf, is an herbaceous shrub belonging to the Asteraceae family, native to northeastern Paraguay, with natural occurrence also in the neighboring regions of Brazil and Argentina (Lemus-Mondaca et al. 2012).

The plant is becoming increasingly important as an agricultural crop due to the production of secondary metabolites named diterpene glycosides or steviol glycosides, with high sweetening potential and no calorific value. The most important commercially available glycosides, stevioside and rebaudioside A, are known to be around 300 and 400 times sweeter than sucrose, respectively (Lemus-Mondaca et al. 2012, Mandal et al. 2013).

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For industrial processing purposes, a higher concentration of rebaudioside A over stevioside is desired because the latter has a strong bitter residual taste, a characteristic not appreciated by the market (Yadav et al. 2011).

Commercial stevia cultivation occurs mainly in Asia and America. As per the regional analysis, North America represents the most lucrative market, followed by Latin America and Asia-Pacific, excluding Japan. China has a lead over other countries regarding production capacity and export of stevia, globally. Low-cost of production and availability of skilled resource is a major factor driving the market growth (Mordor Intelligence 2017).

Globally, stevia market was estimated to value at US\$ 347.0 million in 2014 and expected to reach US\$ 565.2 million by 2020. In terms of volume of consumption it is expected that stevia may reach 8 506.9 tonnes by the end of 2020, registering an annual growth of around 7-8% during the forecast period (Future Market Insights 2014). Considering the Brazilian context, production is not enough to supply domestic demand, with imports of more than US \$ 3 million registered in stevioside in 2016 (Brasil 2017).

Considering the economic importance of the crop, studies have been carried out to define the most appropriate management practices to increase biomass yields and contents of compounds of interest (Jarman et al. 2010, Mandal et al. 2013, Moraes et al. 2013, Pal et al. 2013, 2015, Serfaty et al. 2013, Kumar et al. 2014a, Barbet-Massin et al. 2015). Among the different management practices for the crop, plant density and harvesting time seem to have a decisive influence on the production of both biomass and diterpene glycosides.

The main factor that affects the content of diterpene glycosides in stevia is flowering (Brandle and Rosa 1992). Radiation is also extremely important and it is considerably affected by planting densities, being determinant in the production of biomass and sweetening compounds (Serfaty et al. 2013).

The highest efficiency in the conversion of assimilates to the production of biomass in the species occurs at the beginning of flowering (Lima-Filho 2004), the same stage in which highest levels of stevioside and rebaudioside A are observed, due to the greater expression of genes involved in the steviol glycosides biosynthetic pathway (Yang et al. 2015).

Regarding the influence of plant spacing, densities between 83 000 and 111 000 plants/ha are recommended for higher yields of biomass and sweetening compounds (Madan et al. 2010, Serfaty et al. 2013, Kumar et al. 2014a). Some authors report, however, that higher stands may still provide biomass increases by area (Kumar et al. 2014a).

The objective of this study was to evaluate the effect of different planting densities and harvesting times on the productive, physiological and phytochemical characteristics of stevia in Southern Brazil.

## MATERIALS AND METHODS

### EXPERIMENTAL LOCATION, FIELD CONDITIONS AND GENERAL PROCEDURES

The experiment was carried out under field conditions at the Cangüiri Experimental Station Center (CEEx), Federal University of Parana (UFPR), Pinhais, Parana, Brazil. The station is located at 25 ° 23'S latitude, 49 ° 07' W longitude and 920m altitude.

The climate of the region is temperate humid with temperate summer, being classified as Cfb in the climatic classification system of Köppen (Köppen 1931). During the experiment, the global radiation registered by the National Institute of Meteorology (INMET) in Curitiba averaged 1007.18, 1164.10, 1386.56 and 1179.82 kJ m<sup>-2</sup> in November and December 2015, and January and February 2016, respectively. The average day lengths for these months were 13.3, 13.6, 13.4

and 12.9 hours, according to latitude and solar declination. The maximum temperature recorded was 32.3 °C and the minimum temperature was 11.6 °C. The climatic data under which the experiment was conducted are shown in Table I. Soil samples were collected at a depth of 20 cm and sent for chemical and granulometric analysis, the results of analyses are presented in Table II.

The results of the soil analysis and recommendation of the Manual of fertilization and liming for the states of Rio Grande do Sul and Santa Catarina for stevia cultivation (Comissão de Química e Fertilidade do Solo 2004), were used to establish the necessity of nutrients supply. The application of 60 kg ha<sup>-1</sup> of nitrogen was proceeded. The application was made by sowing, using urea (45% of N) as a source of nitrogen. Also according to the recommendation of the manual,

the application was parcelled out, being the first application of 1/3 of the nitrogen at the time of planting and the remainder after 20 days.

The experiment was installed in November, 2015, with plants of approximately 15 cm in height at different spacings according to treatments. After 15 days of the installation, the replanting was carried out, in order to maintain the planting densities without variations. A mortality rate of approximately 8% was observed, probably due to problems in plants adaptation when transferred from greenhouse to field conditions.

During the whole period of the experiment, weekly manual control of weeds were proceeded. No phytosanitary procedures were required to control pests and diseases. Irrigation was not necessary either.

**TABLE I**  
Climatic conditions during the experiment. Pinhais, Parana, Brazil, 2015/2016.

Variables	Month/year			
	NOV/2015	DEC/2015	JAN/2016	FEB/2016
Precipitation (mm) <sup>1</sup>	112.70	200.40	164.20	289.30
Relative humidity (%) <sup>1</sup>	90.98	88.57	86.35	89.46
Average Maximum Temperatures (°C) <sup>1</sup>	24.47	26.46	26.06	27.78
Average Minimum Temperatures (°C) <sup>1</sup>	15.61	17.48	16.67	18.16
Average temperature (°C) <sup>1</sup>	18.81	20.91	20.42	21.55
Global radiation (kJ m <sup>-2</sup> ) <sup>2</sup>	1007.18	1164.10	1386.56	1179.82
Day length (h) <sup>3</sup>	13.3	13.6	13.4	12.9

<sup>1</sup>Source: Paraná Meteorological System (SIMEPAR), 2016. <sup>2</sup>Source: National Institute of Meteorology (INMET), 2017 (Data from the Municipality of Curitiba). <sup>3</sup>Data calculated as a function of latitude and solar declination.

**TABLE II**  
Chemical and granulometric characteristics of the soil used in the experiment. Pinhais, Parana, Brazil, 2015/2016.

Granulometry											
Clay	Silt		Total Sand		Thick Sand		Fine Sand				
----- g kg <sup>-1</sup> -----											
513	175		313		141		172				
Chemical Characteristics											
pH	Al <sup>+3</sup>	H <sup>+</sup> +Al <sup>+3</sup>	Ca <sup>+2</sup>	Mg <sup>+2</sup>	K <sup>+</sup>	SB	T	P	C	V	
CaCl <sub>2</sub>	SMP		----- cmol <sub>c</sub> dm <sup>-3</sup> -----						mg dm <sup>-3</sup>	g dm <sup>-3</sup>	%
6.0	6.3	0.0	4.00	6.50	2.90	0.65	10.05	14.05	46.20	28.9	72

#### PLANT MATERIAL AND PLANTLETS PRODUCTION

A total of seven accessions of *S. rebaudiana*, identified by the numbers 4, 6, 7, 8, 9, 12 and 14 were provided by Brazilian Company of Agricultural Research (EMBRAPA) and evaluated by Francisco et al. (2018) regarding productive performance in the edaphoclimatic conditions of the metropolitan region of Curitiba, state of Paraná.

Accessions 6, 7 and 14, were identified with rebaudioside A/stevioside ratio close to 1, desirable for the industry. The accession 6, selected for use in the present study, yielded a dry mass of leaves of 4008.3 kg ha<sup>-1</sup>, in two harvests, with rebaudioside A/ stevioside ratio of 0.95, and yield of 9.7% of Rebaudioside A in the average of the two harvests (Francisco et al. 2018).

The plant material was multiplied *in vitro* by nodal segments, according to protocol reported by Das et al. (2011). Plantlets with two pairs of leaves were transferred to 120 cm<sup>3</sup> plastic tubes filled with Tropstrato® commercial substrate. The tubes were placed in plastic supports and remained for 15 days for acclimatization in a greenhouse with intermittent mist of 5 seconds every 30 minutes. After this period the tubes with plants were transferred to greenhouse without mist, with daily manual watering for a period of 30 days. At the end of the period, the plantlets with 15 cm height and five pairs of leaves were selected for the field experiment.

#### EXPERIMENTAL DESIGN

The experimental design was in randomized blocks with a split-plot scheme, evaluating the effect of three planting densities (166 667, 83 333 and 33 333 plants ha<sup>-1</sup>) in the plots and three harvesting periods (before, at the beginning and at full flowering) in the subplots, with 4 replicates. Each plot contained 65 plants, being used nine plants in each subplot.

The plant densities were calculated as a function of different spacings: 0.60 x 0.50 m;

0.40 x 0.30 m; and 0.30 x 0.20 m, between lines and plants, respectively. Harvesting times were performed according to plant phenology. The before flowering harvest took place 75 days after transplanting, when less than one half of the plants showed flower bud formation, and with no open flower. Harvesting at the beginning of flowering was carried out when the plants had about 5% of capitula with the presence of open flowers, which occurred 96 days after transplanting. The full flowering harvest occurred when more than 50% of the plants had most of the capitula with open flowers, on the 110th day after transplanting.

#### BIOMETRIC EVALUATIONS AND CALCULATION OF PHYSIOLOGICAL INDEXES

The plants were harvested by cutting stems at 5 cm from the soil. After this procedure the leaves were separated from the branches and flowers manually, weighed for determination of fresh mass of leaves and branches, and then stored in Kraft® multifoil paper bags. The stems and leaves remained in dryer with forced air circulation at 65 ° C until constant weight and were again weighed to determine the dry mass of leaves and total dry mass of the aerial part.

A total of 60 discs of 0.785 cm<sup>2</sup> of leaves were weighted from each experimental subplot for the measurement of leaf area (LA) calculated in proportion to the total weight of leaves. Leaf area index (LAI), specific leaf area (SLA), leaf area ratio (LAR) and leaf mass ratio (LMR) were calculated.

Leaf area index was determined by the ratio between leaf area and soil area (SA) occupied by the plants obtained in each subplot:  $IAF = LA / SA$ . LAR was calculated by the ratio between total leaf area and total dry mass (TDM):  $RAF = LA / TDM$ . SLA was calculated by the ratio between leaf area and leaf dry mass (LDM):  $SLA = LA / LDM$ . LMR was calculated by the ratio between dry leaf mass and total dry mass:  $RPF = LDM / TDM$ .

## BIOCHEMICAL EVALUATIONS

To determine the levels of *a*, *b* and total chlorophylls, twenty disks of 0.785 cm<sup>2</sup> of the central region of leaf tissues were removed and weighed. It was macerated in a mortar with 10 ml of 80% acetone and placed in test tubes, which were centrifuged for 10 minutes at 12000 rpm with refrigeration at 4°C. 1 ml of the supernatant was transferred to cuvettes and subjected to spectrophotometer (UV-1601-Shimadzu, Kyoto, Japan) readings at the wavelengths of 645 and 663 nm using 80% acetone as a control. For the quantification of chlorophylls, the equations described by Arnon (1949) were used.

Total protein content was determined by Bradford (1976) method. An amount of 0.5 g of fresh leaf tissue from each sample was macerated in a mortar with 10 ml of 0.2 M phosphate buffer (pH 7.5) and filtered. An aliquot of 0.05 ml of the filtrate was placed in a test tube along with 0.45 ml of distilled water and 1.0 ml of the Bio-Rad® reagent (Sigma-Aldrich®). The material was transferred to quartz cuvettes and subjected to spectrophotometer reading at 630 nm, with bovine serum albumin as control.

Total sugar contents were determined by the phenol-sulfuric method, described by Dubois et al. (1956). The amount of 0.1 g of fresh leaf tissue was macerated in a mortar containing 10 ml of 0.2 M phosphate buffer (pH 7.5) and filtered. 0.01 ml of the filtrate was placed in the test tube with 0.049 ml of distilled water. In this solution it was added 0.5 ml of 5% Phenol and 2.5 ml of concentrated sulfuric acid. After cooling to room temperature the material was transferred to quartz cuvettes and subjected to spectrophotometer reading, at 490 nm. The concentration of total sugars was determined by standard glucose curve.

The analyses of stevioside and rebaudioside A levels were performed according to methodology adapted from Kolb et al. (2001). For the extraction of the diterpene glycosides 0.5 g of dry leaf tissue

was crushed and transferred to containers containing 50 ml of 70% ethanol. The solution was subjected to constant stirring for 30 minutes in a water bath at 70°C. The material was filtered through nylon syringe filters (porous 0.2 µm), transferred to Eppendorf tubes and stored in a freezer (-18°C) until quantification.

The extract was injected into HPLC (High performance liquid chromatography) apparatus (SPD-10A, CTO-10A, CBM-10A and LC-10<sup>a</sup>, Shimadzu®), NH 2 column (250 x 4.6 mm), mobile phase composed of acetonitrile and ultrafiltered water (80:20), flow of 2 ml/min, temperature of 30°C and detection with UV reader at 210 nm (0.04 AUFS).

The quantification was made through a calibration curve created by dilution of stevioside and rebaudioside A analytical standards, trade mark Sigma-Aldrich®. The levels of stevioside and rebaudioside A (mg g<sup>-1</sup>), ratio between rebaudioside A and stevioside (R / E), and yields of stevioside and rebaudioside A were determined.

## STATISTICAL ANALYSES

Data were submitted to the Bartlett test to verify homogeneity of variances and, when homogeneous, ANOVA variance analysis was performed. When significant, the means were compared by Tukey's test at 1 and 5% probability, using the statistical software ASSISTAT® (Silva and Azevedo 2016).

## RESULTS

For the variables height, number of branches, LAI, SLA, LMR and LAR there was no interaction between planting densities and harvest period. Evaluating the factors isolated, planting density was only significant for the LAI ( $p > 0,005$ ) while the harvesting time did not show significance only for the LMR. LMR ranged from 0.48 to 0.56 (Table III) and was not affected by the evaluated cultivation conditions.

The height of plants reached maximum values in the full flowering harvest, with an average increment of more than 15 centimetres compared to the harvest before flowering. The number of branches was also significantly lower ( $p < 0,001$ ) in the harvest before flowering compared to the other periods. The same was observed for LAI, SLA and LAR, with higher means on harvests on the beginning and at full flowering. For the LAI, although, the plant density was the factor that presented greater influence. The highest indexes (average 1.45) were observed in the highest plant density, whereas the lowest values were found in the density of 33 333 plants ha<sup>-1</sup> (0.59).

Regarding variables related to biomass accumulation, there was interaction between plant densities and harvesting times for aerial part dry mass yield. Evaluating the factors in isolation, both harvesting times and planting densities were significant for dry mass of leaves per plant, dry mass of aerial part per plant and leaves dry mass of leaves yield.

Considering harvesting times, dry mass of leaves per plant, dry mass of aerial part per plant and leaves dry mass of yield presented similar behavior, with superiority in the beginning and full flowering harvests. The lower planting density (33 333 plants ha<sup>-1</sup>) resulted in higher accumulation of leaf biomass and total biomass per plant, but the number of plants at the highest density (166 667 plants ha<sup>-1</sup>) compensated for the lower production per unit, providing the highest leaf dry matter yields (Table IV).

For aerial part dry mass yield, it was observed an interaction effect ( $p \leq 0,005$ ) between harvesting time and plant density. It was noticed that in the pre-flowering harvest there was no effect of planting densities. At the beginning of flowering, the densities of 166 667 and 83333 plants ha<sup>-1</sup> promoted greater accumulations of biomass per area. At the harvest in full flowering, the density of 166 667 plants ha<sup>-1</sup> was superior to the other densities (Table V).

TABLE III

Average height, number of branches, leaf area index (LAI), specific leaf area (SLA), leaf mass ratio (LMR) and leaf area ratio (LAR) in *Stevia rebaudiana* grown at different planting densities and harvesting times (Pinhais, Parana, Brazil, 2016).

Variable		166 667 plants ha <sup>-1</sup>	83 333 plants ha <sup>-1</sup>	33 333 plants ha <sup>-1</sup>	LSD
Planting densities	Height (cm)	54.05 <sup>ns</sup>	57.97	55.79	7.43
	Number of branches	14.66 <sup>ns</sup>	18.77	18.28	6.46
	Leaf area index	1.45 a	1.05 b	0.59 c	0.39
	Specific leaf area (cm <sup>2</sup> g <sup>-1</sup> )	24.75 <sup>ns</sup>	19.61	24.71	8.29
	Leaf mass ratio (g g <sup>-1</sup> )	0.50 <sup>ns</sup>	0.51	0.55	0.04
	Leaf area ratio (cm <sup>2</sup> g <sup>-1</sup> )	13.71 <sup>ns</sup>	11.18	14.54	4.54
Variable		Before flowering	Onset of flowering	Full flowering	
Harvesting times	Height (cm)	44.54 b	61.41 a	61.85 a	2.54
	Number of branches	9.74 b	20.96 a	21.05 a	2.60
	Leaf area index	0.62 b	1.32 a	1.15 a	0.36
	Specific leaf area (cm <sup>2</sup> g <sup>-1</sup> )	14.63 b	28.61 a	25.82 a	10.02
	Leaf mass ratio (g g <sup>-1</sup> )	0.56 <sup>ns</sup>	0.48	0.51	0.01
	Leaf area ratio (cm <sup>2</sup> g <sup>-1</sup> )	8.33 b	16.40 a	14.70 a	5.76

Means followed by the same letter in the rows do not differ from each other by the Tukey's test at 5% probability. LSD: Least significant difference. <sup>ns</sup>non-significant.

There was no significant interaction between treatments for total chlorophyll, chlorophyll *a* and *b*, proteins and total sugars. Evaluating the factors isolated, planting density influenced only the production of chlorophyll *a* and total chlorophyll, while harvesting time was significant for protein and total soluble sugars. Chlorophyll *a* and total chlorophyll content were significantly higher at planting densities of 33 333 and 88 333 plants ha<sup>-1</sup>.

The protein contents in stevia leaves varied between 44.59 and 62.09 mg g<sup>-1</sup>, with higher level at the before flowering harvest when compared to harvests at the beginning and full flowering. A similar pattern was observed for sugar contents with an average of 462.81 mg g<sup>-1</sup> in leaves harvested before plant flowering (Table VI).

For stevioside and rebaudioside A contents, rebaudioside A/stevioside ratio and rebaudioside A yield there was interaction between plant densities and harvesting times. Only for stevioside yield there was no interaction. For this variable, although, there was effect of plant densities and harvesting times as isolated factors.

The interaction between harvest at the beginning of flowering and the adoption of planting density of 33 333 plants ha<sup>-1</sup> promoted the highest contents of stevioside and rebaudioside A.

The highest rebaudioside A/stevioside ratio was obtained when the plants in densities of 33 333 and 166 667 plants ha<sup>-1</sup> were harvest at the beginning of flowering. In relation to the productivity of the compounds, greater yield of stevioside was observed in the beginning and full flowering harvests. Regarding planting densities, the stand of 166 667 plants ha<sup>-1</sup> promoted higher yields, due to the greater accumulation of biomass per area. For rebaudioside A productivities, interaction between treatments was observed, and the harvest at the beginning of flowering at the highest planting density reached higher values (43.22 kg ha<sup>-1</sup>) (Table VII).

The yields of steviol glycosides (stevioside + rebaudioside A) obtained at the beginning of flowering and density of 166 667 plants ha<sup>-1</sup> reached 116.43 kg ha<sup>-1</sup>, with a rebaudioside A/stevioside ratio of 0.60.

## DISCUSSION

There was an evident increase in plant vegetative development from the harvest before flowering, mainly characterized by plant height, branches number, leaf area and dry mass. The increase in the branches number at harvests in full and at the beginning of flowering can be attributed to the

TABLE IV  
Average dry mass of leaves and aerial part per plant and leaves dry mass yield in *Stevia rebaudiana* grown at different planting densities and harvesting times. (Pinhais, Parana, Brazil, 2016).

Planting densities	Variable	166 667 plants ha <sup>-1</sup>	83 333 plants ha <sup>-1</sup>	33 333 plants ha <sup>-1</sup>	LSD
	Dry mass of leaves (g plant <sup>-1</sup> )		6.32 b	10.65 ab	14.30 a
Dry mass of aerial part (g plant <sup>-1</sup> )		12.69 b	21.22 ab	26.31 a	10.82
Leaves dry mass yield (kg ha <sup>-1</sup> )		1053.67 a	887.40 ab	476.83 b	534.12
Harvesting times	Variable	Before flowering	Onset of flowering	Full flowering	
	Dry mass of leaves (g plant <sup>-1</sup> )	5.46 b	11.63 a	14.18 a	4.94
Dry mass of aerial part (g plant <sup>-1</sup> )	9.69 b	24.07 a	26.31 a	6.15	
Leaves dry mass yield (kg ha <sup>-1</sup> )	433.40 b	943.82 a	1040.67 a	334.02	

Means followed by the same letter in the rows do not differ from each other by the Tukey's test at 5% probability. LSD: Least significant difference. <sup>ns</sup>non-significant.

**TABLE V**  
Interaction values of aerial part dry mass yield in *Stevia rebaudiana* grown at different plant densities and harvesting times. (Pinhais, Parana, Brazil, 2016).

Harvesting time	Planting densities (plants ha <sup>-1</sup> )			Mean
	166 667	83 333	33 333	
Before flowering	1061.55 bA	870.80 bA	408.22 bA	780.19
Onset of flowering	1985.01 aA	2213.87 aA	984.24 abB	1737.63
Full flowering	2596.49 aA	1549.52 aB	1223.51 aB	1789.84
Mean	1881.01	1655.65	871.99	

LSD for columns 665.63; LSD for rows 855.94. Significant interaction ( $P=0.0193$ )

Means followed by lowercase letters in the columns and capital letters in the rows do not differ from each other by the Tukey's test at 5% probability. LSD: Least significant difference. <sup>ns</sup>non-significant. LSD: Least significant difference.

**TABLE VI**  
Average contents of chlorophyll a, chlorophyll b, total chlorophyll, proteins and sugars in *Stevia rebaudiana* grown at different plant densities and harvesting times (Pinhais, Parana, Brazil, 2016).

Planting densities	Variable	166 667 plants ha <sup>-1</sup>	83 333 plants ha <sup>-1</sup>	33 333 plants ha <sup>-1</sup>	LSD
		Chlorophyll a (mg/cm <sup>2</sup> )	0.065 b	0.080 a	0.078 a
	Chlorophyll b (mg/cm <sup>2</sup> )	0.035 <sup>ns</sup>	0.041	0.036	0.009
	Total Chlorophyll (mg/cm <sup>2</sup> )	0.100 b	0.121 a	0.115 a	0.014
	Proteins (mg/g)	49.03 <sup>ns</sup>	49.59	53.20	13.41
	Sugars (mg/g)	422.30 <sup>ns</sup>	382.47	467.62	105.37
Harvesting times	Variable	Before flowering	Onset of flowering	Full flowering	LSD
		Chlorophyll a (mg/cm <sup>2</sup> )	0.068 <sup>ns</sup>	0.074	0.081
	Chlorophyll b (mg/cm <sup>2</sup> )	0.036 <sup>ns</sup>	0.037	0.039	0.009
	Total Chlorophyll (mg/cm <sup>2</sup> )	0.105 <sup>ns</sup>	0.111	0.120	0.025
	Proteins (mg/g)	62.09 a	44.59 b	45.14 b	11.96
	Sugars (mg/g)	462.81 a	428.85 ab	369.72 b	76.31

Means followed by the same letter in the rows do not differ from each other by the Tukey's test at 5% probability. LSD: Least significant difference. <sup>ns</sup>non-significant.

longer time for the shoots and roots development. Since the synthesis of cytokinins occurs mainly at the root / radicular apices, a more developed root system allows a greater production of these phytohormones and their export to the stems, where they stimulate the production of lateral branches (Pal et al. 2015). In a study carried out with different harvesting regimes for stevia, Pal et al. (2015) also verified the influence of plant life cycle on the number of branches and pointed out a positive relation of this variable with the leaf area index. Similar behaviour was observed in the

present study, with an average increase of more than two fold in the LAI when the harvest occurs at the beginning of flowering compared to the before flowering.

In spite of the difference observed during the crop cycle in relation to the harvesting times, the spacing was the factor that had the greatest influence on LAI, that increased with higher planting density, as observed by *S. rebaudiana* grown in Colombia (Jarma et al. 2010) and in the Himalayas (Kumar et al. 2014b). Kumar et al. (2014b) report maximum LAI of 1.21 in spacing of 30 x 30 cm and minimum



**TABLE VII**  
**Averages of stevioside and rebaudioside A contents, rebaudioside A/stevioside ratio and stevioside and rebaudioside A yields in *Stevia rebaudiana* grown at different plant densities and harvesting times (Pinhais, Parana, Brazil, 2016).**

Harvesting time	Plant densities (plants ha <sup>-1</sup> )			Mean
	Stevioside content (mg g <sup>-1</sup> )			
	166 667	83 333	33 333	
Before Flowering	71.02 aA	62.36 aA	67.81bA	67.06
Onset of Flowering	56.76 bB	48.52 bB	86.98 aA	64.08
Full Flowering	43.51 cA	46.29 bA	55.48 bA	48.42
Mean	57.09	52.39	70.09	
LSD for columns 12.98; LSD for rows 13.44. Significant interaction ( $P=0.0004$ )				
	Rebaudioside A content (mg g <sup>-1</sup> )			
Before Flowering	26.34 aA	24.82 aA	30.19 bA	27.11
Onset of Flowering	33.60 bB	22.56 bB	47.31 aA	34.48
Full Flowering	17.65 cA	20.99 bA	30.06 bA	22.90
Mean	25.86	22.80	35.86	
LSD for columns 8.45; LSD for rows 9.08. Significant interaction ( $P=0.0037$ )				
	Rebaudioside A/stevioside ratio			
Before Flowering	0.37 bB	0.40 bAB	0.45 bA	0.40
Onset of Flowering	0.60 aA	0.47 aB	0.54 aA	0.53
Full Flowering	0.41 bB	0.45 abB	0.54 aA	0.46
Mean	0.46	0.43	0.51	
LSD for columns 0.06; LSD for rows 0.07. Significant interaction ( $P=0.0002$ )				
	Stevioside yield (kg ha <sup>-1</sup> )			
Before Flowering	40.76	30.46	15.97	29.06 b
Onset of Flowering	73.21	51.94	39.65	54.94 a
Full Flowering	54.60	50.47	39.89	48.32 a
Mean	56.19 A	44.29 B	31.84 C	
LSD for columns 9.42; LSD for rows 10.35. Non-significant interaction ( $P=0.3713$ )				
	Rebaudioside A yield (kg ha <sup>-1</sup> )			
Before Flowering	15.14 bA	11.91 bA	7.05 bA	11.36
Onset of Flowering	43.22 aA	24.20 aB	21.35 aB	29.59
Full Flowering	22.68 bA	23.70 aA	21.68 aA	22.69
Mean	27.02	19.93	16.69	
LSD for columns 9.14; LSD for rows 8.97. Significant interaction ( $P=0.0033$ )				

Means followed by lowercase letters in the columns and capital letters in the rows do not differ from each other by the Tukey's test at 5% probability. LSD: Least significant difference. <sup>ns</sup>non-significant. LSD: Least significant difference.

of 0.48 in spacing 0.45 x 0.30 cm, values similar to the present work. Lima-Filho (2004) in analysis of crop growth reported LAI of 0.35, considered very low. Jarma et al. (2010) reported a maximum value of 3.15, according to the authors, still far from the critical LAI estimated for the crop. These variations

indicate the importance of using other indicators to understand the vegetative development of the plant.

SLA considerably increased in the beginning and full flowering harvests in comparison to the pre-flowering harvest. This fact can be attributed to the greater development of plants and leaf area

expansion that promotes greater shading, and consequently, reduction of radiation along the canopy. Under low radiation conditions, the plants increase the leaf expansion and, consequently, SLA. In general, these changes result in an increase in the interception of radiation, rising the photosynthetic efficiency (Lambers et al. 2008). Considering that no alterations were observed in LMR, the values for LAR had a similar behaviour compared to SLA, as an adaptation to lower levels of radiation through the canopy with the increase of vegetative development in plants harvested at beginning and full flowering. Similar pattern was reported by Jarma et al. (2006) for *S. rebaudiana* cultivated in Colombia, where an increase in LAR was observed as the incident radiation decreased.

The increase in leaf and aerial part dry mass and leaf yield on beginning and full flowering as a function of greater plant development was also reported by Lima-Filho (2004) when performing analysis of stevia growth in Brazil. For stevia grown in Israel, the interaction between planting densities and three harvest indicators showed that flowering is actually the most appropriate parameter to indicate the harvest period of the species. The best results for leaf biomass were obtained at the start of flowering (Serfaty et al. 2013). Regarding planting densities, the authors reported that a stand of 100 000 ha<sup>-1</sup> plants resulted in higher yields of leaf biomass (3000 kg ha<sup>-1</sup>).

This value is similar to those recommended by Donalisio et al. (1982). Increases in biomass values were also reported by Madan et al. (2010) with densities between 83 000 and 111 000 plants ha<sup>-1</sup>. The authors, however, have not evaluated higher planting densities, as in the present work. In spite of lower leaf mass yields than those observed by the authors, in this study, the cultivation with 166 667 plants ha<sup>-1</sup> presented higher yields than the lower densities. However, an increase of more than 5-fold in planting density resulted in an increase of two times in productivity. In field conditions

where seedlings or seeds are not a limiting factor, increase in density could be considered not only to improve productivity but also as a strategy for the cultural control of weeds (Basuki 1990, Taleie et al. 2012, Kumar et al. 2014b).

Higher production of biomass per plant in the lower density can be associated with more radiation and soil resources (Jarma et al. 2006, Kumar et al. 2012, 2014a). Higher yields in high densities are a function of the greater number of plants per area, which compensates the great biomass production per plant (Kumar et al. 2014a).

The highest planting density promoted an average leaf yield of 1053.67 kg ha<sup>-1</sup>. This value is close to the maximum reported by Pal et al. (2013, 2015), Kumar et al. (2014b), González et al. (2007) and Mejegi et al. (2005) for the first cut in the first year of cultivation.

Yield values obtained in the present work, notwithstanding, are lower than those reported by Francisco et al. (2018) for the same genetic material under similar cultivation conditions on a density of 80 000 plants ha<sup>-1</sup>, with planting carried out in September 2014, when it was reported a dry leaf yield of 3255.1 kg ha<sup>-1</sup>. This reduction in productivity may be associated with a decrease of 48 days in the vegetative period caused by differences in the transplant period, which occurred in November in the present experiment.

The late planting (November) reduces the period of vegetative growth, since, as at the end of December there is a constant photoperiod decrease, which stimulates the flowering of the crop, that occurs usually between January and March in the southern hemisphere (Ahmed et al. 2011, Yadav et al. 2011). In Brazil, stevia planting made until mid-October are recommended to increase the duration of the growing season, when plants reach the maximum economic yields (Lima-Filho 2004, Lima-Filho et al. 2004).

Regarding to photosynthetic pigments, it was observed an increase in chlorophyll *a* and

total chlorophyll higher values were observed in lower plant densities. Shaded leaves minimize light limitation through increases in light capture capacity and decrease in the carboxylation capacity and mesophyll conductance, but this does not invariably lead to higher concentrations of chlorophyll per unit area of leaf. In most plant species, higher levels of chlorophyll per unit of fresh mass and per chloroplast in shaded leaves are compensated by the lower number of chloroplasts and a lower fresh mass per area (Lambers et al. 2008). This fact results in a practically constant level of chlorophyll per area unit or even in higher levels of chlorophyll per leaf area in leaves submitted to lower shading, as observed in the present study at lower planting densities. Similar behaviour was observed in *Myrtus communis* L., where higher concentrations of chlorophyll per unit of leaf area was found in leaves grown in full sun (Mendes et al. 2001).

Although there is not always a positive correlation between chlorophyll content and liquid photosynthesis it is common for a plant with higher chlorophyll content to reach higher photosynthetic rates, due to its higher potential to capture photons per unit of time (Porra et al. 1989, Chappelle and Kim 1992, Rêgo and Possamai 2004). In stevia, Wu et al. (2013) reported that increases in chlorophyll *a* levels increase the solar energy capture capacity and may consist of a method to increase net photosynthesis in the species.

Higher levels of chlorophyll were positively related to accumulation of biomass and biosynthesis of enzymes associated to electrons transport, proteins and carbohydrates in plants of *S. rebaudiana* (Jain et al. 2009), reinforcing the idea of greater photosynthetic activities when there is a higher content of chlorophylls by leaf area. In the present work it was also observed that the highest values of chlorophyll *a* and total chlorophyll in the lowest planting densities are related to the higher biomass productions per plant.

In addition to the increase in biomass, it is worth mentioning that a relationship between the accumulation of steviol glycosides, both *in vivo* and *in vitro*, is also described with the extension of the development of the chloroplast membrane system and the content of photosynthetic pigments (Ladygin et al. 2008, Wu et al. 2013). According to Jain et al. (2009), the relationship between the activity of the photosynthetic apparatus and the production of diterpene glycosides suggests that the synthesis of isoprenoids in chloroplasts is a determining factor for the production of the sweetening substances. The biosynthesis of steviol glycosides and chlorophyll comes from the same precursors, isopentenyl diphosphate and dimethylallyl diphosphate of the methylerythritol phosphate pathway in plastids (Guleria et al. 2014). Thus, the ideal conditions for the production of chlorophyll molecules at the beginning of the biosynthetic pathway are the same as those regulating the production of the sweetening substances in stevia.

Considering the relationship between higher chlorophyll content and higher biosynthetic activity, it is possible to associate the production of steviol glycosides with the greater availability of carbohydrates from photosynthesis since the precursor molecules for the MEP pathway (glyceraldehyde-3-phosphate and pyruvate) derive directly from photosynthesis or glycolysis (Barbet-Massin et al. 2015).

Proteins and sugars are also important molecules in stevia metabolism. For both it was observed a similar pattern regarding plant flowering, with higher contents before flowering following constant decrease after that. The reduction of protein levels occurs due to redistribution of nitrogenous compounds from leaves to reproductive organs throughout the plant cycle (Reis et al. 2009, Xu et al. 2012). During vegetative growth, nitrogen is rapidly absorbed by the roots and transported to the leaves. In leaves, nitrate reduction produces amino acids that are incorporated into leaf protein

(Masclaux-Daubresse et al. 2010). During flowering remobilization of leaf nitrogen protein occurs and amino acids translocation to the reproductive organs is high, decreasing the contents of these compounds in the leaf (Imsande and Touraine 1994, Bredemeier and Mundstock 2000). This remobilization of reserves to the reproductive organs also occurs in sugar metabolism. The intense consumption of carbohydrates at the beginning of flowering for the formation of floriferous shoots results in the reduction these compounds contents (Cruz et al. 2007, Moreira et al. 2014).

Other important aspect of carbohydrate remobilization in stevia is its role in the composition of diterpene glycosides. After the formation of the steviol molecule, a series of glycosylations occur in the cytosol, which leads to the formation of the various sweetening compounds in the plant. These reactions are catalyzed by cytosolic UDP-dependent glycosyltransferases (UGTs), enzymes that transfer a sugar residue from a donor (mainly UDP-glucose) to an acceptor molecule (Brandle and Telmer 2007, Ceunen and Geuns 2013c, Yang et al. 2015). In this way it is possible to infer that the decrease of the total sugar contents, observed at the beginning and full flowering, is also related to the greater biosynthesis of steviol glycosides.

In a study performed by Ceunen and Geuns (2013a) it was observed that ontogenetic variations in glucose levels were markedly different on short days and long days. During long days, the glucose content increased about two times in the beginning of the flower bud formation. In short days, however, the authors reported that there was stagnation or a small reduction in glucose contents, as observed in the present study for total sugars. This relation reinforces the hypothesis that the late planting greatly reduced the vegetative period of the crop, causing it to have a cycle and development model similar to that described for the species when submitted to cultivation on short days.

Concerning steviol glycosides, the higher contents per plant at the lower planting density might be related to the lower competition of plants for radiation, water and/or nutrients, as these factors are directly related to plant populations and are reported by several authors as important conditions for production of sweetening compounds in stevia (Jarma et al. 2012, Mandal et al. 2013, Pal et al. 2013, Kumar et al. 2012, 2014b, Parris et al. 2017). Considering the abovementioned relationship between chlorophyll *a* and total chlorophyll contents and a higher photosynthetic capacity it is possible to infer that the best use of the radiation per individual promoted greater accumulation of precursors and energy for the biosynthesis of these molecules.

According to Brandle and Rosa (1992) the main factor affecting the content of diterpene glycosides in stevia is flowering. The content of sweetening compounds in stevia tends to increase until the beginning of flowering, decreasing continuously until the production of seeds, therefore, it is advisable to harvest the leaves at the beginning of the opening of the flowers (Kang and Lee 1981, Lima-Filho et al. 2004). Corroborating with these results, it is observed in the present work the reduction in both stevioside and rebaudioside A contents at the full flowering harvest, especially at higher planting density.

It seems to be a consensus that the physiological mechanisms associated with the ontogenetic variation in steviol glycosides accumulation involves a change in the transcription profile of the genes involved in the synthesis pathway (Ceunen and Geuns 2013a). According to Yang et al. (2015) most of the genes involved in steviol glycosides biosynthesis present expression peaks after the appearance of the floral bud, among them *SrUGT74G1*, which encodes the UGT responsible for stevioside synthesis. This ontogenetically regulated overexpression can be related with the higher contents of glycosides at the harvest at the

beginning of flowering but does not explain the decreased level at full flowering.

Some hypotheses for reduction in steviol glycosides accumulation with the progress of flowering are related to the presence of senescent leaves with low levels of these metabolites (Kalandia et al. 2004), the remobilization to the reproductive organs (Bondarev et al. 2003), and/or even one catabolism *in situ* by endogenous glycosidases, although most of these assumptions have been recently refuted (Ceunen and Geuns 2013b). Accordingly the regulation of the production of these compounds appears to be a more complex mechanism that still need to be characterized (Yang et al. 2015).

Considering the industrial preference of rebaudioside A compared to stevioside, the endogenous rebaudioside A/stevioside ratio is an important factor to evaluate the quality of genetic material and cultivation practices for commercial extraction (Yadav et al. 2011, Ceunen and Geuns 2013c). The present study shows that the lowest ratio occurred at the pre flowering harvest and with the highest planting density. This behavior might be justified especially by the higher productivity of stevioside in this phase coupled with the greater competition between plants. A greater competition between plants can reduce the radiation availability per plant, and, consequently reduce photosynthesis (Jarma et al. 2010, Kumar et al. 2014b). Considering that the molecule of rebaudioside A bears one more glucose moiety in its structure in comparison with stevioside (Brandle and Telmer 2007, Ceunen and Geuns 2013a, c), a lower availability of glucose, a product from photosynthesis, can reduce the amount of rebaudioside A in relation to stevioside. A lower rebaudioside A/stevioside ratio was also related by Mandal et al. (2015) in plants with decreased photosynthetic rates.

Since stevioside is a direct precursor of rebaudioside A, there is a limitation in the metabolic flux of rebaudioside A by the levels

of stevioside (Ceunen and Geuns 2013b). It is possible that the increase in rebaudioside A contents observed in the harvest at the beginning of flowering was a function of both the conversion of stevioside synthesized before the opening of the flowers, and not only of enzymatic regulation.

In relation to the stevioside accumulation, it was observed an increase in the beginning and full flowering harvests, as a function of the higher contents of the compounds in the beginning phase of flowering and the increase of the biomass accumulation in the full flowering phase. Regarding planting densities, the population of 166 667 plants ha<sup>-1</sup> promoted higher yields due to the greater accumulation of biomass per area.

For rebaudioside A yield, it was observed that the higher number of plants per area and harvesting at the beginning of flowering compensated for the better individual performance of the plants in the lower density, as observed by Serfaty et al. (2013) and by Kumar et al. (2014a), which reached a more than 2-fold increase in steviol glycoside accumulation in the density of 111 111 plants ha<sup>-1</sup> compared to lower density (37 037 plants ha<sup>-1</sup>).

In a previous evaluation of the same accession under similar cultivation conditions (same region and soil conditions) a rebaudioside A/stevioside ratio of 0.95 and productivity of 695 kg ha<sup>-1</sup> of steviol glycosides was obtained in the first cut (Francisco et al. 2018). This result is considerably higher than those reported in the present work. The previous discussed decrease in the vegetative period due to late planting may explain this drastic reduction and draw attention to the physiological and productive importance of the adequate planting period.

## CONCLUSIONS

Harvesting at the beginning of flowering provides higher contents and yields of diterpene glycosides as a function of the dynamics of the biosynthesis flow

of these compounds and the increase in biomass values. The adoption of a density of 166 667 plants ha<sup>-1</sup> associated to the harvest at the beginning of flowering promotes higher yields of biomass and steviol glycosides in the genotype evaluated in the present study.

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