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Stevia rebaudiana Bertoni: The Interaction of Night Interruption on Gas Exchange, Flowering Delay, and Steviol Glycosides Synthesis

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Abstract: The *Stevia* market is estimated to be USD 1.14 billion in 2028 due to its acceptance in the food and beverage industry. *Stevia rebaudiana* and its two more relevant edulcorants: stevioside (St) and rebaudioside A (Reb-A) can reach 450-fold sweeter than sucrose. The species is considered a long night plant, promoting flowering and shortening vegetative growth. Thus, to increase the leaf area and St and Reb-A increase, we broke the long night with a short light pulse, here called night interruption (NI). In this study, three NI times and two *S. rebaudiana* genotypes were tested to promote larger vegetative growth, flowering delay, and higher synthesis of steviol glycosides (SvGly). The main goal of this study was to demonstrate that NI increased net photosynthesis (9% to 20%), the internode length (59%), the leaf area (25%), while delays in 4 to 10 days of the flowering phase, impacting in 17% to 25% more St and Reb-A, respectively. Here we describe an inexpensive flowering delay, elongation of vegetative growth, allowing extended harvesting, which could yield four to five annual harvesting of leaves, increasing the production in 21% to 24% more St and Reb-A yield (kg ha^{-1}).

Keywords: *Stevia*; phytochromes; growth parameters; gas exchange; COVID-19

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1. Introduction

Stevia rebaudiana Bertoni is a perennial shrub belonging to the Asteraceae family. It is a subtropical wild plant, native to the northwest of the province of Misiones, in Paraguay [1–3].

The *S. rebaudiana* leaves contain the largest percentage of steviol glycosides (SvGlys), most of which are comprised of stevioside (4–20%, DW), rebaudioside A (3%, DW), dulcoside A (0.5%, DW), and steviolbioside (trace) [4]. Due to its industrial importance, *Stevia* is commercially cultivated in several parts of the world, such as Argentina, Brazil, Canada, China, India, Indonesia, Japan, Korea, Mexico, Russia, Tanzania, and the USA, mainly for its therapeutic and sweetening properties as a non-caloric sweetener [5]. North America dominates the steviol glycoside market because of the enhanced administration and of the huge need for artificial sweeteners in the region. The global Stevia market was valued at USD 620.8 Million in 2020 and is projected to grow at a compound annual growth (CAGR) of 8.0% to USD 1.14 billion in 2028 [6].

SvGlys are mainly extracted from *S. rebaudiana* leaves. SvGlys have a sweetening power 50–450-fold sweeter than sucrose, providing an enormous advantage in the sweetener market [3]. The global rebaudioside A (Reb-A) market includes forecast data, demand, application details, price trends, and Reb-A actions by geographic region [6]. Thus, the SvGly has been offered as a substitute for sucrose in the pharmaceutical and food industries [7]. *Stevia* is safe for use by both diabetics and hypoglycemics. Its benefits include anti-hypertensive, anti-hyperglycemic, anti-carcinogenic, and anti-human rotavirus activities, including a new coronavirus named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), which cause acute atypical respiratory diseases with a huge number of deaths worldwide [3,8,9]. The sweetener industry is interested in *Stevia* leaves with a high

concentration and high ratio of rebaudioside A/stevioside since stevioside has a bitter and strong flavor, a characteristic that is not interesting for the market [10]. Thus, many research groups around the world have carried out feasibility studies for new genotypes with the purpose of obtaining varieties with a higher concentration of Reb-A [11–14].

World consumption of powder-based Stevia is expected to reach 10,254.93 metric tons by 2027, with a growth of 7% to 8% per year [15]. Such growth is due to the rapid expansion of the beverage industry, which prefers natural sweeteners over synthetic ones for sugar-free use and products [16]. Often, the highest concentration of SvGly in *Stevia* leaves is seen in the pre-flowering stage. In some *S. rebaudiana* cultivars, the concentration of SvGly can reach up to 25% of the dry weight (DW) of the leaves [12]. Soon after flowering, SvGly levels can reach 6% to 7% DW, but these concentrations are cultivar-specific and vary according to the cultivation conditions and analysis methodology [17].

S. rebaudiana is a long night (LN) plant with a critical day length of about 11 to 12 h [7,8]. However, supplemental light can easily modulate the photoperiod creating a short night (SN) environment [8]. It is well known that night interruption (NI) would be provided by weak light by fluorescent or light-emitting diode (LED) [7,18]. NI has an inhibitory effect on floral induction, favoring vegetative growth and plant development in the period in which flowering would naturally increase [4,7,8,12]. Ceunen and Geuns [8] investigated the effect of NI during the dark period after an LN photoperiod (8 h) in *S. rebaudiana* and showed that NI promoted an increase in the total content of SvGly compared to plants grown under LN. These results suggest that NI is a possible alternative to the cultivation of *S. rebaudiana* plants under a naturally SN photoperiod.

A wide range of literature covers various aspects of SvGly production in *S. rebaudiana*. The main studies involve ontogeny [12], different spectra of red (R), distant red (FR), and blue [7,13,19], effects of light intensity [4,20], as well as the effect of light quality on the growth and development of in vitro seedlings [19,21], quality of light in the content of chlorophylls and carotenoids [22]. However, the role of night interruption (NI) on net photosynthesis (A_N), linked to relative growth rate/biomass accumulation and delayed flowering caused by NI and SvGly production, has rarely been studied in a single paper. This study hypothesizes that net photosynthesis has a positive correlation with NI treatment, morphological data, and some SvGly content. The main objective of this study was to evaluate two non-commercial genotypes of *S. rebaudiana* and test whether NI influences photosynthesis and, consequently production of SvGly, the production of leaf biomass and the delay in flowering.

2. Materials and Methods

2.1. Plant Material and Environmental Conditions

In this study, two *S. rebaudiana* genotypes (2 and 4) were studied, obtained by natural pollination in a controlled greenhouse, after selection among 25 distinct started genotypes [23]. The study was carried out under biospace conditions [20] at the experimental farm of the Faculty of Agricultural Sciences at the University of Cordoba (Monteria-Colombia), located at 8°47′37″ N; 75°51′51″ W, 15 m a.s.l., experiencing a mean annual rain of 1346 mm, relative humidity of 84%, and mean annual temperature of 27.4 °C [24]. During the winter, where the experiments were carried out, the night length is close to day length, with a slight tendency to a shorter night, approximately 11.5 h night. So, during the experiment, the nights were always interrupted by the pulse of light.

Montería is characterized as a tropical wet climate according to the Köppen climate classification. The experiment started on 27 November 2018 and finished on 21 February 2019 or in 2019 Julian days (−35 to 63 days). During the experiment, the air temperature was practically stable. However, the solar radiation was a strong variable with daily values ranging from 4.81 at 39 days to 9.41 at −6 days (Figure 1). Therefore, there were never long nights, even in the control experiment, that did not receive additional light.

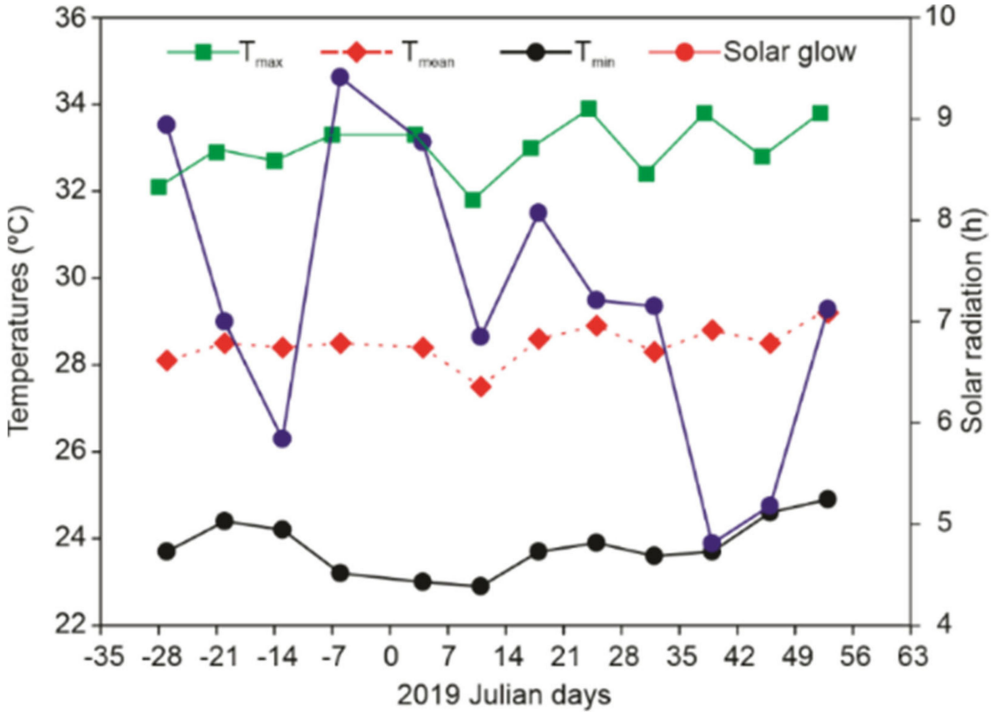

Figure 1. Maximum (**green**), minimum (**black**), and mean temperature (**dotted red**) registered during the experiment. The blue line denotes the solar radiation in hours.

Cuttings of genotype 2 and genotype 4 of *S. rebaudiana* were grown in a greenhouse with a type I biospace (1000 m^2) subdivided into four blocks of 0.3 × 0.3 m and a population density of 111,000 plants ha^{-1}, as recommended by plant cultivation [25]. The blocks were subdivided into plots of 5 m^2 each, containing only one treatment in each plot (5 m^2) with chambers designed with 800 caliber black plastics to artificially separate each treatment. A line of 6 fluorescent 50-W white fluorescent lamps (Sylvania Led T5 Tubes, Sylvania Portugal Lda, Lisboa, Portugal) that provided a light intensity of 250 µmol photons m^{-2} s^{-1}, and an R/FR of 11.4 were placed inside each chamber. The experimental setup was a 2 × 3 completed blocks design, containing the two genotypes (2 and 4) and three times NI times (0, 10, and 20 min). More specifically, throughout the experiment, every day between 11:45 p.m. and 00:15 a.m., the lights were turned on for either 0, 10, and 20 min controlled by a 2.4 GHz smart light switch (Gosund, NY, USA) programmed before NI treatment. Thus, all plants received one pulse of light during the night, which is referred to as night interruption or NI in the current study. The treatment duration was chosen based on recommendations of Hajihashemi and Geuns [26].

2.2. Timeline of the Experiments, Leaf Harvesting, and Flower Induction

The rooted seedlings were obtained and developed as described above (day 0). Fourteen days after transplanting, all plants were subjected to first leaf harvest since this process has proved to promote flowering [27]. After the first leaf harvest, all *S. rebaudiana* plants were stayed to promote the first flower budding. Three days after the first flowering, a second leaf harvest was performed, and after that, the plants were stayed to promote the second flower budding. In a similar manner, four days after the second flowering, a third leaf harvest was performed, which promoted the third flower budding. The final experiment occurred at 100 days for genotype 2 and 120 days for genotype 4, where no flower was promoted (Figure 2).

2.3. Morphological and Physiological Parameters

During the NI treatment, all plants were supervised, and then morphological data were collected, such as plant height, internode length, total biomass, leaf area (LA), specific

leaf weight (SLW), leaf area ratio (LAR), flowering delay, RGR, net photosynthesis (A_N), stevioside (St), and rebaudioside A (Reb-A) concentration in leaves. Each feature was collected in a different timeline. The plant height and internode length were measured by a metric tape. Total biomass was weighed after 72 h in an oven at 75 °C when the mass remained stable after cooling. The LA was measured by the non-destructive method as previously reported [28]. Finally, for the estimation of SLW, 20 disks of 1.2 cm in diameter were randomly removed along the leaf surface.

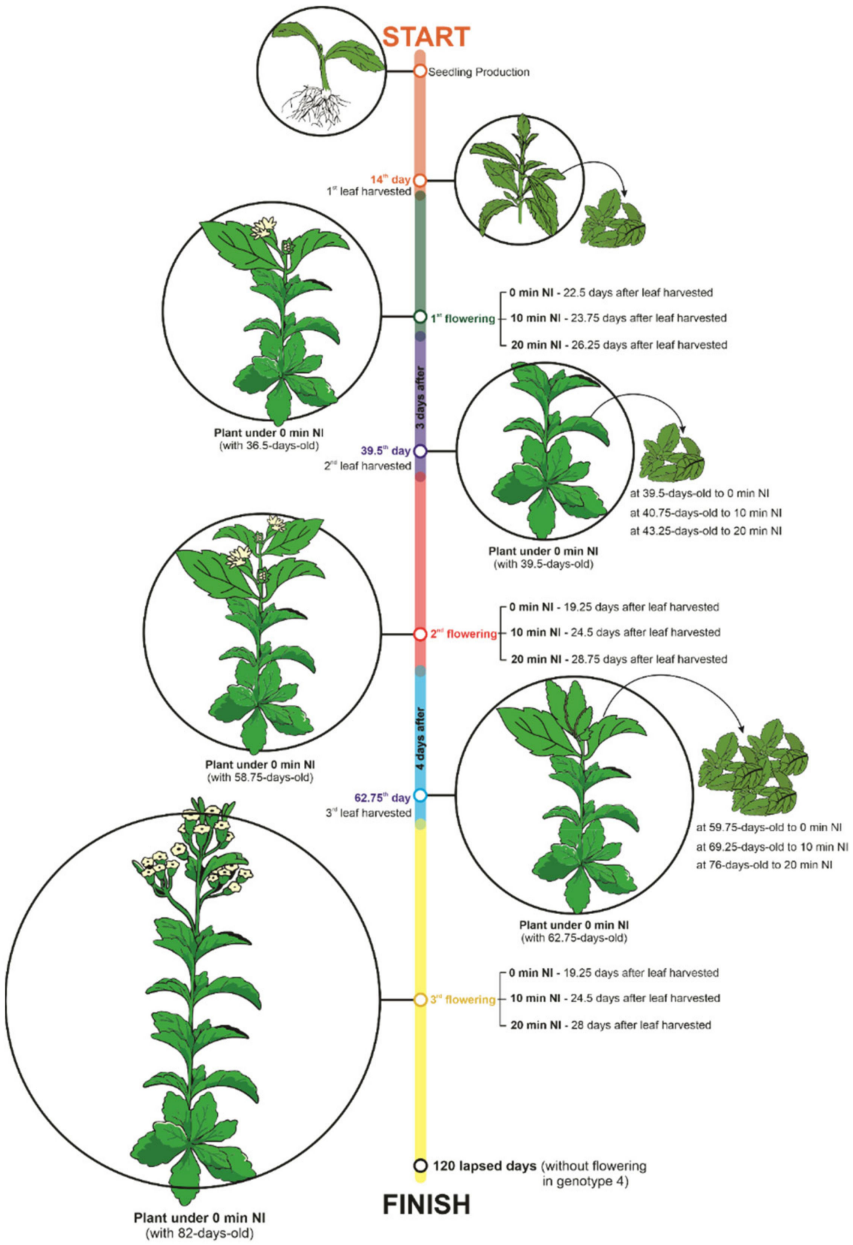

Figure 2. Timeline showing all steps of this study that encompassing three-leaf harvest (in plants of at 14, 39.5, and 62.75 days old) and three-leaf harvest (in 36.5, 58.75, and 82 days old), considering only 0 min NI. Figure drawn by authors, supported by Vecteezy [29].

These disks were oven-dried (72 h, at 75 °C) and weighed on a Unibloc analytical balance, mod. Shimadzu ATX224 PSC (Shimadzu Corporation, Kyoto, Japan). With dry disks mass, measured analytically and the area measured by the conventional formula, the specific leaf mass was obtained as $SLW = \frac{dry\ weight\ of\ each\ disc}{\frac{leaf\ disc\ area}{10{,}000}}$ where the SLW was expressed

by g m^{-2}. The LAR was calculated as $LAR = \frac{leaf\ discs\ area}{SLW}$ and expressed by cm^2 g^{-1}. The RGR was calculated as $RGR = \frac{\log_n(dry\ weight_2 - dry\ weight_1)}{days\ lapsed\ between\ 1^{st}\ and\ 2^{nd}\ harvest}$. For flowering, the days to flowering after the formation pruning until the moment of registering 50% of the plants in the flowering stage for each experimental unit were considered. Net photosynthesis was conducted exactly described in Dos Santos, Mendes [30].

2.4. Analysis of Steviol Glycosides (SvGly)

The SvGly concentration varies widely when comparing young leaves [12]. Thus, leaves were chosen from the fourth internode from the top to the base plant, collected after the third flowering, and stored in paper bags, protected from light and moisture until use. A total of 100 mg of dehydrated leaves were extracted 3-times with 20 mL of HPLC-grade water and combined at the end. The extraction of St and Reb-A was carried out in a water bath at 90 °C for 20 min. Gaseous N2 was injected into the tubes to prevent the formation of air bubbles. After 30 min, the tubes were centrifuged at 15,000× *g*, 10 min, 4 °C. The obtained supernatant was filtered using a Millipore 0.45 µm filter (MF-Millipore™ MCE Membrane Filters, Part number 32031602, Millipore, Darmstadt, Germany) and 20 µL of the extract was injected on reverse phase HPLC (Hewlet Packard, Houston, TX, USA, series 1050, USA) with a 2 Alltima C18 columns in series (250 × 4.6 mm ID, 5 particle size; Grace, Lokeren, Belgium). The elution of SvGlys was performed at 25 °C over 25 min, with a 1 mL min^{-1} flow rate, using an optimized non-linear gradient of AcCN: acidified H_2O (1 mM H_3PO_4; 34% AcCN (*v*/*v*), 4 min, 35%, 10 min, 42%, 16 min, 42%, 16–25 min, 34%, 25 min, stop). The compounds in each sample were identified and quantified by comparing their retention times with those of standards. The calibration curve was obtained with standards of St (Sigma-Aldrich Chemical Co, Darmstadt, Germany, part number 19345) and Reb-A (Sigma-Aldrich, part number 01432). The results were expressed in g kg^{-1} DW.

2.5. Statistical Data Analysis

A Shapiro–Wilk test [31] was used to test for normality, and a Brown–Forsyth test [32] was used to test for equal variances using the statistical software package SigmaPlot Version 14.0 (Systat Software Inc., Chicago, IL, USA). All data are presented as means plus standard errors (SE; n = 10). All results were analyzed with a two-way ANOVA, and means were compared using a Student test Newman-Keuls (SNK) with the statistical software package SigmaPlot. The parametric correlations were made with the statistical software Statistica Version 8.0 (StatSoft, Tulsa, OK, USA). The results were considered to be significant when $p \leq 0.05$. However, when necessary, the data were appropriately transformed to improve a normal distribution [33].

3. Results

This section may be divided into subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

3.1. Net Photosynthesis and Relative Growth

Net photosynthesis varied widely over time and between NI treatments. In genotype 2, on 0 min of NI, photosynthesis ranged from 7.65 µM CO_2 m^{-2} s^{-1} (at 73-days-old plants) to 11.44 µM CO_2 m^{-2} s^{-1} (at 85-days-old plants). These values were moderately increased under 10 min of NI, where the A_N ranged from 8.72 to 11.16 µmol CO_2 m^{-2} s^{-1} (at 43-days-old plants). In 20 min NI, the A_N varied in the same intensity as 10 min, ranging from 8.86 µmol CO_2 m^{-2} s^{-1} (at 36-days-old plants) to 11.44 µmol CO_2 m^{-2} s^{-1} (at 85-days-old plants). In genotype 4, the A_N was appreciably greater than in genotype 2, ranging from 9.52 µmol CO_2 m^{-2} s^{-1} (at 50-days-old plants under 0 min NI) to 15.33 µmol CO_2 m^{-2} s^{-1} (at 85-days-old plants under 20 min NI) (Figure 3A,B).

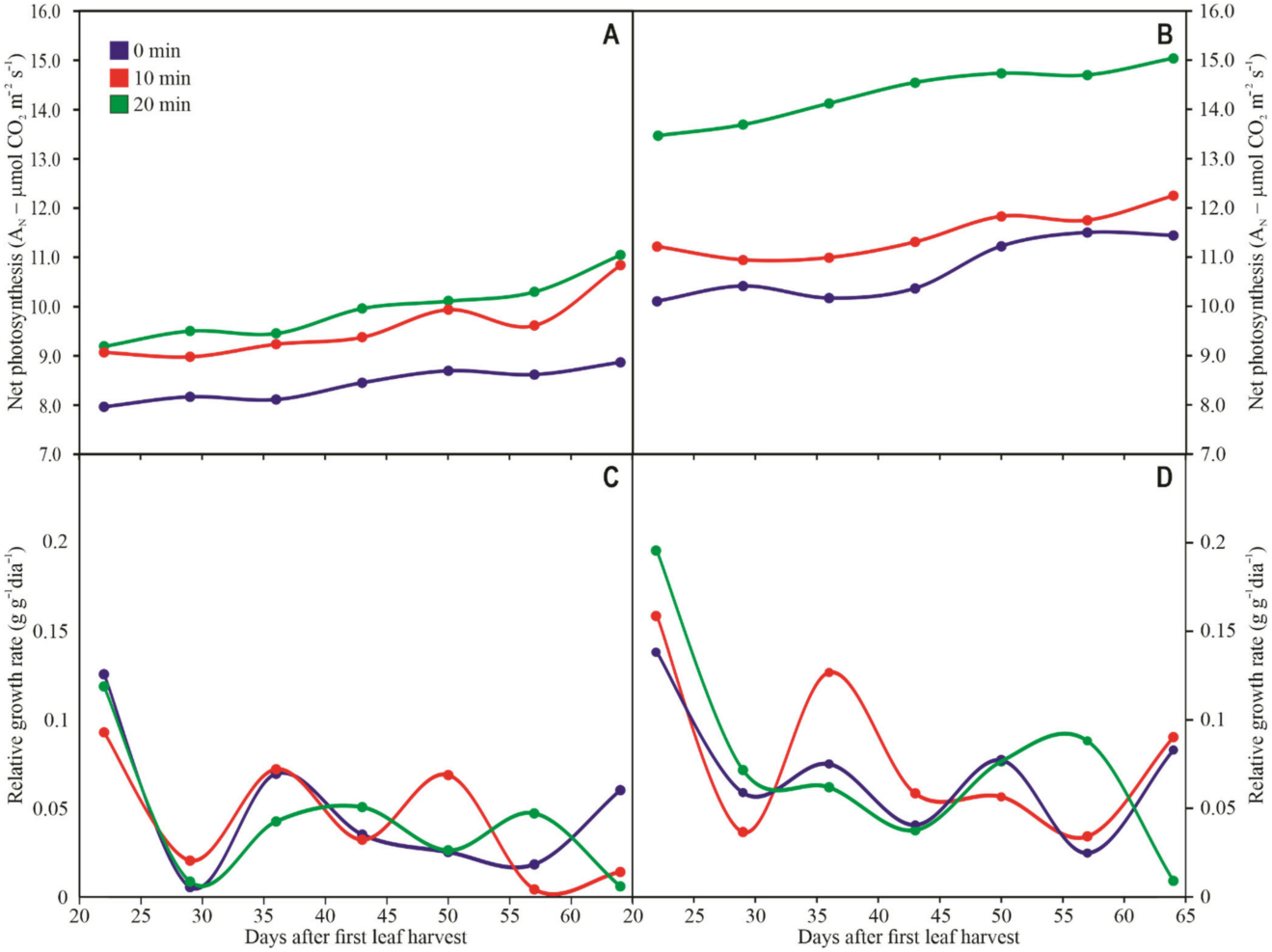

Figure 3. Net photosynthesis (**A**,**B**) and relative grown rate (**C**,**D**) in plantlets of genotype 2 (**A**,**C**) and 4 (**B**,**D**) of *Stevia* rebaudiana under three times of light exposure in night-break. All data are expressed as means ± SE, n = 10.

In all times of NI and independently of the genotype, the values of RGR were exceptionally low. For both genotypes, the RGR growth was inversely proportional to the time of light in NI. In genotype 2, the RGR was 0.0401, 0.0350, and 0.0345 g g^{-1} day^{-1}, respectively, at 0, 10, and 20 min of NI (Figure 3). For genotype 4, the means were 0.0945, 0.0929, and 0.0711 g g^{-1} day^{-1}, respectively, for 0, 10, and 20 min of NI. The student *t*-test showed that the means are statistically significant for all NI times analyzed between two analyzed genotypes.

3.2. Flowering Delay

First, we must contextualize that genotype 2 is a short-cycle plant, while genotype 4 is a long-cycle plant. Then, this study will only describe data for genotype 2 because genotype 4 does not bloom during the 120 days elapsed from the beginning to the end of this study. The NI showed a weekly significant effect on flowering delay ($r = 0.213$, $p = 8.94 \times 10^{-7}$; Supplementary Data File) and lengthening the vegetative period of *S. rebaudiana* (Figure 3). In 14-day-old *S. rebaudiana* plants, the first leaf harvest was performed (Figure 2). After this time, the differential light treatments promoted flowering. So, the flower budding occurs in 22.5, 23.75, and 26.25 days after first leaf harvest, respectively in 0, 10, and 20 min NI plants. Three days after first flowering, a second leaf harvest was performed (at 39.5-days after transplantation) where plants returned to the generative period, and the 0, 10, and 20 min NI plants flowered after 19.25 days, 24.5 days, and 28.75 days, after second leaf harvest, resulting in 58.75-days-old control plants (Figure 4). Four days after the second flowering

(plants 62.75 days old after transplantation), a third leaf harvest was performed. At this time, the 0, 10, and 20 min NI plants delayed their flowering by 19.25 days, 24.5 days, and 28 days, respectively, resulting in 82-days-old *S. rebaudiana* control plants. Analyzing only the flowering delay, it was shown that after the first harvest, NI-treated plants for 10 and 20 min delayed their flowering on 1.25 and 3.75 days. On the second and third harvest, the delay was 5.25 to 9.5 days (under 10 min NI) and 5.25 to 8.75 days (under 20 min NI) (Figure 4).

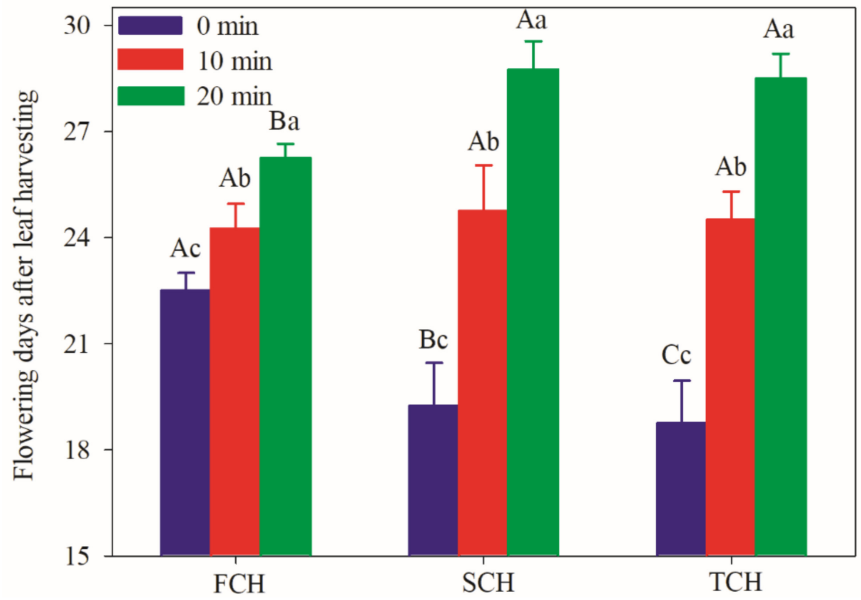

Figure 4. Days to flowering of the first harvested (FCH), second harvested (SCH), and third harvested (TCH) in plantlets of genotype 2 of *Stevia* rebaudiana under three times of light exposure in night-break. All data are expressed as means ± SE, n = 10. Different lowercase letters denote significance within times of light exposure in night interruption for each genotype, and different capital letters denote significance within the type of cutting (FCH, SCH, and TCH).

3.3. Plant Development

The internode length increased in accordance with increasing NI; however, for plant height and total biomass, these differences were not significant to NI treatments (Figure 5). Moreover, there were no significant changes in the internode length in genotype 2. Compared to genotype 4, the plant height and total biomass appeared to be independent of the internode length. For plant height and total biomass, there were no significant changes in the time-course of light (or NI). The height and total biomass per plant of genotype 4 were, respectively, 1.6 and 2.1 times higher than those of genotype 2 (Figure 5).

The leaf area (LA) of genotype 2 was 1.6-fold higher under 10 min of NI compared to control. However, the LA was decreased by 21% under 20 min of NI (Table 1). Genotype 4 showed the same trend as genotype 2, where under 10 min of NI, the LA was 1.4-fold higher compared to control; however, under 20 min of NI, the LA of genotype 4 was 36% less than those at 10 min of NI.

The specific leaf weight (SLW) that represents the biomass produced in each square meter of leaf trend to be higher in genotype 4 in comparison to genotype 2. However, this superiority of genotype 4 was rarely statistically significant (asterisks in Table 1). Similar to the other morphological features, the leaf area ratio (LAR) trended to be higher in 10 min of NI when compared to control or 20 min NI.

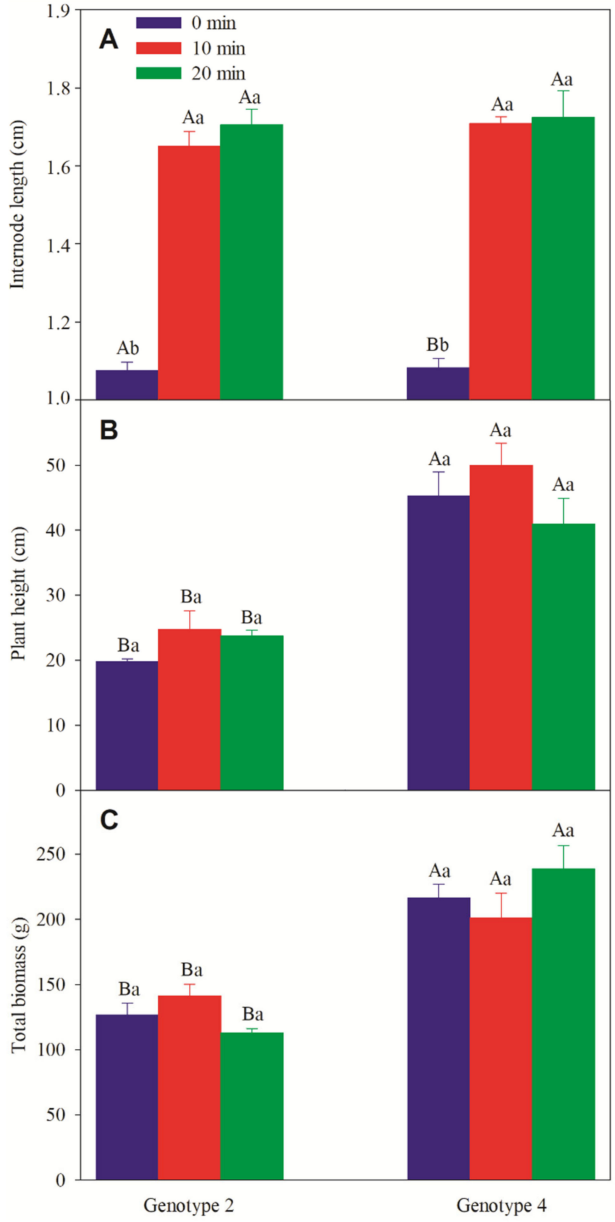

Figure 5. Internode length (**A**), plant height (**B**), and total biomass (**C**) in plantlets of genotype 2 and 4 of Stevia rebaudiana under three times of light exposure in night-break. All data are expressed as means ± SE, n = 10. Different lowercase letters denote significance within times of light exposure in night interruption for each genotype, and different capital letters denote significance within genotype for the same light exposure in night interruption.

3.4. Steviol Glycosides

The St content had a significant increase as NI increase (r = 0.839, $p = 1.12 \times 10^{-6}$; Supplementary Data File). In both genotypes, NI increased St, irrespective of light conditions. In genotype 2, 20 min of NI promoted a significant increase of 17.6% in St, while the increase shown at 10 min of NI is not significant (Table 2). For genotype 4, the increase was strongest than genotype 2, where 10 min promoted a weakly significant increase of 8.4% St.

On 20 min NI plants, the increase was more acute, with 24.6% more St compared to its control plants. The level of Reb-A in genotype 2 trended to be like St, where 10 min and 20 min promoted 7.3% and 20% more Reb-A than control. For genotype 4, 10 min and 20 min of NI promoted a significant increase of 9.7% and 28.5% of Reb-A, respectively (Table 2). In both genotypes, the ratio between Reb-A and St was not influenced by NI

time. However, genotype 4 plants under 0 min and 20 min of NI showed a higher ratio on average 7% and 9% when compared to genotype 2. Both genotypes showed a slight-to-moderate increase in St and Reb-A under NI treatments. The increase in Reb-A, in specific, would be relevant to industry because of the productivity that can be obtained in one hectare. So, this study verified that on genotype 2, the estimated St yield was increased from 986.68 ± 16.22 kg ha^{-1} (on control) to 1160.00 ± 17.46 kg ha^{-1} (on 20 min NI plants), an increase of 17.6%. In genotype 4, the comparison between 20 min NI and control returned a moderate increase of 24.6% (Table 2). The comparison of estimated Reb-A between 20 min NI and 0 min NI returns an increase of 20.4% (on genotype 2) and 27.3% (on genotype 4). Thus, we verify that the accumulation of St was genotype- and time-dependent because genotype 4 on average accumulated, respectively, 1.2-, 1.3-, 1.2-, and 1.3-fold St, Reb-A, St yield, and Reb-A yield when compared to genotype 2. In a similar manner, plants under 20 min accumulated approximately 1.3-fold higher St and Reb-A than control plants.

Table 1. Leaf area, specific leaf weight, and leaf area ratio measured in plantlets of genotype 2 and 4 of *Stevia rebaudiana* under three times of light exposure in night interruption (NI) evaluated in different plant ages (PA) in relation to 0 min of NI (to other treatment, please consult Figure 2 to clarify).

	Leaf Area (cm^2)					
PA	**Genotype 2**			**Genotype 4**		
	0 min	**10 min**	**20 min**	**0 min**	**10 min**	**20 min**
29	140.2 ± 29.3 Af	112.9 ± 41.3 Af	109.4 ± 33.8 Ag	99.8 ± 28.1 Ah	55.7 ± 31.8 Af	58.9 ± 14.8 Ag
36	321.0 ± 24.1 Ad	374.5 ± 60.5 Ad	330.6 ± 56.2 Ae	265.4 ± 12.8 Ag	236.9 ± 20.2 Ae	175.2 ± 30.4 Bf
43	273.2 ± 44.5 Ae *	261.3 ± 39.0 Ae *	237.7 ± 10.9 Af *	496.3 ± 74.3 Af	405.6 ± 52.9 Ad	490.0 ± 33.3 Ae
50	466.6 ± 45.2 Ad *	371.2 ± 54.1 Ad *	381.9 ± 38.5 Ad *	856.0 ± 22.3 Be	930.8 ± 32.5 Ac	897.8 ± 75.4 Ad
57	498.1 ± 127.2 Bc *	704.7 ± 126.7 Ac *	622.1 ± 108.0 Ac *	1144.0 ± 209.1 Ad	1222.5 ± 172.9 Ab	923.3 ± 143.3 Ac
64	946.1 ± 169.2 Bb *	1305.0 ± 202.4 Aab	1363.3 ± 175.2 Ab*	1953.9 ± 111.5 Ac	2151.8 ± 551.2 Aab	2131.5 ± 165.5 Ab
71	1152.2 ± 327.0 Ba *	1146.2 ± 288.4 Bb *	1558.0 ± 367.6 Aa *	2800.9 ± 454.1 Aa	2554.8 ± 389.6 Aa	2679.9 ± 344.9 Aa
78	1676.8 ± 299.8 Aa *	1546.8 ± 452.5 Aa *	1363.4 ± 214.4 Bb *	2484.0 ± 184.4 Bb	2999.8 ± 709.2 Aa	2982.5 ± 470.5 Aa
85	1906.0 ± 148.5 Bb *	2993.4 ± 558.2 Aa	2375.2 ± 552.3 Aa	2843.3 ± 226.7 Bb	3904.4 ± 609.5 Aa	2488.2 ± 357.7 Bb
PA	Specific leaf weight (g m^{-2})					
	0 min	10 min	20 min	0 min	10 min	20 min
29	36.4 ± 2.6 Ac	38.5 ± 4.1 Ab *	41.4 ± 10.2 Ac	44.5 ± 3.5 Ad	19.0 ± 2.4 Cd	30.2 ± 4.6 Bc
36	49.7 ± 7.7 Ab	43.6 ± 7.2 Ab	52.9 ± 15.8 Aa	60.6 ± 11.2 Ac	51.3 ± 7.7 Ab	53.1 ± 8.6 Ab
43	40.6 ± 1.1 Bb	47.0 ± 14.3 Aa	39.7 ± 0.4 Bc *	44.4 ± 2.9 Ad	40.5 ± 2.0 Cc	46.4 ± 2.9 Bb
50	44.0 ± 2.8 Ab	47.5 ± 4.9 Aa	44.4 ± 1.7 Ab	50.6 ± 1.2 Ac	45.3 ± 2.0 Bc	45.3 ± 0.6 Bb
57	62.1 ± 3.6 Aa	39.4 ± 2.1 Bb	58.7 ± 14.1 Aa	50.3 ± 7.0 Ac	61.9 ± 11.8 Ab	52.4 ± 20.4 Ab
64	46.3 ± 8.9 Bb *	50.6 ± 5.1 Aa	38.9 ± 5.1 Bc	61.7 ± 4.2 Ac	54.3 ± 7.4 ABb	48.5 ± 13.2 Bbc
71	43.7 ± 7.0 Ab	39.3 ± 2.6 Ab	45.1 ± 5.7 Aa	50.6 ± 2.7 Ac	51.2 ± 6.3 Ab	77.1 ± 16.0 Aa
78	52.7 ± 6.0 Aa *	49.6 ± 6.5 Ba *	52.4 ± 7.7 Aa	94.8 ± 5.1 Aa	80.7 ± 4.2 Ba	72.0 ± 10.9 Ba
85	46.8 ± 3.7 Ab *	39.4 ± 4.17 Bb *	38.6 ± 7.3 Bc *	75.2 ± 5.8 Ab	53.8 ± 2.1 Bb	70.0 ± 5.9 Aa
PA	Leaf area ratio (cm^2 g^{-1})					
	0 min	10 min	20 min	0 min	10 min	20 min
29	4.0 ± 1.0 Ae	3.2 ± 1.2 Ae	3.5 ± 1.5 Ae	2.4 ± 0.7 Ag	2.5 ± 1.1 Af	1.9 ± 0.3 Af
36	6.7 ± 0.6 Bd	8.6 ± 0.5 Ad *	7.5 ± 1.8 ABcd	4.7 ± 0.7 Af	5.0 ± 1.0 Ae	3.4 ± 0.7 Ae
43	6.8 ± 1.3 Ad	12.3 ± 8.3 Ac	6.0 ± 0.3 Ad *	11.4 ± 1.9 Ae	10.3 ± 1.8 Ad	10.6 ± 0.8 Ad
50	10.9 ± 1.6 Ac *	8.0 ± 1.4 Ad *	8.7 ± 1.0 Ac *	17.0 ± 0.8 Bd	20.7 ± 1.1 Acd	19.8 ± 1.8 Ac
57	7.8 ± 2.6 Bd	18.3 ± 3.9 Ac	14.5 ± 5.6 ABc	27.7 ± 7.0 Abc	21.4 ± 5.8 ABc	14.0 ± 5.4 Bcd
64	24.5 ± 2.6 Bb	27.6 ± 6.5 ABb	37.7 ± 8.1 Aab	31.9 ± 1.8 Ab	27.4 ± 2.5 Abc	36.5 ± 8.7 Ab
71	31.0 ± 11.8 Ab	30.7 ± 9.1 Ab	37.3 ± 11.8 Aab	56.1 ± 9.6 Aa	58.6 ± 7.0 Aa	49.7 ± 4.2 Aa
78	34.9 ± 9.2 Aab	38.4 ± 18.5 Ab	29.3 ± 8.3 Ab	26.6 ± 2.9 Bc	27.7 ± 3.2 Bb	47.4 ± 13.8 Aab
85	42.3 ± 6.9 Ba	117.1 ± 5.0 Aa *	38.7 ± 2.7 Ba	39.3 ± 6.5 Bb	64.2 ± 11.1 Aa *	37.0 ± 8.0 Bb

All data are expressed as means ± SE, n = 10. Different lowercase letters denote significance within distinct DABF for each light time. Different capital letters denote significance within light quality for the same PA, and asterisks (*) denote significance within genotype for the same time and same PA.

Table 2. Stevioside and rebaudioside A contents, rebaudioside A/steviosides ratio and estimative of stevioside and rebaudioside A yields in plantlets of genotype 2 and 4 of *Stevia rebaudiana* under three times of light exposure in night interruption evaluated after third flowering.

Genotype 2			**Genotype 4**		
Stevioside (g kg^{-1} DW)					
0 min	10 min	20 min	0 min	10 min	20 min
109.63 ± 1.80 Bb	112.83 ± 3.05 Bb	128.89 ± 1.28 Ba	124.81 ± 1.94 Ac	135.32 ± 0.51 Ab	155.55 ± 2.22 Aa
Rebaudioside A (g kg^{-1} DW)					
83.67 ± 1.06 Bc	89.75 ± 1.10 Bb	100.73 ± 1.31 Ba	102.85 ± 1.52 Ac	112.86 ± 0.56 Ab	132.07 ± 1.27 Aa
Rebaudioside A/Stevioside ratio					
0.77 ± 0.02 Ba	0.80 ± 0.02 Aa	0.78 ± 0.01 Ba	0.83 ± 0.02 Aa	0.83 ± 0.01 Aa	0.85 ± 0.02 Aa
Stevioside yield (kg ha^{-1})					
986.68 ± 16.22 Bb	1015.51 ± 27.42 Bb	1160.00 ± 17.46 Ba	1123.32 ± 17.46 Ac	1217.85 ± 4.60 Ab	1399.93 ± 20.02 Aa
Rebaudioside A yield (kg ha^{-1})					
752.99 ± 9.57 Bc	807.74 ± 9.89 Bb	906.55 ± 11.79 Ba	925.65 ± 13.65 Ac	1015.78 ± 5.01 Ab	1178.64 ± 11.46 Aa

All data are expressed as means ± SE, n = 10. Different lowercase letters denote significance within times of light exposure in night interruption for each genotype, and different capital letters denote significance within genotype for the same light exposure in night interruption.

4. Discussion

The light signals for long and short days are transferred to the plant by a wide range of photoreceptors, including phytochromes and cryptochromes, absorbing light at specific wavelengths [34]. Noteworthy that short night conditions prolonged the vegetative growth stage in *S. rebaudiana* [12]. In terms of RGR, when we compare genotype 2 and genotype 4, we perceive the superiority of genotype 4. This is probably due to a genetic advantage in terms of the duration of its vegetative cycle, which does not cease during the NI, that without flowering, maintains its vegetative growth. The growth of a plant organ consumes carbon substances, and as a result, its concentration in the adjacent supply channels decreases, establishing a concentration gradient that implicates a fine-tuned regulation of carbon allocation across plant organs through the phloem, which move other materials from the organs that incorporate either the building block substances, or they simply give them up with age [35]. The more active the growth of one part, the more the available carbon goes to it, and the more the growth in other parts will be restricted. The lesser RGR described here to genotype 2 suggests that young *Stevia* tissues subjected to radiation for a long time were less efficient in biomass production compared to plants that are under the stimulus for a shorter time. Van Iersel [36] described that a decrease in A_N with the increase in dry biomass was correlated with a decrease in the RGR. These data are like those in this study, which showed a negative correlation between A_N and RGR, either when both genotypes were analyzed in a joined form ($r = -0.975$, $p = 3.37 \times 10^{-13}$), or as for genotype 4 ($r = -0.938$, $p = 1.93 \times 10^{-14}$) was analyzed independently. The importance of maintenance respiration in the carbon balance of plants increases with increasing plant size so that maintenance respiration is responsible for 25% of total respiration in small plants and 90% in large plants [36]. Similar results were also added for sweet pepper [37], where an RGR, A_N, LAR, and LWR decreased sharply with plant age.

The effects of the NI treatment on the internode length were positive ($r = 0.866$, $p = 2.18 \times 10^{-6}$). Specific leaf weight (SLW) is one of the few morphological characteristics of plants that show large changes over a single day. This value is one of the easiest components of growth to measure, whereas LAR indicates the efficiency with which a plant uses its leaves to produce photoassimilates [38]. Thus, it is expected to be a negative correlation between LAR and SLW. In this study, we show a non-significative ($p = 0.529$) correlation between LAR and SLW when both genotypes were joined analyzed. However, when we analyzed genotype 2 and genotype 4 individually, the correlation is negative, from

−0.285 (genotype 2) to −0.724 (genotype 4). These results were in accordance with those presented by Nilwik (1981) that also showed a strong negative correlation between LAR and SLW ($r = -0.920$, $p = 1.25 \times 10^{-3}$). In *S. rebudiana* [12] and in three species of the *Betulaceae* family [39], RGR decreased with ontogeny, which may be due to differences between the rate of division and cell growth in the different parts of the plant and Nilwik [37], who described that RGR, A_N, LAR and SLW sharply decreased with plant age.

The results of the internode length presented in this investigation coincide with Erwin and Heins [40]. In accord with Schmitt and Wulff [41], internode length is modulated by the action of phytochromes so that the longer the internodes, the lower ratio between red light (R) and far-red light (FR) in which the plants grow. Moreover, the positive interaction between NI and internode length ($r = 0.866$, $p = 1.61 \times 10^{-2}$) and the positive interaction between NI and St accumulation ($r = 0.839$, $p = 9.76 \times 10^{-4}$) or NI and Reb-A ($r = 0.751$, $p = 1.21 \times 10^{-4}$) can be explained by the biosynthetic SvGly pathway, which in parts is similar to the biosynthetic route of gibberellins (GAs) through the precursor ent-kaurenoic acid [13,42]. The accumulation of ent-kaurene at 2- to 3-fold higher in LD conditions than in SD conditions [43] is circumstantial evidence that SvGly and GAs are linked through their precursors genanylgeranyl diphosphate and ent-kaurene acid. Moreover, Hajihashemi, Geuns [44] demonstrated that in *S. rebaudiana,* an external GA treatment changes transcription of SvGly-related genes. Yoneda, Shimizu [45] showed that in some lifetime moments, *S. rebaudiana* may be preferred to accumulate its ent-kaurene acid in SvGly overwhelmed the gibberellin-biosynthetic route. This preference is more acute in pre- and during bud flowering since *S. rebaudiana* is a self-incompatible plant and the pollination behavior is entomophilous [10] when the plant becomes more vulnerable to attack by pathogens. Under herbivory the *S. rebaudiana* leaves may elicit plant defense responses, using SvGly, which may act as a defense mechanism against pathogens or be quickly mobilized for synthesis of other molecules with anti-herbivory action, such as protease inhibitors [46].

This study described the effects of NI, promoted by fluorescent lamps of 250 µmol photons m^{-2} s^{-1} at 0, 10, and 20 min, on plant features. It was perceived that NI did not promote the plant height and total biomass. Its data contradicts Yoneda, Shimizu [4], which describe NI of 50 µmol m^{-2} s^{-1} for 4 h is adequate to increase the biomass production in *S. rebaudiana*. This result seems plausible to speculate that the non-effect of NI on these features was because the times used in this study were not sufficient to achieve a stimulus in the hormones involved in cell elongation, as previously described by Park, Muneer [47] and Somers, Kim [48]. However, the expressive increase in the synthesis of St and Reb-A may have channeled the ent-kaurene to the biosynthetic pathway of SvGly to the detriment of the biosynthetic pathway of GAs, which act more in the elongation of the internodes as reported by Yoneda, Shimizu [45]. Moreover, the SvGly would be a carbon skeleton to return to gibberellin biosynthesis, promoting the elongation of the internode [45]. By the way, Yoneda, Nakashima [13] described that 400 µmol photons m^{-2} s^{-1} can cause photodamage in the leaves of *S. rebaudiana*. In this study, our plants have remained healthy until the end of the experiment, with no photodamage symptoms.

Usually, densities of 40,000 and 250,000 plants ha^{-1} were described in the literature [11], but in accord with Serfaty, Ibdah [49], 100,000 plants ha^{-1} proved to be better than 60,000 or 80,000 plants ha^{-1}. Other densities above 150,000 plants ha^{-1} have already been successfully exceeded in the literature [20,50,51]. While lower plant densities can generate more leaf per plant, higher densities can compensate for lower leaf production per plant due to the greater accumulation of biomass by area [11,51]. With an increase in density to 166,667 plants ha^{-1}, 1053 kg ha^{-1} of leaves can be produced, values close to those already existing in the literature [25,52–54]. These data are 74% of the values in the conditions of the Colombian Caribbean [20], which indicates a yield of 1420 kg ha^{-1} of leaf per harvest This led this study to speculate that in these Caribbean conditions, up to 6900 kg ha^{-1} $year^{-1}$ of the leaf can be produced. The capacity to produce between three and five harvests per year is due to the climatic conditions of the Colombian Caribbean (Af type climate; tropical

forest climate—Köppen-Geiger climate classification system, Martonne aridity index of 40.6 [26]). The increase in productivity of *S. rebaudiana* linked to environmental conditions corroborates previously published data [16,55,56], which demonstrates the climate factor as a preponderant for the increase in leaves and SvGly production.

The analysis of the biomass of the leaves showed that it tended to increase with the intensity of additional light [4]. These authors described the effect of the R/FR ratio on the growth of *S. rebaudiana* plants and showed that the lower ratio (0.16) obtained higher plant height, a fact that differs from our study where we used an R/FR ratio of 11.4. Yoneda, Shimizu [4] demonstrated that an R/FR condition of 7.60 showed plants with a higher leaf area than plants under 0.16 R/FR. On the other hand, Ceunen and Geuns [12] found that SN plants had a prolonged vegetative stage and late flowering. However, these plants showed higher biomass when compared to LN plants; a fact that differs from our data where both genotype did not show a significant difference in the plant height or in the total biomass. However, plants under 20 min NI showed an internode length on average 1.6-fold greater than the control plants.

Yoneda, Shimizu [4] described that 50 µmol m^{-2} s^{-1} red LED in 6-week-old plants was better to show higher foliar expansion in *S. rebaudiana*. The time of NI influences the morphological characters that control the phytochromes. Interpreting all data presented in this study, we can be able to argue that the extensive leaf harvest described in this study may have reduced the leaf area in some moments in the timeline of the experiment, and so, overwhelmed the effect of light to activate the physiological mechanisms to promote new leaves. In accord with Ceunen and Geuns [12], the LA was higher in SN plants when compared to LN plants. In genotype 2, the LA was strongly and positively correlated to LAR ($r = 0.909$, $p = 6.03 \times 10^{-31}$), moderately and positively correlated with St ($r = 0.695$; $p = 3.64 \times 10^{-4}$), Reb-A ($r = 0.656$, $p = 3.10 \times 10^{-3}$), and Reb-A yield ($r = 0.651$, $p = 9.99 \times 10^{-4}$), and negatively correlated with RGR ($r = -0.795$, $p = 8.16 \times 10^{-5}$), plant height ($r = -0.744$, $p = 1.89 \times 10^{-2}$), and SLW ($r = -0.285$, $p = 0.01$). Contrasting, LA of the genotype 4 was strongly and positively correlated with LAR ($r = 0.866$, $p = 5.17 \times 10^{-9}$), and moderately and negative correlated with SLW ($r = -0.724$, $p = 9.16 \times 10^{-7}$).

Some scholars described that NI can increase flowering [7,12,13], data that are corroborated by the data presented in this investigation, where we verified a strong positive correlation ($r = 0.892$, $p = 1.21 \times 10^{-4}$) between the time of exposure to fluorescent lamps of 250 µmol photons m^{-2} s^{-1} and flowering buds. Flowering can be induced by blackouts on long days and inhibited by day length extension or NI with artificial lighting [55]. Studies demonstrated that the effect of NI on locus T de chrysanthemums (FT—CmFTL3), red light (655 nm), and white light inhibited the expression of CmFTL3 by inhibiting flowering, while blue (485 nm) and FR did not [56]. The results in this study corroborate with previous studies [57] that prevent a NI from increasing the concentration of the active form of phytochrome (PFR) and thus have been in the vegetative phase and promote flowering. This study described NI of 20 min delay flowering by 3.75, 9.5, and 9.75 days after the first, second, and third leaf harvest. The high rate of flowering described in this study may be due to our high R/FR ratio (11.4), which, in accord with Sumitomo, Higuchi [56] raises the Pfr/P ratio, known to increase flowering, after NI. The results showed here also are in accord with those described by Ceunen and Geuns [12] that described that the flowering time was about 3-fold shorter in LN compared to SN plants.

In accord with Mohamed, Ceunen [42], in an LN plant, the photoperiod increases the percentage of total SvGlys content without increasing total biomass, and total SvGlys yield would increase if leaf biomass were increased under an LN photoperiod. These data were in accord with those presented here, and ads more information, i.e., the positive interaction between leaf area and St ($r = 0.752$, $p = 6.18 \times 10^{-6}$), Reb-A ($r = 0.792$, $p = 1.06 \times 10^{-3}$), St yield ($r = 0.570$, $p = 1.92 \times 10^{-3}$), and Reb-A yield ($r = 0.752$, $p = 6.18 \times 10^{-6}$). Ceunen and Geuns [58] described that, per unit biomass, free SvGly was present in greater quantities under LN compared to SN (e.g., 19 and 6 g/g DW, respectively). Ceunen and Geuns [12] described 28% more SvGlys produced per g biomass at the end of vegetative growth

under LN, compared to SN (69 and 53 mg, respectively [12]). These results contradict our data because genotype 4 showed 1.3-fold higher St and/or Reb-A than genotype 2. Kumar, Sharma [59] revealed that lower light environments did not result in significant changes in St and Reb-A, results that also contradict those presented in our study where the duration of the NI showed a positive correlation with the St ($r = 0.839$, $p = 1.12 \times 10^{-6}$), Reb-A ($r = 0.751$, $p = 1.21 \times 10^{-4}$), St yield ($r = 0.657$, $p = 7.06 \times 10^{-4}$) and Reb-A yield ($r = 0.569$, $p = 5.19 \times 10^{-15}$). Similarly, Hernández, Combatt [60] described that there is no interaction between the levels of environmental radiation in the synthesis of Reb-A. These data contradict our results where 20 min of NI promoted, on average, 25% more Reb-A than plants cultivated in 0 min of NI. One explanation for this may be that small flashes of light are sufficient to activate phytochromes [61] and then the synthesis of mRNA leads to the synthesis of rebudioside [4,13]. However, too much light can cause photoinhibition of photosynthesis and less carbon availability for the synthesis of other organic molecules [60,62,63].

Francisco, Pereira [14] described that in Brazilian *S. rebaudiana* plantations, the level of Reb-A varies between 0.46% and 12.23%, a variation that according to Giraldo, Marín [64] may be strongly linked to the genotype used in the production of SvGly, so that the search for genotypes with higher yield or genetic manipulation to increase the production of SvGly, in particular, Reb-A is a task well appreciated by the market and by industries production of sweeteners. Ceunen, Werbrouck [7] described that the highest concentration of St was 9.13% or 91.3 g kg^{-1}, which contradicts the data presented in this study, where St values were rarely below 100 g kg^{-1} DW, i.e., under the conditions described in this study, the St levels was 0.92- to 1.45-fold higher when compared to Ceunen, Werbrouck [7] or Ceunen and Geuns [12]. The level of St and Reb-A described by Kumar, Sood [50] are on average 80% and 20.5% of the values described in this study. However, the values of St and Reb-A described in this study are in agreement with the values obtained by Hernández, Combatt [60]. Other studies described a lesser level of St and Reb-A. In accord with values showed by Francisco, Pereira [14], Parris, Shock [65], and Pereira, Storck [66], the levels presented in this study to Reb-A were, respectively, 4.2- 6.4-, and 11-fold higher.

Francisco, Pereira [14] described productivity of up to 457 kg ha^{-1} of Reb-A in the first harvest, values that are 24% of those presented in this study, considering that in this study, the St and Reb-A were measured only in the last harvest, i.e., in 84-days *S. rebaudiana* plants. The lower yield of Reb-A described by Francisco, Pereira [14] is due to the Brazilian climate that allows between two and three leaf harvests per year, a fact that differs from the conditions of the Colombian Caribbean, where it is possible to make between three and five, sometimes six harvests of leaves per year [20].

When we compare our results of other countries, we show that in Ontario, Canada, the Reb-A productivity reaches 500 kg ha^{-1} [65]. Sun, Yang [67] describes that Chinese genotype of *S. rebaudiana* increase the productivity of St may reach 1% to 3% and Reb-A productivity reach to 8% to 10% under increased nitrogen source in the soil. These relative values represent 10 g to 30 g kg−1 of St and 80 to 100 g kg−1. The values presented in this study in the Caribbean region were 5.2-fold higher to St and 1.5-fold higher to Reb-A. In Morocco, with a plant density of 71,428 plants ha^{-1} (a density 36% lesser of this study), the mean production of St and Reb-A was 6.22% and 3.45%, respectively [68]. These data represent 62.20 of St and 34.5 g kg^{-1} of Reb-A, or 60%, and 74% less than described in this study. All these studies was carried out in other countries out of the Caribbean region; therefore, it is matchless than those values described in this study. Thus, when we compare the data presented in this study with other studies carried out in the Colombian Caribbean [20], it appears that the values presented here are in accordance with those previously published in this region of the world.

Finally, the most important feature in commercial terms is the production of St and Reb-A per hectare per year. So, in considering an average concentration of 155 g kg^{-1} of St and 132 of Reb-A (Table 2) in a plantation with a density of 111,000 plants per hectare, producing an average of 9000 kg of ha^{-1} y^{-1} of leaf [11,60], we will have a volume of

approximately 1400 kg ha^{-1} y^{-1} of St and 1190 ha^{-1} y^{-1} of Reb-A, a condition exceptionally found in Colombian plantations. In this sense, we can infer that Colombia has great chances of becoming one of the countries that produce most St and Reb-A, not necessarily in terms of cultivation by area, but in USD per area, a fact that is of great interest to world investors.

In summary, the supplemental light significantly delayed flowering and increased in a similar proportion to the St and Reb-A levels. This led us to speculate that the application of night interruption could promote greater biomass accumulation and greater leaf production available to the St and Reb-A extraction industry. Additionally, *S. rebaudiana* plants grown under the NI regime can be easily multiplied by micropropagation [69], providing fresh plant material with large amounts of SvGlys. Recently some scholars have applied LED modules to promote higher SvGly and delay in the generative development [4,7,12,70,71]. The effects of light on the SvGly accumulation and the delay in flowering presented in this study carried out with fluorescent lamps were like the results of the other studies; therefore, we can argue that fluorescent lamps can show effects similar to those demonstrated with the use of LED lamps. It should be noted that energy consumption is lower with the use of LED lamps compared to fluorescent lamps. Here, this study also described that the effect of NI on net photosynthesis, RGR, St, and Reb-A is photoperiod- and cultivar-dependent, as previously reported by Ceunen, Geuns [18].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7120543/s1, Supplementary Data File: Correlation matrix, showing all parwise between physiological and morphological features. The color code following the scale. Asterisks (*) denote.

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