VEGETATIVE PHASE EXTENSION FOR STEVIOL GLYCOSIDE ACCUMULATION IN STEVIA: PHOTOPERIOD, *IN VITRO* AND *EX VITRO* CULTURES MANIPULATION

Catherine C. Arradaza[1*], Ma. Lourdes O. Cedo[2], Evalour T. Aspuria[2], Constancio C. De Guzman[2], Lourdes B. Cardenas[2], Rocelie R. Zara[2], Rachel Ann Gonzaga[2]

[1]Department of Horticulture, College of Agriculture and Food Science, Visayas State University, Baybay City, Leyte 6521, Philippines

[2]University of the Philippines Los Baños, College, Laguna 4031, Philippines

*Corresponding author: catherine.arradaza@vsu.edu.ph

(Received: July 7, 2021; Accepted: September 9, 2022)

ABSTRACT

The growing consumers' concern over excessive sugar intake leading to obesity and diabetes has created a huge demand for an alternative, low-calorie non-synthetic sweetener. *Stevia rebaudiana* Bertoni is a good sugar alternative that produces sweet-tasting leaves owing to their steviol glycoside (SG) content. *In-vitro* and *ex-vitro* experiments were conducted to determine the effect of different photoperiods [24h/natural daylength (control), 11-h, 13-h and 15-h] on flowering and steviol glycoside (stevioside and rebaudioside A) accumulation in *Stevia rebaudiana* Bertoni. Under *in-vitro* conditions, 11-h photoperiod did not induce flowering of stevia except in a few cultures which exhibited flower bud formation (2%). *In vitro*-grown stevia had lower SGs (average of 0.88% stevioside and 0.28% rebaudioside-A) compared to the *ex vitro*-grown plants derived from tissue culture. Exposure to non-inductive 15-h photoperiod effectively inhibited flowering in tissue culture-derived stevia. Highest stevioside (6.62%) and rebaudioside-A (3.84%) content of *ex vitro*-grown stevia was obtained in the leaves under 15-h photoperiod.

Key words: tissue culture, stevia, photoperiod, steviol glycosides percentage, flowering

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni), a perennial herb of the Asteraceae family, is known to contain steviol glycosides (SGs), which are reported to be about 300 times sweeter than sucrose at their concentration of 4% (w/v) (Kinghorn and Soejarto 1985). SGs are largely used as a natural sweetener and some of the compounds present in stevia are known to be therapeutic, non-toxic, non-carcinogenic and non-mutagenic (Brusick 2008). The huge demand for an alternative sweetener such as stevia increased because of growing concerns over excessive sugar intake leading to obesity. Commercially, stevia is consumed either fresh or in processed form as a sweetener for tea, chocolate, jam, cookies, ice cream, juice, soft drinks and yoghurt (Ibrahim et al. 2008).

Stevia growth and their production of secondary metabolites are known to be influenced by external and internal factors. SG accumulation pattern in stevia leaves is specifically shown to vary with cultivar (Serfaty et al. 2013; Bondarev et al. 2003), phenological stage (Brandle and Rosa 1992), growth conditions like photoperiod (Ceunen and Geuns 2013), temperature and available nutrients (Pal et al. 2013). The leaves are the most economically important part of stevia since the sweet compounds SGs are predominantly found in this organ, but when flowering commences, leaf production stops. Therefore, it is crucial for stevia growers to extend the vegetative phase of the plant so that more leaves will be produced. The maximum production of SGs in the leaves occurred just before or during the formation of flower buds (Kang and Lee 1981). Young leaves contained more SGs than senescent leaves (Jain et al. 2014). Moreover, rebaudioside-A and stevioside contents were highest when 50% of the plants harvested was at the flower bud stage (Kumar et al. 2011). Stevia has been established to be a short-day plant whose flowering is induced at photoperiods shorter than 13-h (Metivier and Viana 1979; Valio and Rocha 1977), thus altering the photoperiod becomes a useful means of prolonging the vegetative growth of the plant. The exposure of plants to long-day conditions was proven to delay flowering thus resulting in the increase leaf biomass and steviol glycoside accumulation in stevia by as much as 50% (Metivier and Viana 1979; Ceunen and Geuns 2013). In the past, research on stevia dealt with the effects of photoperiod on steviol glycoside accumulation (Ceunen and Geuns 2013; Zaidan et al. 1980; Metivier and Viana 1979; Valio and Rocha 1977).

The daylength under Philippine conditions renders a constantly favorable conditions for the flowering of stevia since it does not exceed 13 h. In this study, cultural intervention such as prolonging the day length to delay flowering to increase SG production was investigated under local conditions. It is known that growing this plant under long-day (LD) condition prolongs vegetative growth. If stevia plants will be exposed to long-day condition, flowering

process can be delayed and its vegetative growth will continue, this could result to greater leaf mass and SG yield (Metivier and Viana 1979; Ceunen et al. 2013, Hossain et al. 2017). The supplementation of light was reported to easily modulate photoperiod by using additional electrical energy required to artificially extend the photoperiod to create a long-day environment (Ceunen et al. 2012, Yoneda et al. 2017). The study sought to determine the influence of different photoperiods on SG accumulation under *in vitro* and *ex vitro* conditions.

MATERIALS AND METHODS

The study was conducted at the Crop Physiology Division, Institute of Crop Science, College of Agriculture and Food Science, University of the Philippines Los Baños (UPLB), College, Laguna, Philippines from August 2015 to February 2016.

In vitro plants. Under *in vitro* conditions, *Stevia rebaudiana* Bertoni plantlets, from the Macapuno Laboratory, University of the Philippines Los Baños, were established initially in Murashige and Skoog's (MS) basal medium following the protocol of Zara et al. (2014). After one month of exposure to continuous light, these were grouped according to size and extent of shoot proliferation and then distributed to the different photoperiod treatments, namely 11-, 13-, 15- and 24-h light. The experiment was laid out in Complete Randomized Design (CRD) having three replications with 15 samples per replicate. The cultures were maintained in a room temperature at 25ºC for 5 months without subculturing. The number of days to first sighting of floral bud formation, flower opening, and percentage flowering were recorded. Data on the number, length, and weight (fresh and dry) of shoots as well as the number and length of roots were collected at the termination of the experiment.

Ex-vitro plants. For the *ex-vitro* experiment, stevia cultures maintained in 15-h photoperiod with well-developed roots were taken out of the culture room and were acclimatized by gradually exposing to ambient room conditions for five days; these were then transferred to the greenhouse gradually exposing to sunlight and removing the cover of the bottles. On the day of transplanting, plantlets were taken out of the culture bottles and the roots were washed thoroughly with tap water to remove traces of the agar medium. The plants were dipped for 2 seconds in 2g/L mancozeb fungicide (Dithane M-45) solution before planting in small polyethylene pots (4x4x7 in) containing garden soil, carbonized rice hull and coconut coir dust mixture (1:1:1 v/v/v). To prevent desiccation, the potted plants were covered with clear polyethylene bags and maintained under intermittent mist, thrice a day. The plastic cover was removed after three weeks and the plants were transferred to bigger polyethylene pots (10x10x17 in) containing the same soil medium. The experiment was laid out in Complete Randomized Design (CRD) having three replications with 10 samples per replicate.

Similar to the *in vitro* experiment, the plants were subjected to the different photoperiods: 11 hr-,13hr-, 15-hr light and natural daylength as control (equivalent to 12.6-h) (Dateandtime.Info.2108). To simulate the different photoperiods, the plants were placed under structures with opaque cover except for the control; during daytime, the cover was removed to allow the plants to be exposed to natural light. Artificial lighting provided by 40W Philips single fluorescent tubes per level of culture shelves was used. The plants were fertilized with 4.18 gram urea per 5 L water every month and watered when needed. Insect infestation (e.g leaf worms, aphids) was controlled manually through hand picking if needed.

The shoot length, number of leaves and biomass production of the plants grown *ex vitro* were determined after five months. The number of days for the appearance of the first flower bud and percentage flowering were also recorded.

Steviol glycoside (SG) analysis. Samples of the shoots, leaves and flowers from the *in vitro* and *ex vitro* experiments were collected, cleaned and air-dried under the shade for 24 h. The air-dried samples were dried further in an oven at 50 ºC for 16 h. The samples were pulverized immediately after drying using mortar and pestle and then stored in sealed polyethylene bags in the refrigerator at 4 ºC until further use. SGs in the powdered samples were extracted using ethyl alcohol (70%, v/v) (Kolb et al. 2001). The extracts were analysed for presence of stevioside and rebaudioside-A through HPLC according to the protocol established by the FAO (2010). Chromatographic conditions of the two columns (Capcell pak C18 MG II- Shiseido Co.Ltd. or Luna 5µ C18(2) 100A (Phenomenex) or equivalent (length: 250 mm; inner diameter: 4.6 mm, particle size: 5µm)} were used to test the most suitable for the analysis of SGs stevioside and reb-A present in the dried samples to be able to achieve a clear separation of the two SGs. Analyses were done at the National Institute of Molecular Biology and Biotechnology (BIOTECH), UPLB.

Statistical analysis. All data were analyzed by using the computer software Statistical Tool for Agricultural Research (STAR) 2013 software based on CRD design employing ANOVA then followed with a post hoc LSD test at 0.05 alpha level.

RESULTS AND DISCUSSION

Effects of photoperiod on *in vitro* grown plantlets. The effects on the flowering of stevia *in vitro* to the different photoperiod treatments became evident at two months exposure. The relatively few number of shoots developed under short photoperiods induced the elongation of these shoots as observed in plants exposed to 11-h photoperiod. The number and length of shoots were showed direct relationship, wherein under relatively short photoperiods (11-13hr), less number of shoots emerged but with higher shoot length. These responses were continuously observed for five months incubation (Table 1 and Fig 1). Continuous 24h photoperiod resulted in significantly higher fresh shoot weight biomass allocation (about 19g plant^{-1}). There were no significant differences for fresh weight of roots regardless of the photoperiod treatments, except for that the 13h of light caused the plant to have significantly low fresh root weight (below 0.4g plant^{-1}). All plants formed roots; the more profuse the shoots in a culture bottle, the fewer and shorter roots formation was observed.

Table 1. Effect of photoperiod on growth and flowering response of *in vitro* grown stevia after 5 mo of incubation in Murashige and Skoog's (MS) medium.

Parameter	Photoperiod (h)			
	11	**13**	**15**	**24**
Number of shoots	17 b	17 b	17 b	22 a
Shoot length (cm)	31.69 a	30.04 a	28.96 a	17.05 b
No. of roots	86 a	72 a	72 a	70 a
Root length (cm)	16.89 a	11.15 b	11.63 b	6.52 c
Fresh weight of shoot (g plant^{-1})	14.41 b	13.44 b	12.47 b	18.86 a
Fresh weight of roots (g plant^{-1})	0.45 a	0.35 b	0.48 a	0.44 a
Dry weight of shoot (g plant^{-1})	1.39 a	1.18 a	1.22 a	1.45 a
No. of days to flowering	79	-	-	-
Percentage flowering (%)	2	NF	NF	NF

* Means followed by the same letter per row are not significantly different at 5% level of significance based on LSD test
**NF-No Flower

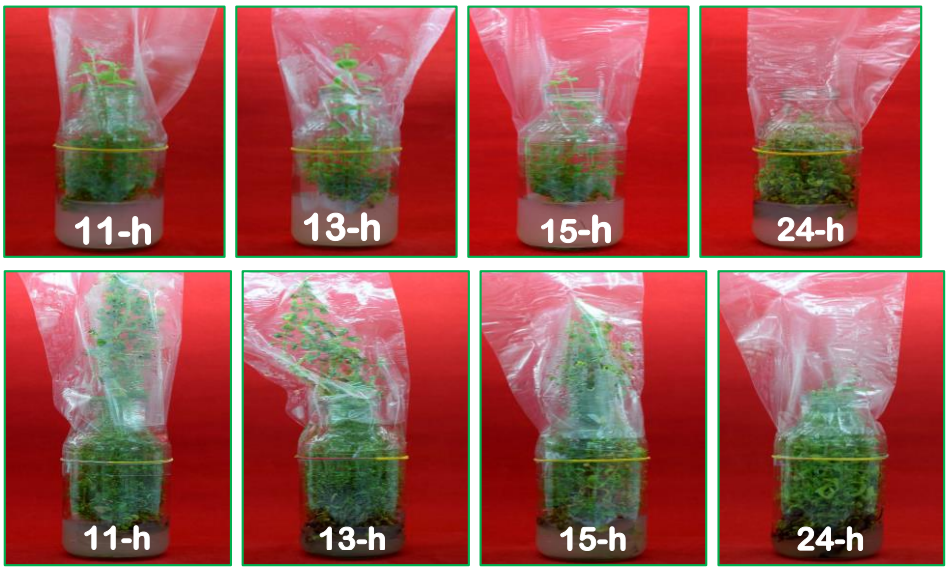

Fig. 1. Stevia shoots exposed to various photoperiods for 1 mo (top) and 4 mo (bottom) under *in vitro* conditions.

Only the shortest photoperiod of 11-h induced flower bud formation at 2% while the first sighting of floral bud formation was observed at 79 d following the photoperiod treatment (Fig. 2). The flowering shoots had noticeably shorter internodes and rosette arrangement of leaves than the non-flowering shoots. So far, no similar reported study on *in vitro* flowering of stevia by other researchers were found.

Fig. 2. Flower buds observed in stevia plants after 79 d of exposure to 11-h photoperiod under *in vitro* conditions. Photographed using camera macro lens (left) and microscope with 20x magnification (right).

Tissue culture-derived plants grown *ex vitro*. Tissue culture-derived plants were established in the greenhouse and exposed to the different photoperiod treatments. Tissue-cultured stevia exposed to the natural day length for 3 mo significantly attained the highest shoot length (44.68 cm) compared to the plants subjected to 11-h, 13-h and 15-h photoperiods (Fig. 3 and Table 2). In terms of biomass production, the fresh weight of stevia shoots grown under the natural daylength was statistically higher than those of the three photoperiod treatments; the lowest values for these parameters were recorded in plants maintained at 11-h photoperiod. All tissue-cultured stevia plants exposed to the different photoperiods flowered after 40-57 d except those kept at 15-h photoperiod where flowering was completely inhibited. This indicates that tissue cultured plantlets have the capability to develop into mature plants that can perceive both inductive and non-inductive photoperiodic condition. Flowering was earliest at the shortest photoperiod of 11-h.

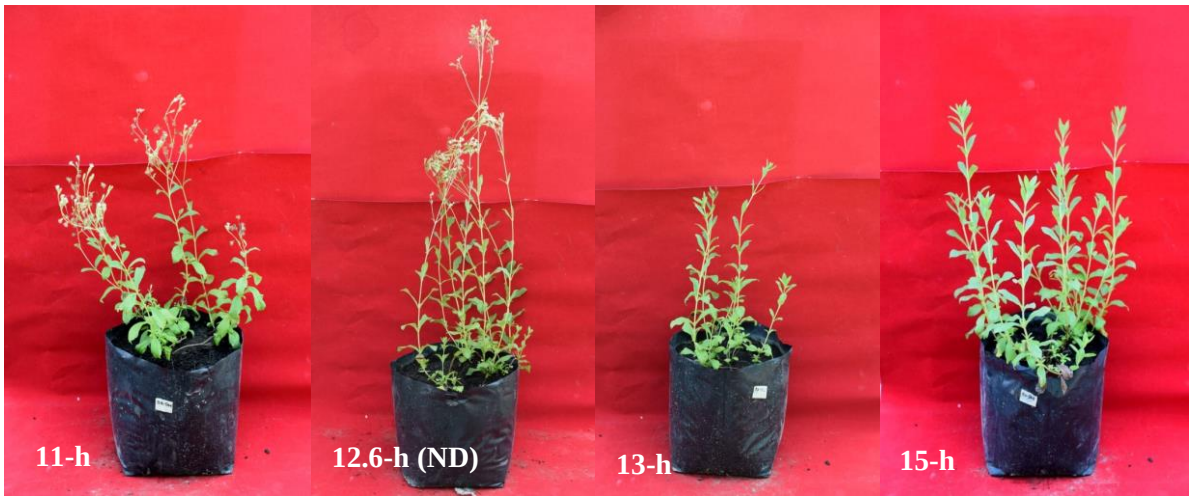

Fig. 3. Tissue culture-derived stevia plants exposed to different photoperiods for 3 months under greenhouse conditions. (*ND -natural daylength)

In matters of the number of flowers, the plants exposed to the shortest photoperiod of 11-h had the tendency to produce the most number of flowers, but statistically comparable to those maintained under the natural daylength (12.6-h), the least was at 13-h photoperiod (Table 2). The significantly low number of flowers obtained in 13-h photoperiod could be due to the few flowering shoots produced by the plants under this treatment. All three photoperiods, 13-h, 11-h and natural daylength (12.6-h) induced stevia plants to flower but not the 15-h photoperiod. Similarly, a photoperiod of 14 h inhibited floral initiation of stevia *(Piquerria trinervia*), with a 16 h photoperiod maintains the plants in a vegetative condition (Healy and Graper 1989). Under the local condition, the prevailing natural daylength is approximately 12.6-h, which is conducive for stevia flowering. Moreover, it was noted that the number of flowering shoot tips and percentage flowering per plant significantly decreased with increasing photoperiod up to 15-h. However, in the experiment, a tissue culture derived stevia was used.

Table 2 . Effect of photoperiod on growth and flowering response of tissue culture-derived stevia after 3 months of culture in pots under greenhouse conditions.

Parameter	Photoperiod (h)			
	Natural daylength (12.6)	11	13	15
Plant height (cm)	44.68 a	38.09 b	37.53 b	40.13 b
Number of leaves	178 b	143 bc	127 c	227 a
Fresh weight of shoot (g plant^{-1})	9.91 b	8.76 b	7.84 b	14.54 a
Fresh weight of leaves (g plant^{-1})	4.21 b	4.13 b	4.37 b	7.23 a
Dry weight of shoot (g plant^{-1})	2.49 b	2.10 bc	1.81 c	3.50 a
Dry weight of leaves (g plant^{-1})	1.23 b	1.15 bc	0.98 c	1.97 a
No. of days to flowering	54 b	40c	57 a	NF
Percentage flowering	97	100	100	NF
No. of flowers	53 a	59 a	24 b	NF
Fresh weight of flowers (mg plant^{-1})	610 a	490 b	300 c	NF
Dry weight of flowers (mg plant^{-1})	190 a	200 a	110 b	NF

* Means followed by the same letter per row are not significantly different at 5% level of significance based on LSD test
** NF - no flower

Steviol glycoside accumulation. Under local screenhouse conditions, the daylength conditions throughout the year is naturally inductive for the flowering of stevia and this limits leaf yield, which is responsible for the bulk of steviol glycosides (stevioside and rebaudioside-A) that may be harvested from the plant. The SG content of stevia shoots exposed to the different photoperiods after 5 months of incubation in MS medium was below 1% and comparable among treated plants (Table 3). Stevioside content ranged from 0.83% - 0.90% while rebaudioside A amounted to only 0.02% -0.42% in shoots exposed to 24h ,13-h, and 15-h photoperiod, respectively. Rebaudioside-A was not detected in the shoots maintained at 11-h. The result indicated that *in vitro* grown stevia had comparable level of stevioside and rebaudioside-A regardless of the length of photoperiod used in the experiment.

Table 3. Effect of photoperiod on steviol glycosides content (percent) of stevia shoots after 5 mo of incubation in Murashige and Skoog's (MS) medium.

Steviol glycoside	Photoperiod (h)			
	11	13	15	24
Stevioside	0.89 ± 0.014	0.90 ± 0.010	0.89 ± 0.010	0.83 ± 0.009
Rebaudioside A	ND	0.39 ± 0.026	0.42 ± 0.016	0.02 ± 0.003

* ± SD
** ND – none detected

When *in vitro* plantlets were potted out and allowed to develop under greenhouse conditions (Table 4), higher SG content of the leaves was obtained compared to the plantlets that were continuously maintained under *in vitro* conditions (Table 3). Furthermore, in this experiment, it was found that photoperiod influenced SG accumulation in stevia leaves and flowers. The highest leaf stevioside (6.62%) and rebaudioside-A (3.84%) contents were obtained from plants exposed to 15-h photoperiod while the lowest values (4.85% stevioside and 2.97% rebaudioside A) were recorded at 11-h photoperiod. The result indicated that exposure of tissue culture-derived stevia plants to 15-h photoperiod successfully inhibited flowering and increased level of the SGs. Ceunen and Geuns (2013) reported that long-day photoperiod (≥14h) prolonged the vegetative growth in *S. rebaudiana* resulting in a greater amount of leaf mass and total SG accumulation. Moreover, Rajasekaran et al (2006) also recorded a highest stevioside contents in 1-month-old greenhouse leaves (64.80g steviolbioside kg^{-1} dried plant material) and *in vitro* leaves (0.99g

rebaudioside-A kg^{-1} dried plant material). As expected, the SG content of plants exposed to natural daylength (12.6h) and 13-h were statistically not significant.

Table 4. Steviol glycosides content of tissue-cultured stevia exposed to different photoperiods after 3 mo of culture under greenhouse conditions.

Steviol Glycoside Content (%)	Photoperiod (h)			
	Natural Daylength (12.6)	11	13	15
Leaves				
Stevioside	5.98 ab	4.85 c	5.12 bc	6.62 a
Rebaudioside A	3.19 b	2.97 b	3.08 b	3.84 a
Flowers				
Stevioside	3.24 b	2.57 b	5.64 a	NF
Rebaudioside A	1.40 ab	0.97 b	2.10 a	NF

*Means followed by the same letter per row are not significantly different at 5% level of significance based on LSD test
** NF – no flowers

None of the plants kept under 15-h photoperiod flowered until the 5th month. These results provide additional evidence that indeed stevia is a short-day plant with a critical daylength of 12-13h (Silva de Andrade et al. 2021). Stevia is indeed an obligate short-day plant, as suggested earlier by Mohede and van Son (1999) since the plants kept under 15-h, *in vitro* and *ex vitro*, remained vegetative up to five months.

CONCLUSION AND RECOMMENDATION

Overall, flowering of stevia under *in vitro* condition was not induced but if grown under *ex vitro* conditions, tissue culture-derived plantlets flowered under short day photoperiods at 11-h, 13-h and natural daylength (12.6-h). However, if plants are subjected to longer photoperiod (15-h), flowering was successfully inhibited and increased the number of leaves thereby increasing their SGs content. The best photoperiod period condition with the highest stevioside (6.62%) and rebaudioside-A (3.84%) content for *ex vitro*-grown stevia from tissue culture was in the stevia leaves grown under 15-h photoperiod.

Relevant studies assessing the presence of other SGs aside from stevioside and rebaudioside-A as well as antioxidants in both *in vitro* and *ex vitro* grown stevia may be considered. Moreover, night-interruption may be applied to the treatments used in the experiment to determine its effect on flowering and leaf biomass.

ACKNOWLEDGMENT

Funding from the Department of Science and Technology – Science Education Institute Accelerated Science and Technology Human Resource Development Program (DOST-SEI ASTHRDP) and Department of Agriculture-Bureau of Agricultural Research (DA-BAR) is greatly acknowledged.

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