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Original Article

Photoperiod Influenced Flowering and Steviol Glycoside Accumulation of Stevia Under *In Vitro* And *Ex Vitro* Condition

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ABSTRACT: The purposive manipulation of photoperiod condition that could delay or inhibit flowering in stevia (*Stevia rebaudiana* Bertoni) both under in vitro and *ex vitro* conditions was investigated. Under *in vitro* condition, stevia did not flower under either short-day or long-day condition; instead, leaf production was promoted. In contrast, *ex vitro* grown plants, whether potted or directly planted to the field, flowered under photoperiods shorter than the established critical daylength of 13 h for stevia and were inhibited from flowering at longer photoperiod of 15 h. Flowering was not prevented in stevia obtained from plants that have previously flowered even when exposed to long photoperiods. The steviol glycoside (SG) accumulation was lower in in vitro grown plants than those of plants maintained under *ex vitro* conditions; the stevioside content ranged from 0.83%−0.90%, while rebaudioside A amounted to only 0.26%−0.42%. Under *ex vitro* conditions, the average leaf stevioside content of tissue culture-derived stevia was 5.64%, while that of the flowers was 3.82%; rebaudioside A was 0.96% and 0.48% in these organs, respectively. The purposeful manipulation of photoperiod may be useful to stevia growing and SG accumulation both under in vitro and ex vitro production systems.

KEY WORDS: flowering, photoperiod, *Stevia rebaudiana* Bertoni, steviol glycoside

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni), a perennial herb of the Asteraceae family, has gained scientific interest worldwide because of the intense sweet taste of its leaves. The plant 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 plant also shows blood pressure lowering properties and has low glycemic index. Due to growing consumers concern over excessive sugar intake leading to obesity, a huge demand for an alternative sweetener such as stevia increased. Nowadays, 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).

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The growth of stevia plants 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) and growth conditions like photoperiod (Ceunen and Geuns, 2013), temperature and available nutrients (Pal *et al*., 2013).

In stevia, the leaves are the most economically important part since the sweet compounds steviol glycosides are predominantly found in this organ. Once the plant produces the terminal inflorescence, leaf production stops. It is therefore important for stevia growers to extend the vegetative phase of the plant so that more leaves will be produced, which will synthesize the SGs. The maximum production of steviol glycosides (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 was 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 increased 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).

Under Philippine conditions, daylength does not exceed 13 h, rendering a constantly favorable condition for the flowering of stevia. However, information on photoperiod affecting stevia production under local condition is limited. In this study, manipulation of daylength as cultural intervention to delay flowering and consequently increase steviol glycoside production was investigated. Experiments were conducted to determine whether the photoperiods that induced flowering in greenhouse-grown plants would bring about the same response in in vitro plants. The influence of different photoperiods on SG accumulation under *in vitro* and *ex vitro* conditions was also determined.

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 2017.

Plant Material

***In vitro* plants.** Under *in vitro* condition, stevia plantlets 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, they 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. A time switch was installed to provide the required photoperiod. Room temperature was maintained at 25 ºC for 5 mo. 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 with well-developed roots were taken out of the culture room and were acclimatized for 5 d at ordinary room conditions; they were then transferred to the greenhouse for the additional 2-day acclimatization. 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 then dipped in fungicide solution before planting them in small pots containing garden soil, burnt 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 mist. After 3 weeks, the plastic cover was removed and the plants were transferred to big pots containing the same soil medium.

Stem cuttings taken from plants that have previously flowered were also used as test material for the ex vitro experiment. The plants were ratooned initially to allow new shoots to develop. Single shoots about 10 cm long with four nodes were excised and planted in small polyethylene bags containing a mixture of garden soil, coconut coir dust and burnt rice hull (1:1:1 v/v/v). The potted plants were maintained under mist for 4 weeks to allow further development of the roots before they were transferred to big pots filled with the same potting mix.

Similar to the *in vitro* experiment, the plants were subjected to the different photoperiods: 11-, 13-, 15-h light and natural daylength as control (equivalent to 12.6-h http://dateandtime.info/index.php). To simulate the different photoperiods, the plants, except the control, were placed under structures with opaque cover; during daytime, the cover was removed to allow the plants to be exposed to natural light. Artificial lighting provided by 40W Philips fluorescent tubes and a time switch were installed in each structure to satisfy the required daylength.

The plants were fertilized with 1 tbsp (14.3 grams) urea per 5 l water, with 350 ml of the fertilizer mixture applied for each plant, every month and watered when needed. Insect (*e.g.* leaf worms, aphids) infestation was controlled manually. The shoot length, number of leaves and biomass production of the plants grown *ex vitro* were determined at the termination of the experiment. The number of days to first flower bud appearance and percentage flowering of stevia were also recorded.

Steviol Glycoside (SG) Analysis

For the steviol glycoside analysis, samples of the shoots, leaves and flowers from the *in vitro* and *ex vitro* experiments were collected, cleaned and air-dried under shade for 24 h. The air-dried samples were dried further in an oven at 50 ºC for 16 h. Immediately after drying, the samples were pulverized using mortar and pestle and then stored in sealed polyethylene bags in the refrigerator with temperature of 0–4 ºC until use.

SGs in the powdered samples were extracted with 70% (v/v) ethyl alcohol as the solvent, following the procedure of Kolb *et al*. (2001). The extracts were then analyzed through High-performance liquid chromatography (HPLC) according to the protocol established by the FAO (2010).

Analyses were done at the National Institute of Molecular Biology and Biotechnology (BIOTECH), UPLB and the Philippine

Institute of Pure and Applied Chemistry (PIPAC), Ateneo de Manila University, Loyola Heights, Quezon City.

Statistical Analysis

All data were subjected to Analysis of Variance (ANOVA) for completely randomized design using the computer software Statistical Tool for Agricultural Research (STAR) 2013. The Least Significant Difference (LSD) test at $P<0.05$ level was applied for comparison of treatment means.

RESULTS

***In Vitro* Stevia Plants**

Stevia plants showed varying responses to the different photoperiods, in terms of growth, flowering and steviol glycoside accumulation.

The effect of photoperiod on flowering of stevia *in vitro* became evident two months after exposing the plants to the different photoperiod treatments. The relatively few number of shoots developed under short photoperiods induced the elongation of these shoots as observed in plants exposed to 11-h photoperiods. In general, plants exposed to 24-h light periods produced the most number of shoots; although, they were shorter than those in the other treatments (Table 1 and Fig. 1). Consequently, the plants in this treatment had the highest shoot fresh weight; however, the dry weight of shoots from the different photoperiods was comparable. All plants formed roots; the more profuse the shoots in a culture bottle, the fewer and shorter were the roots formed.

Table 1. Growth and flowering respone of *in vitro* grown stevia to different photoperiods after 5 mo of incubation in Murashige and Skoog's (MS) medium.

Parameter	Photoperiod (h)			
	11	**13**	**15**	**24**
No. 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	86.00	72.00	72.00	70.00
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})a	0.45 a	0.35 b	0.48 a	0.44 a
Dry weight of shoot (g plant^{-1})	1.39	1.18	1.22	1.45
No. of days to flowering	79	-	-	-
Percentage flowering (%)	2	0	0	0

*Numerica values were taken from average values. Values with similar letters has no significant difference with each other

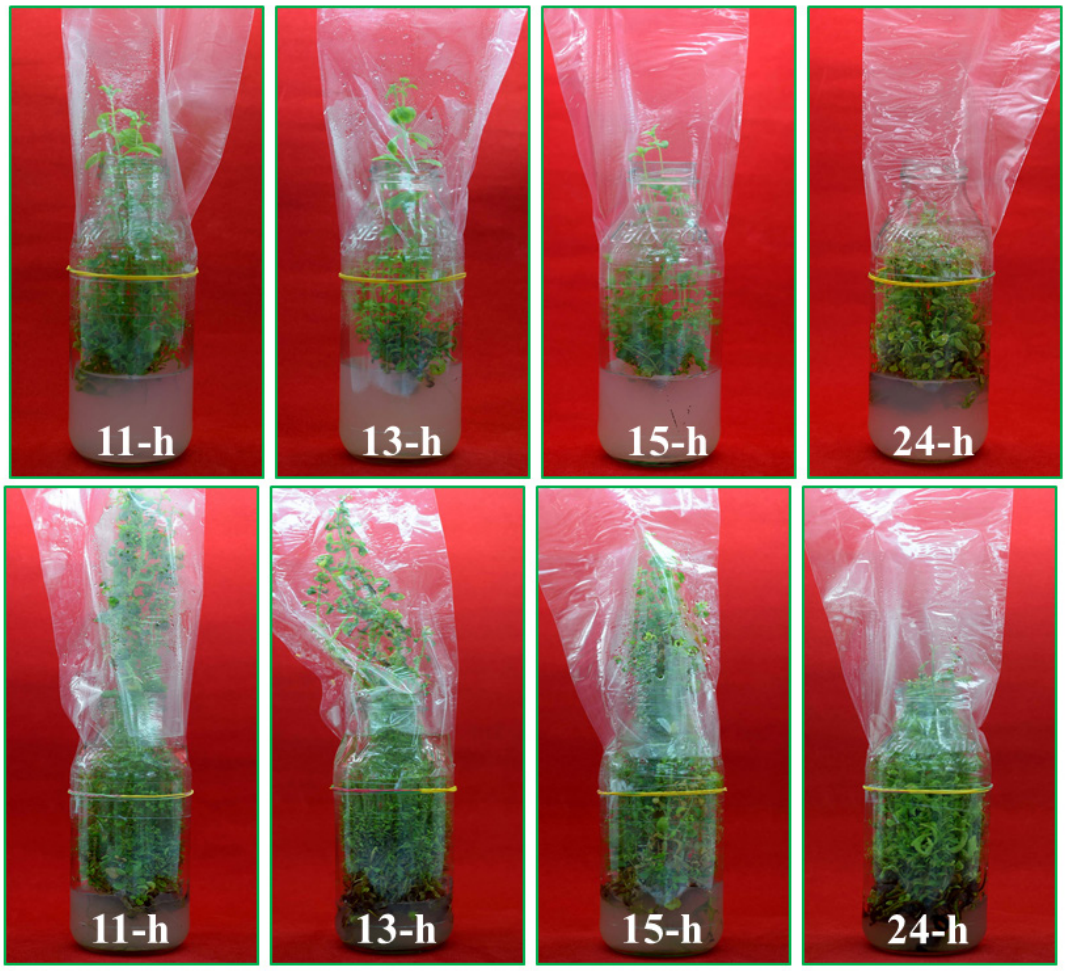

Figure 1. Stevia shoots exposed to 11-h, 13-h, 15-h and 24-h photoperiods for 1 mo (top) and 4 mo (bottom) under in vitro condition.

On the photoperiod effect on flowering, only the shortest photoperiod of 11-h induced flower bud formation at a very low percentage of occurrence at 2%; the first sighting of floral bud was recorded after 79 days of exposure to the inductive photoperiod (Fig. 2). The flowering shoots had noticeably shorter internodes and rosette arrangement of leaves than the non-flowering shoots.

Tissue Culture-Derived Plants Grown *Ex Vitro*

Parallel lots of tissue culture-derived plants and plants propagated from stem cuttings were established in the greenhouse and exposed to the different photoperiod treatments. Tissue-cultured stevia exposed to the natural daylength for 3 mo attained the highest shoot length (44.68 cm) compared to the plants subjected to 11-h, 13-h and 15-h photoperiods; the same plants also produced the most number of secondary branches (Table 2a and Fig. 3a). In terms of biomass production, the fresh weight of the roots and shoots of stevia grown under the natural daylength was statistically higher than those of the three photoperiod treatments. The lowest

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

Table 2a. Growth and flowering respone of tissue culture-derived stevia to different photoperiods after 3 mo of culture in pots under greenhouse condition.

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
No. 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	40	57	-
Percentage flowering	97	100	100	0
No. of flowers	53 a	59 a	24 b	0 c
Fresh weight of flowers (mg plant^{-1})	610 a	490 b	300 c	0 b
Dry weight of flowers (mg plant^{-1})	190 a	200 a	110 b	0 c

values for these parameters were recorded in plants maintained at 11-h photoperiods. All tissue-cultured stevia plants exposed to the different photoperiods flowered after 40−57 d except those kept at 15-h photoperiods, where flowering was completely inhibited. In contrast, flowering was earliest at the shortest photoperiod of 11 h.

In terms of the number of flowers, the plants exposed to the shortest photoperiod of 11-h produced the most number of flowers, but this was comparable with those maintained under the natural daylength (12.6-h); the least was at 13-h photoperiods. The significantly low number of flowers obtained in 13-h photoperiods could be due to the few flowering shoots produced by the plants under this treatment.

Stevia Plants Propagated from Stem Cuttings

The terminal shoots of the plants derived from stem cuttings were decapitated before exposure to the different photoperiod treatments to ensure that no pre-formed floral buds were present. As a result of the decapitation, apical dominance was removed, thus releasing the axillary shoots at the nodes of the main stem and of the branches, and subsequently producing more leaves. The main stem of the plants grown from stem cuttings was slightly woody, indicating that the plants were in a more advanced stage of maturation.

The exposure of stevia grown from stem cuttings to 11-h photoperiods also produced the smallest plants as reflected in the plant's height and biomass production, differing significantly from the other photoperiod treatments (Table 2b and Fig. 3b). This observation may be explained by the fact that the 11-h photoperiods induced these plants to flower early, thereby inhibiting further

Figure 3a. Tissue culture-derived stevia plants exposed to 11-h, 13-h and 15-h photoperiods and natural daylength for 3 mo under greenhouse conditions.

Table 2b. Growth and flowering respone of stevia derived from stem cuttings to different photoperiods after 3 mo of culture in pots under greenhouse condition.

Parameter	Photoperiod (h)			
	Natural daylength-12.6	11	13	15
Plant height (cm)	48.59 a	39.38 b	47.08 a	45.08 a
Fresh weight of shoot (g plant-1)	38.42 a	21.78 b	38.23 a	35.29 a
Dry weight of shoot (g plant-1)	6.80 a	2.87 b	6.68 a	5.69 a
No. of days to floral bud formation	34	29	32	34
Percentage flowering	100	100	100	58
No. of flowers	153 b	426 a	172 b	17 c
Fresh weight of flowers (mg plant-1)	1640 b	4990 a	2090 b	210 c
Dry weight of flowers (mg plant-1)	350 b	900 a	370 b	30 c

*Numerical values were taken from average. Values with similar letters has no significant difference with each other.

growth in height and inducing the shoot tips to produce flowers. The plants exposed to natural daylength and 13-h and 15-h photoperiods, on the other hand, were comparable in growth response. Unlike the tissue culture-derived stevia, more than 50% of the plants obtained from stem cuttings flowered when exposed to 15-h photoperiods. The earliest to flower and producing the most number of flowers were those exposed to 11-h photoperiods; the number of days to floral bud formation and flower opening were recorded to be 29 d and 35 d, respectively in these plants.

The number of days to floral bud formation did not differ among photoperiod treatments. It appeared that the plants derived from stem cuttings were already capable of flowering even if kept under non-inductive photoperiods. The stem cuttings used in this experiment were taken from mature mother plants, which had flowered earlier; possibly, the entire shoot of the plants were already in the induced state. These results seem to indicate that once flowering is induced in stevia, the whole plant becomes committed to flower and that excision of a shoot part from the mother plant does not cause the plant part to revert back to the juvenile or non-committed state.

DISCUSSION

The results of the present experiment confirmed that long photoperiods favor vegetative growth and inhibit flowering in stevia plants. Growing stevia in vitro may be more advantageous in terms of prolonging the vegetative growth, compared to growing them in the greenhouse where flowering was easily induced even with the removal of flower buds, which was done in stem cuttings. Under local conditions, the plants were continuously exposed to natural daylength of <13-h, which was inductive for stevia flowering.

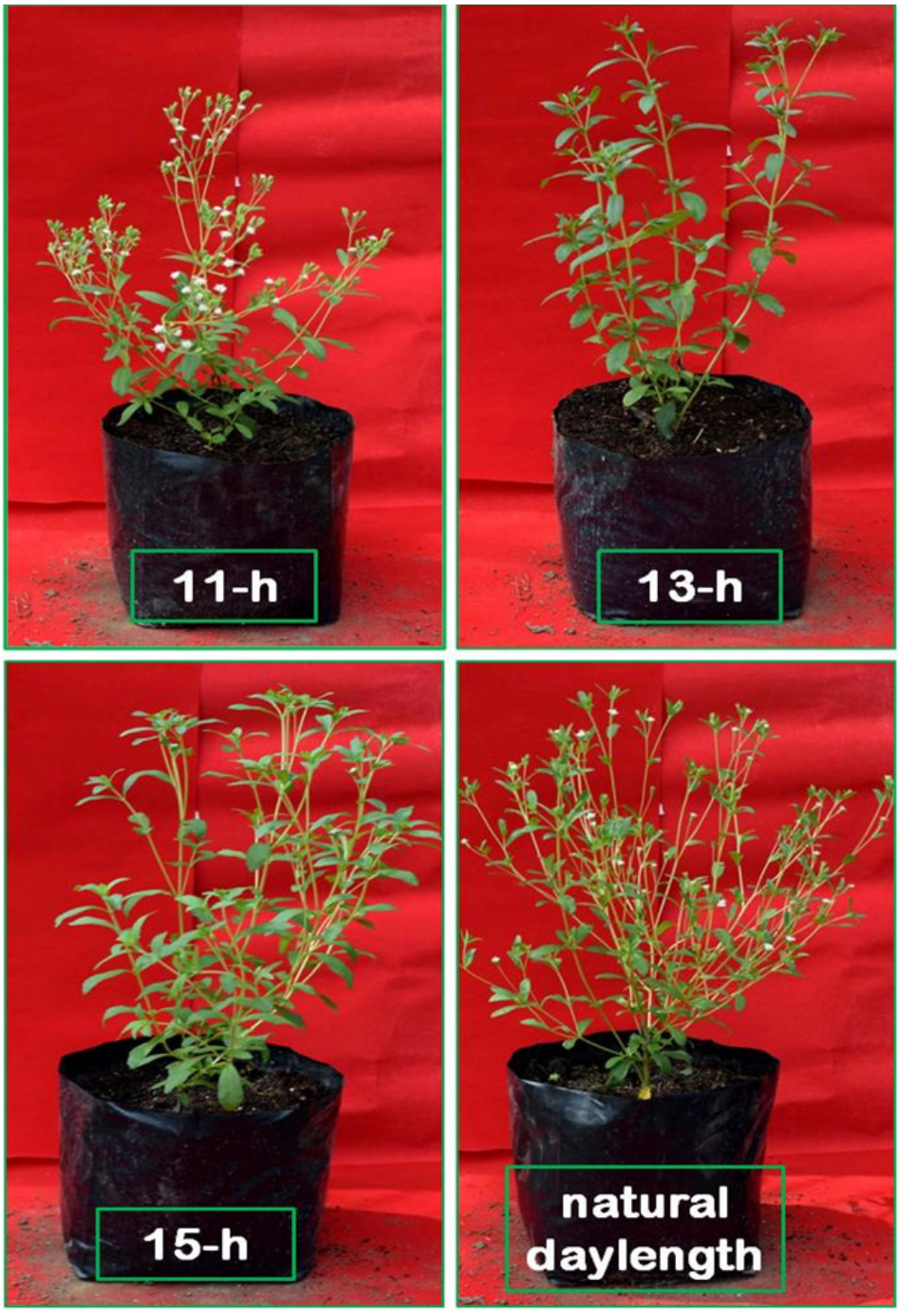

Figure 3b. Stevia plants derived from stem cuttings exposed to 11-h, 13-h and 15-h photoperiods and natural daylength for 3 months under greenhouse conditions.

The exposure to 15-h photoperiod effectively inhibited flowering in tissue culture-derived stevia under greenhouse condition. This response was parallel to those of the *in vitro*-grown plants subjected to 15 h, which remained vegetative throughout the experiment. The result was also consistent with the previous studies on stevia where flowering was not observed under long-day conditions (Metevier and Viana, 1979; Ceunen and Geuns, 2013).

All three photoperiods, i.e. 13-h, 11-h and natural daylength (12.6-h), induced stevia plants to flower, but not the 15-h photoperiods. These results provide additional evidence that stevia is indeed a short-day plant with a critical daylength of > 13-h. It may also be said that stevia is really 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 the termination of the experiment.

Steviol Glycoside Accumulation as Affected by Photoperiod

The daylength condition in the country throughout the year is naturally inductive for the flowering of stevia and this limits leaf yield, which in turn accounts 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 0.26% and 0.42% in shoots exposed to 13-h and 15-h photoperiods, respectively. Rebaudioside A was not detected in the shoots maintained at 11-h and 24-h photoperiods.

When in vitro plantlets were potted out and allowed to develop under greenhouse conditions, higher SG content of the shoots was obtained compared to the plantlets that were continuously maintained under in vitro condition (Table 4). Moreover, 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 photoperiods, while the lowest values (4.85% stevioside and 2.97% rebaudioside A) were recorded at 11-h photoperiods. As expected, the SG content of plants exposed to natural daylength (12.6h) and 13-h were almost the same.

Incidentally, none of the plants kept under 15-h photoperiods flowered until the termination of experiment.

CONCLUSION

The present study investigated the manipulation of the factors that could delay or inhibit flowering in stevia, like photoperiod. The exposure of stevia to long photoperiods induced shoot production both under in vitro and ex vitro conditions. *In vitro* grown stevia did not flower under either short-day or long-day conditions; the 2% flowering observed under 11-h photoperiods was considered insignificant. Thus, *in vitro* production of stevia under any photoperiod eliminates flowering and allows continuous leaf production.

The *ex vitro* grown stevia plants, potted or directly planted to the field, exhibited the typical response of a short-day plant to different photoperiods, i.e. they flowered under photoperiods shorter than the established critical daylength of 13-h for stevia and were inhibited from flowering by photoperiods longer than 13-h. The present study shows that tissue-cultured plantlets

Table 3. Steviol glycosides content of stevia shoots exposed to different photoperiods after 5 mo of incubation in Murashige and Skoog's (MS) medium.

Steviol Glycoside Content (%)	Photoperiod (h)			
	11	13	15	24
Stevioside	0.89	0.90	0.89	0.83
Rebaudioside A	ND	0.26	0.42	ND

ND-none detected

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

Steviol Glycoside Content (%)	Photoperiod (h)			
	11	13	15	24
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

NF-No flowers

*Numerical values were taken from average values. Values with similar letters has no significant difference with each other.

have the capability to develop into mature plants that can perceive both inductive and non-inductive photoperiodic conditions. When stem cuttings taken from mother plants that have previously flowered were used, long photoperiods did not prevent flowering, but reduced flower production.

The steviol glycoside accumulation was lower in in vitro grown plants than those of plants maintained under ex vitro conditions. SG accumulation of tissue culture-derived plants was also higher under long days than under short days.

Growing of stevia and steviol glycoside production under Philippine conditions may benefit from purposeful manipulation of photoperiods both under in vitro and ex vitro production systems.

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