アマハステビアの開花と体内ステビオサイド含量に対する日長の影響

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Effect of Photoperiod on Flowering and Stevioside Content in Plants of *Stevia rebaudiana* Bertoni

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Stevia rebaudiana Bertoni is an endemic herb from Paraguay and the Brazilian border with that country[3]. Its leaves are sweet tasting and are used by local people as a sweetner[4]. The substance responsible is known as stevioside and BRIDEL and LAVIEILLE[1,2] and METIVIER and VIANA[8] obtained 60 to 65 g of stevioside per kilogram dry weight of leaves. During recent years stevioside has been considered to be a possible substitute for synthetic sweetners and a great amount of research has appeared concerning the plant and stevioside[3].

Using plants, introduced from Paraguay in the 60's to the Instituto de Botânica de São Paulo. VÁLIO and ROCHA[12] determined the effect of photoperiod on flowering in *Stevia rebaudiana*. They showed that it was a short-day plant with a critical daylength between 13 and 14 h. The plant flowered in the 8, 10, 12 and 13 h photoperiods, though the highest percentage of flowering occurred in the 13 h photoperiod.

Recently, new plants from Paraguay were introduced to the Instituto de Botânica de São Paulo. This paper reports a study comparing the flowering behaviour of these plants regarding photoperiod and their leaf stevioside content with the original plants studied by VÁLIO and ROCHA[12]. The stevioside content of different plant organs that have been cultivated in the Instituto de Botânica de São Paulo for several years are also reported.

Materials and Methods

Plants of *Stevia rebaudiana* Bert., introduced from Paraguay in the early 60's to the Instituto de Botânica de São Paulo were used in this paper; these plants will be referred to as IBt plants.

Recently, fresh plants of *Stevia rebaudiana* were introduced from Paraguay. Examination of these plants revealed certain subjective morphological differences such as the shape and size of leaves, as well as stem and flower colour. According to their distinctive morphological characteristics these plants were classified into 14 lots, from which material was collected and included in the Herbarium of Instituto de Botânica. A taxonomic study of these 14 lots was done recently by MONTEIRO[10]. The numeration of these 14 plants is given in Table 1; these plants will be referred to by their respective numbers 1 to 14.

Daylength All treatments were subjected daily to 8 h of natural light in a greenhouse. Photoperiods longer than 8 h (10, 12, 14 and 16 h) were obtained by complementary incandescent light (240 lx) in darkrooms. This has previously been shown to be effective in supplementing photoperiod[12]. The plants used were invariably obtained from the germination of achenes from the plants referred to in Table 1. Six or eight plants cultivated in pots were used in these experiments. The plants were examined weekly from planting.

Stevioside To determine stevioside content, plant material was extracted in methanol and crystallized according to KOHDA et al.[5]. In all experiments the procedure extraction was carried out under the same conditions, so that results were comparable. Stevioside crystals thus obtained were weighted and a small amount was redissolved in methanol, and applied to Whatman number 1 chromatography paper together with pure

stevioside standard. Descendent chromatography was used, solvent system being *n*-butanol: piridine: water (6:4:3). The chromatograms were stained with silver nitrate reagent[11]. The Rf for stevioside in this system was 0.63. The crystalls obtained gave one spot with this same Rf.

In some experiments IBt plants grown in natural conditions and thus flowering were used. In one experiment mature leaves, the whole stem, flowers and the root system were used.

VIANA and METIVIER[13] have shown that the extraction of stevioside was more efficient with dry material. Thus, in the present work dry material was always used.

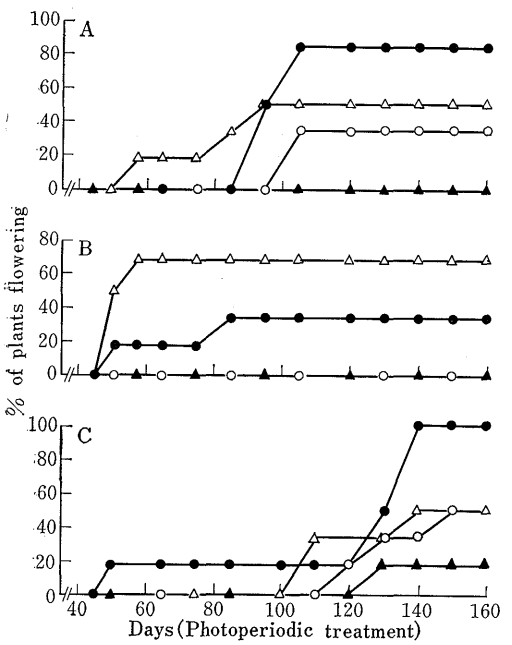

Fig. 1. Effect of photoperiod upon plants of *Stevia rebaudiana*.
1A: plant number **5**.
1B: plant number **6**.
1C: plant number **7**.
Symbols used: photoperiods 8 h ○; 10 h △; 12 h ●; 14 h ▲

Some of the new plants introduced from Paraguay (see Table 1) were grown in short days (SD, 8 h photoperiod) together with IBt plants and stevioside content was assayed in the leaves. The results are given in percentage of stevioside from the new plants in relation to IBt plants (for comparison facilities). Three of the plant lots from Paraguay were also grown in long days (LD, 16 h photoperiod) and their leaves extracted, and the amount extractable in LD was compared to that of the same plants in SD. The results are expressed as percentage.

Results

1. *Effect of photoperiod on flowering*

The results of the effect of different photoperiods upon the 14 different plant lots of *Stevia rebaudiana* are shown in Fig. 1.

The 14 plant types can be grouped into three lots according to their response to photoperiod. These three groups were termed A, B and C and one plant representative of each group is shown in Fig. 1A, 1B and 1C. Plant number **5** represents the A group: this plant flowers in the 8, 10 and 12 h photoperiods. It did not flower with a 14 h daylength, and the highest percentage of plants flowering occurred in the 12 h photoperiod. The critical daylength was between 12 and 14 h. In addition to plant **5**, the following plant lots also belong to this group: numbers 1, 2, 3 and 4.

Only plant number **6** was included in group B. It can be seen from Fig. 1B that this plant type only flowered in two photoperiods: 10 and 12 h, i.e., the intermediate photoperiods. No flowering occurred in 8 or 14 h daylengths.

Fig. 1C showed the results for plant **7**. This type flowered in 8, 10 and 12 h photoperiods, but also flowered in 14 h photoperiod, thus the critical daylength was longer than that of group A. The highest percentage of plants flowering occurred in the 12 h photoperiod. The following plants, besides 7, belong to this group: lots 8, 9, 10, 11, 12, 13 and 14.

Thus, according to their response to photoperiod the distinguishable plant lots of *S. rebaudiana* studied were physiologically different and could be divided into three distinct groups (Fig. 1 and Table 1).

2. *Stevioside content*

(1) *IBt plants* In the first experiment it was determined whether there was any difference in the amount of stevioside in leaves on flowering or vegetative plants

(more precisely plants already induced to flower, but not yet in flower) growing under natural conditions. Table 2 shows the percentage of stevioside per dry weight in the two types of plant. No noticeable differences existed between the two lots. Thus, the appearance of developed flowers does not decrease the amount of stevioside in the leaves.

Different parts of flowering plants, grown under natural conditions, were examined for stevioside, and the results are given in Table 3. Stevioside could be extracted and crystallized from the leaves and the inflorescences but not from the stems and roots. Stevioside was more abundant in the leaves than in the inflorescences of *S. rebaudiana*. The non detection of crystals of stevioside in the extracts of stem and roots does not necessarily indicate that stevioside is absent in these organs, but that the quantity was too low for crystallization in our experimental conditions. Thus 50 μl of either stem extract (0.160 g dry weight $\cdot$ ml^{-1}, final concentration) or root extract (0.195 g dry weight $\cdot$ ml^{-1}, final concentration) were analysed by paper chromatography. Stevioside was clearly present in the stem extract, but none could be detected in the root extract. Thus, it may be concluded that stevioside is present in leaves, inflorescences and stems of *S. rebaudiana*, but not in roots.

Leaves of IBt plants grown in short day conditions (8 h) in a greenhouse and plants growing in natural conditions (but short days) were extracted for stevioside; it was found that the content of this compound in leaves of plants grown under natural conditions was 375 percent higher than in the leaves of plants grown in SD conditions in a greenhouse.

(2) *New plants from Paraguay* Nine of the plant lots from Paraguay were grown together with IBt plants in short day conditions (8 h photoperiod), and the leaves extracted for stevioside. Lots 2 and 5, belonged to group A (according to flowering response), lot number 6 belongs to group B (not induced for flowering with a 8 h photoperiod), and plants 7, 8, 9, 11, 13 and 14 belonged to group C. The amount of stevioside found in IBt plants was considered as 100 percent; the results can be seen in Table 4. The plants in group A had a stevioside content similar to IBt plants. The only plant in group B, number 6, had lower content of stevioside than IBt plants. In group C, plant 7 had approximately half the content of stevioside in relation to IBt plants. Plants 9, 11 and 13 had similar amounts to IBt plants. Plant 14 had a higher stevioside content than IBt plants. However, plant 8 had the highest amount of stevioside: 236.3 percent higher than IBt plants. Thus, two things may be concluded here: a) no correlation exists between flowering (or the three flowering groups) and the amount of stevioside present in the plant (for example, group C has plants with the lowest: 50.6% and highest: 236.3% amounts of stevioside), and b) the optimal plant in terms of stevioside production is plant 8.

Three of these plants (numbers 2, 5 and 7) were also grown in a 16 h photoperiod (LD conditions) and their leaves extracted for stevioside. The amount of stevioside in

Table 1. Relation and Herbarium Number of the plants used in this work. The plants will be called all the way through as IBt and the new plants from Paraguay as 1 to 14.

Plants*	Herbarium number SP	Photoresponse type**
IBt	154971	A
1	154976	A
2	154977	A
3	154979	A
4	154980	A
5	157872	A
6	154972	B
7	154973	C
8	154975	C
9	154978	C
10	154983	C
11	154984	C
12	154985	C
13	154986	C
14	154987	C

* Details of morphological characteristics are given fully in MONTEIRO[10].

** According to Fig. 1.

LD was compared to the amount present in SD in the same plants (Table 4). In all cases there was an increase in stevioside when the plants were grown in LD conditions. In the case of plant 7, the amount in LD was 347 percent higher than that found in the same plant in SD conditions. Thus, there is an increase in stevioside in LD conditions.

Discussion

VÁLIO and ROCHA[12] studied the effect of the photoperiod upon flowering in plants now termed IBt. The 14 new plants now tested according to their response to photoperiod could be grouped into three classes in relation to flowering. Lot A showed the same behaviour as plants studied by VÁLIO and ROCHA[12]; this behaviour was similar for group C, but here the critical daylength was longer, as plants flowered with a 14 h daylength. These plants are, thus, distinct from the IBt plants studied by VÁLIO and ROCHA[12] and the material used by KUDO and KOGA[6]. The plants in group B behaved differently from the plants studied by the above mentioned workers; these flowered only in the two intermediate photoperiods, 10 and 12 h, being, thus, stenophotoperiodic plants, according to LANG[7]. Thus, the 14 plants may be grouped in three different classes according to their physiology in flowering. It should be mentioned that in all classes under optimum daylength flowering started between 50 to 60 days from sowing, which agrees with the data from KUDO and KOGA[6]. However, these physiological differences could not be correlated with taxonomic varieties as MONTEIRO[10], studying the morphological differences present in these plants, was unable to separate them into valid taxonomic varieties. Differences between the 14 plants studied and the IBt plant were also found in the stevioside content, though this could not be related to the flowering behaviour. From a practical point of view, however, plant 8 would be the best for cultivation, as it contains the highest stevioside content and also flowers in a photoperiod of 14 h. Studies could be commenced to obtain a new plant from this that will flower in 14 h photoperiods or more, so that plants will remain vegetative and produce more leaves (the most productive organ in terms of stevioside production) in Brazil.

Also demonstrated here is the fact that stevioside was not found in roots; this corroborates METIVIER and VIANA's hypothesis[8] that stevioside may protect the aerial portions of the plant from herbivore predators.

METIVIER and VIANA[8] found for IBt plants

Table 2. Extraction of stevioside of leaves of plants IBt growing in natural conditions, but from plants in two stage of development: already flowering and vegetative (but already induced, see text). F5% not significant.

Leaves from:	Stevioside (% dry weight)
Vegetative plants	6.88
Flowering plants	6.05

Table 3. Extraction of stevioside from different organs of plants IBt growing in natural conditions.

Organ	Stevioside (% dry weight)
Leaves	6.88
inflorescence	2.72
roots	0.00
stem	0.00

Table 4. Stevioside content in leaves of *S. rebaudiana* (new plants from Paraguay). Six or eighth plants were used. The overall variation for these determinations was in the range of ±15%

Flowering groups	Plant	Short days (8 h) % stevioside in relation to plant IBt	Long days (16 h) % stevioside in relation to the content in SD (short days) in the same plants
	IBt	100.0	—
	2	83.3	156.4
	5	110.1	137.3
	6	75.6	—
	7	50.6	347.0
	8	236.3	—
	9	84.5	—
	11	108.3	—
	13	91.6	—
	14	138.1	—

that plants in LD had relatively higher concentrations of stevioside in the tissues than plants in SD, even though their assay method (METIVIER and VIANA[9]) was different from the one used in the present paper. Their findings agree with the present ones, in which it was also demonstrated that plants contained more stevioside under LD than under short day conditions. It was also shown here that plants grown under natural conditions had higher stevioside levels than the same plants maintained in a greenhouse, where the irradiance was certainly lower. Thus, it seems that the content of stevioside is related to the total irradiance received by the plant rather than the photoperiod itself. This corroborates the hypothesis of METIVIER and VIANA[8] that synthesis of stevioside is linked with total carbon fixation and the allocation of photosynthates so that in LD conditions or high irradiance, leaves with a greater dry weight are produced which are able to allocate relatively more carbon into stevioside production, as a possible defense mechanism, compared with SD of lower irradiances.

Summary

The effect of photoperiod of plants recently introduced from Paraguay was studied. The plants can be divided in three groups according to their behaviour in varying photoperiods: A) flowering occurs in 8, 10 and 12 h photoperiods; B) flowering occurs only in 10 and 12 h photoperiods; C) flowering occurs in 8, 10, 12 and 14 h photoperiods. The stevioside content of these plants was determined. It was also shown in flowering plants that stevioside could be crystallized from extracts from leaves and inflorescences. Stevioside could be detected in extracts of stems, but not in the roots of *Stevia rebaudiana*.

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〔和 文 摘 要〕

アマハステビアの開花と体内ステビオサイド含量に対する日長の影響

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パラグアイから最近導入されたアマハステビアにおける日長の影響について研究した．それらは日長時間に対する反応によって，A) 8，10 および 12 時間の日長で開花，B) 10 および 12 時間の日長だけで開花，C) 8，10，12 および 14 時間の日長で開花，の 3 グループに分けることができた．開花中の植物の葉と花序の抽出物からステビオサイドが結晶化できることが示され，茎の抽出物中にも検出されたが，根には認められなかった．