BIOLOGIA PLANTARUM 57 (2): 390-394, 2013 DOI: 10.1007/s10535-012-0289-6

BRIEF COMMUNICATION

Glucose, sucrose, and steviol glycoside accumulation in *Stevia rebaudiana* grown under different photoperiods

S. CEUNEN and J.M.C. GEUNS*

Laboratory of Functional Biology, Kasteelpark Arenberg 31, BP 2436, B-3001 Heverlee, Belgium

Abstract

The amount of free glucose and sucrose within the leaves of *Stevia rebaudiana* has been evaluated under different photoperiods and related to steviol glycoside (SVgly) accumulation. The diurnal dynamics of glucose and sucrose in leaves, internodes, apices, and roots revealed considerable fluctuations. Within leaves, glucose and sucrose content decreased up to three-fold during the night whereas SVgly amounts did not show any significant diurnal fluctuations. Ontogenetic variations in glucose content was markedly different under long-day (LD) and short-day (SD) regimes. Under LD, glucose content increased two-fold after the onset of flower bud formation, whereas under SD, a stagnation or small decrease was observed. This increase under LD was already noticed in the upper sink leaves at the end of the vegetative stage, possibly acting as a metabolic trigger for the phase transition to reproductive development. Under SD, glucose content appeared more controlled resulting in a better linear relationship with dry matter and SVgly content. The increased sink demands of the transitional apex led to a rapid decline in leaf sucrose which showed little or no correlation with SVgly amounts. From our results, it becomes clear that the correlation between glucose or sucrose as substrates and SVglys as products is very dynamic and is significantly influenced by day-length and ontogeny.

Additional key words: *Asteraceae*, diterpene glycosides, diurnal course, ontogeny, saccharides.

The sweet diterpene steviol glycosides (SVglys) of the Paraguayan short-day (SD) plant *Stevia rebaudiana* Bertoni have already been extensively studied (for a review, see Kinghorn 2002). Most SVglys accumulate in the leaves, in which more than thirty have so far been described, including stevioside (ST) and rebaudioside A (Reb A; Bondarev *et al*. 2003/4, Chaturvedula *et al*. 2011). Within the leaves, maximal SVgly content is reached near the end of flower bud formation, after which it gradually declines. However, this pattern is dependent on genotype, photoperiod (Ceunen and Geuns 2012), and irradiance (Ermakov and Kochetov 1996).

Under long-day conditions (LD), vegetative growth is prolonged, resulting in a greater accumulation of dry matter, total sugar, protein, and ST content in leaves (Metivier and Viana 1979). During SD regime, LD conditions can easily be simulated by a short application of red irradiation during the night to activate the phytochrome (Ceunen *et al*. 2012).

Steviol glycosides are synthesised *via* the plastidial methylerythritol 4-phosphate (MEP) pathway resulting in a close relationship between SVgly accumulation and chloroplast differentiation (Totté *et al*. 2000, Ladygin *et al*. 2008). In *Stevia* leaves, the carbon flow is mainly directed between the MEP and the shikimic acid pathways (Teo *et al*. 2011). Although SVglys constitute a large carbon pool, they have a very small turnover rate rendering them unsuitable for use as a short-term carbon reservoir (Ferraresi *et al*. 1985, De Guzman 2010). The physiological function of SVglys is still unknown. Some reports suggest a role as protectants against insect herbivory (Metivier and Viana 1979), yet this was refuted by field observations (Fuente 2001).

As part of our ongoing studies on the metabolism of SVglys in *S. rebaudiana*, the relationship between glucose, sucrose, and SVgly content was investigated

Received 13 June 2012, *accepted* 19 September 2012.

Abbreviations: LD - long-day; MEP - methylerythritol 4-phosphate; Reb A - rebaudioside A; RLP - rate of leaf production; SD - short-day; ST - stevioside; SVgly - steviol glycoside.

Acknowledgments: We wish to thank Mr. Tom Struyf and Profs. Wim Van den Ende and Filip Rolland for helpful discussions. We further acknowledge Mr. Emerik de Saedeleer for the gift of invertase and Mrs. Hilde Verlinden for technical support. Funding was supplied by a KU Leuven FLOF Scholarship to S.C.

* Corresponding author; fax: (+32) 16 321509, e-mail: jan.geuns@bio.kuleuven.be

under different photoperiods. Glucose and sucrose were chosen because they are precursors of SVglys. Together with fructose, they constitute the majority of soluble sugars in *S. rebaudiana* (Darin Peshev, unpublished results). Sucrose also acts as a signalling molecule in SVgly biosynthesis (Guleria *et al*. 2011), though this influence is probably modulated in multiple layers with response to environmental stimuli (Pathre *et al*. 2004). Diurnal and ontogenetic variations of glucose, sucrose, and SVgly content were also measured along with their mutual correlations.

In order to assess diurnal variations in SVgly, glucose and sucrose content, 20 pots with one-year-old, originally *in vitro* propagated *Stevia rebaudiana* Bertoni cv. Criolla plants were grown in a greenhouse under a 16-h photoperiod with day/night temperature of 25/18 °C, and relative humidity of 60 % for 5 weeks. During 24 h, 3 morphologically similar plants having 12 nodes were collected every 4 h and separated into lower, middle, and upper leaves, internodes, apices, and roots.

To measure ontogenetic variations in glucose and sucrose content, 70 pots containing one 2-year old plant were cut above the third node and further cultivated in a greenhouse under 16-h (LD) or 8-h (SD) photoperiods. Leaves were collected during the vegetative stage, flower bud formation, and flowering, and pooled per 2 nodes. Under LD, plants formed more nodes than under SD (22 or 8 for LD and SD, respectively). Therefore, vegetative growth was arbitrarily classified into different phases according to nodal number. Under LD, "early" and "mature vegetative" plants were grown until they had 10 or 20 nodes, respectively. Under SD, plants were collected when they had 5 ("early vegetative") or 8 nodes ("mature vegetative"). Nodes were counted starting from the bottom in order to measure ontogenetic accumulation patterns within leaves of the same absolute age. The experiment was conducted for 210 d or 105 d in LD or SD regimes, respectively. All plant samples were lyophilised, pulverised, and stored in a desiccator until further use.

Extraction and analysis of SVglys was done as described before (Ceunen *et al*. 2012). The pooled supernatant fractions obtained after water extraction of 20 mg of dry and pulverised plant material were used for glucose and sucrose determination. Glucose analysis was done in a 96-well plate using a modified version of the sensitive 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method (Bruss and Black 1978). This method is glucose-specific because it uses glucose oxidase. Each well contained 50 mm^3 of supernatant to which 300 mm^3 of a reagent was added; it contained horseradish peroxidase (2.5 U cm^{-3}, *Sigma*, Bornem, Belgium), glucose oxidase type X-S from *Aspergillus niger* (3.6 U cm^{-3}, *Sigma*), and 0.75 mM ABTS dissolved in 0.12 M Na-phosphate buffer, pH 6.0 (*Sigma*). Control samples did not contain ABTS. The mixture was incubated at room temperature for 30 min after which absorbance at 405 nm (A_{405}) was measured with a microplate reader (*Multiskan Ascent*, *Thermo*, Aalst, Belgium). Calibration curves for glucose were linear up to 1.04 mM concentration ($R^2 > 0.999$). Sucrose content was calculated from the glucose release after enzymatic degradation. This was done by adding 1 mm^3 of concentrated invertase, dissolved in 50 mM Na acetate buffer, pH 5.0, to 50 mm^3 of supernatant, together with 1 mm^3 of 1 M Na-acetate buffer, pH 5.0. Samples were incubated at 30 °C for 1 h after which 300 mm^3 of ABTS reagent was added and protocol was followed as described before. Due to the efficient sucrose degradation, the calibration curve for glucose could also be used to quantify sucrose-derived glucose formation.

Two-tailed *t*-tests for independent variables were applied to determine significant spatio-temporal changes of glucose and sucrose content in either LD or SD groups. In case of heterogeneity of variances, Welch corrections were applied. Differences were considered as statistically significant at $P \leq 0.05$. Basic regression analysis was used to find linear correlations between the measured parameters, with $n = 129$ or 202 for SD and LD, respectively. Pearson's correlation coefficients were calculated. Slopes of the different curves for the LD and SD groups and vegetative, flower budding, and flowering stages were compared using a one-way analysis of covariance (*ANCOVA*) for independent samples.

Plants grown under LD clearly showed a prolonged vegetative growth which lasted around 130 d during which, on average, 22 nodes were formed. Under SD, vegetative growth was relatively short (35 d) with only 8 nodes formed. Nevertheless, the rate of leaf production (RLP) was greater under SD with new leaf pairs appearing roughly every 4.4 d compared to 6 d under LD.

Total SVgly content did not reveal any significant diurnal variations (results not shown) in contrast with glucose and sucrose content which varied considerably in all analysed tissues (Fig. 1). In general, glucose and sucrose content was greater during daytime whereas a two- to three-fold decrease was measured during the night ($P \leq 0.01$). In middle and lower leaves, a transient increase of glucose content occurred after the onset of the night ($P \leq 0.05$). In upper leaves, sucrose peaked around 04:00 ($P \leq 0.05$). Corresponding to the changes observed in leaves, similar variations were observed in the related stem sections where sugar content was greater compared to leaves. For example, in upper internodes, glucose content was almost 2 mg g^{-1}(d.m.) compared to only 200 µg g^{-1}(d.m.) in upper leaves. Concurrently, the largest diurnal deviations were seen in these upper internodes where maximum glucose content was reached around midnight ($P \leq 0.01$) whereas that of sucrose was smallest around 02:00 ($P \leq 0.01$). In the middle and lower internodes, sucrose peaked around midnight ($P \leq 0.05$). In roots, sucrose content increased sharply during the night. A second maximum was reached around 16:00 ($P \leq 0.05$; Fig. 1).

In order to minimise intra-day-related glucose and sucrose variations, it was necessary to apply strict sampling periods during ontogenetic development. The content of glucose was quite variable with no large spatial

differences across the stem. During LD, free glucose tended to increase two-fold to about 600 µg g^{-1}(d.m.) in most leaves when the plant began to form buds ($P \leq 0.01$). This was markedly different from the plants grown under SD where stagnation in glucose content was seen and even a decline in the middle and upper leaves after flowering ($P \leq 0.05$; Fig. 2).

Under LD, the greatest sucrose content was found in lower leaves [up to 1.5 mg g^{-1}(d.m.)] except during flowering when no significant spatial differences were measured. Small increases were observed in some leaves

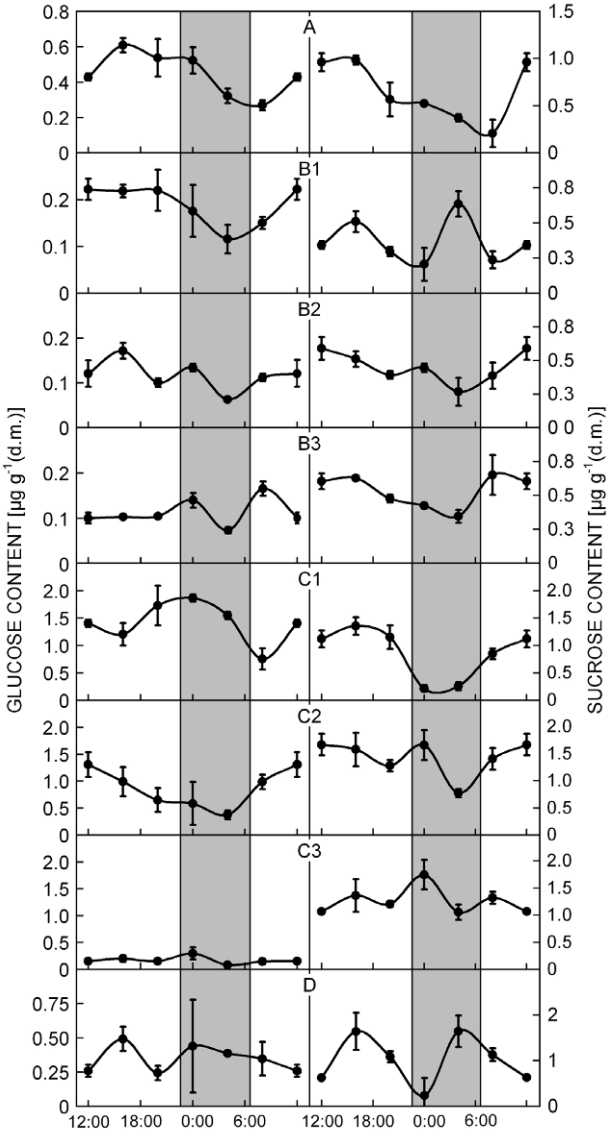

Fig. 1. Diurnal variation of glucose and sucrose content measured in 1-year-old *Stevia rebaudiana* plants grown in greenhouse (a 16-h photoperiod). All plants were at mid-vegetative stage and had 12 nodes. Plants were divided into apices (*A*), leaves (*B1 - B3*), stems (*C1 - C3*) and roots (*D*). Leaves and internodes were collected per 4 nodes allowing the following division: upper (*B1*, *C1*), middle (*B2*, *C2*) and lower (*B3*, *C3*) sections. Day and night are indicated by the *white* or *grey* area, respectively. Means ± SE, $n = 3$.

during the transition to reproductive development (*e.g.*, nodes 17 - 18, $P \leq 0.05$; Fig. 2). In plants grown under SD, no significant spatial differences were seen in all stages. However, sucrose content significantly decreased under SD during flower bud formation ($P \leq 0.05$) followed by an increase during flowering ($P \leq 0.01$).

The diurnal glucose and sucrose variations revealed some aspects of the sink-source relationship in *S. rebaudiana*. It is possible that the transient glucose peak in the middle and lower leaves was due to leaf starch degradation as there is no photosynthetic activity during the night (Zeeman *et al*. 2004). In *S. rebaudiana*, De Guzman (2010) measured a five-fold decline of leaf starch during the night, from 3.3 to 0.7 % (m/m). As source organs, these leaves then exported sucrose to the roots. This was indicated by transient sucrose peaks in the lower and middle internodes around midnight followed by a sharp increase in root sucrose around 04:00 (Fig. 1). At the same time, no peak of sucrose was detected in upper internodes. Presumably, most sucrose synthesised in the upper sink leaves was used for their own growth with little or no transport to other sink organs, such as roots. In sink leaves, photosynthetic rate is usually still insufficient to meet their own sugar demands necessary for growth. Earlier research already suggested that leaf development in *S. rebaudiana* is divided in two phases: at first, dry matter remains fairly constant whereas fresh mass and leaf area increases. When leaf area reaches 80 % of full expansion, *in situ* carbon fixation becomes sufficiently developed resulting in an increase of dry mass and leaf protein content, both having doubled by the time leaves are fully expanded (Viana and Metivier 1980).

In Angiosperms, the transition from vegetative to reproductive development coincides with complex alterations in plant physiology and biochemistry. The increased sink requirements of the apical region are marked by an increased influx of sugars from source leaves (Périlleux and Bernier 1997) and transient increases in sucrose content have been linked to flower induction (Corbesier *et al*. 1998).

Under LD, a sharp increase in glucose content was observed at the end of the vegetative stage in middle-to-upper leaves (Fig. 2). These leaves are situated near the sink-to-source boundary as derived from the diurnal course of sugars (Fig. 1) and from the hypothesis that *in situ* photosynthesis becomes significantly contributory at 80 % of full leaf expansion (Viana and Metivier 1980). Possibly, the glucose peak in those leaves acted as a metabolic trigger for the phase transition to reproductive development influencing cell division in the apical regions in a similar way as was observed, *e.g.* in *Vicia faba* (Borisjuk *et al*. 1998).

Floral induction is often accompanied by increased starch mobilisation (Corbesier *et al*. 1998) and under LD, this may be the source of the increased glucose amounts preceding flower bud formation (Fig. 2). SD conditions, however, generally require increased rates of starch biosynthesis during the day whereas starch mobilisation during the night occurs at a lesser, more controlled rate

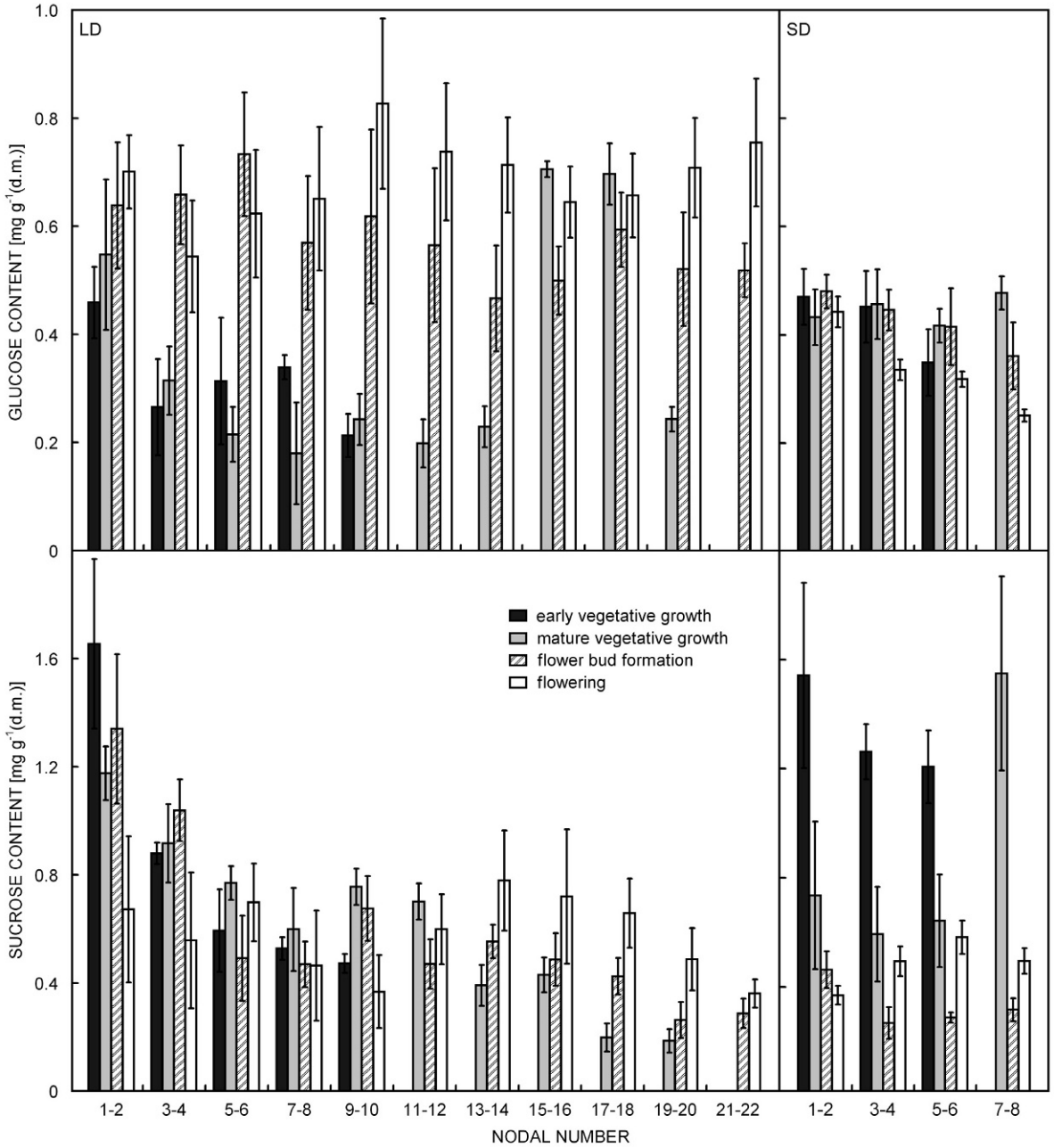

Fig. 2. Spatial accumulation patterns of glucose and sucrose during ontogeny of *Stevia rebaudiana*, grown in both LD and SD regimes. Top leaves correspond to the greatest nodal number. Means ± SE, $n = 3$ (SD, early and mature vegetative growth), 6 (SD, flower bud formation; LD, vegetative and reproductive stages) or 21 (SD, flowering). Total n depends on the amount of independent samples which had to be pooled in each ontogenetic stage.

(Smith and Stitt 2007). In vegetative stages, *Stevia* leaf glucose content was up to two-fold greater under SD compared to LD ($P \leq 0.01$) which might further be linked with the increased RLP under SD. Mitotic activity in the apical region is known to increase after 5 SD cycles indicating an early formation of the transitional apex in this species (Monteiro and Gifford 1988) which is likely to increase sugar influx.

In both LD and SD groups, significant linear correlations ($P \leq 0.001$) were found between leaf dry matter and glucose or sucrose content per leaf ($r = 0.81$ or 0.65 for SD and LD, respectively, $P \leq 0.05$). An opposite pattern was observed for sucrose ($r = 0.47$ or 0.72 for SD and LD, respectively). The relationship between glucose or sucrose and amount of SVglys per leaf also showed a positive correlation. However, variance was quite large ($R^2 < 0.2$). The main exception was the close correlation between glucose and SVgly content in plants under SD ($r = 0.80$; $P \leq 0.001$).

A linear relationship was found between total soluble

sugars and SVgly content in leaves of *Stevia* grown in field under photoperiod of 12 to 13 h (Nishiyama *et al*. 1992). Plants grown from seeds under a 16-h photoperiod showed an inverse correlation between total soluble sugar content and leaf dry matter (Viana and Metivier 1980). From our data, it appears that the relationship between glucose, sucrose and SVgly content was affected by a photoperiod. Correlations between glucose and total SVgly levels were more linear under SD, possibly due to the more controlled glucose flux in these conditions. However, SVgly biosynthesis might be less homeostatically balanced under SD during vegetative growth (Ceunen and Geuns 2012). In the current study and under the same conditions, such variations were mainly observed between sucrose and dry matter or total SVgly content. This may be logical because sucrose is mainly used as transport vehicle and storage for glucose. The putative role of sucrose as an enhancer of SVgly biosynthesis (Guleria *et al*. 2011) might partially explain the less balanced flux of SVglys during vegetative growth under SD as the result of large sucrose fluctuations at this stage. How exactly sugars influence SVgly accumulation still needs further clarification.

References

Borisjuk, L., Walenta, S., Weber, H., Mueller-Klieser, W., Wobus, U.: High-resolution histographical mapping of glucose concentrations in developing cotyledons of *Vicia faba* in relation to mitotic activity and storage processes: glucose as a possible developmental trigger. - Plant J. **15**: 583-591, 1998.

Bondarev, N.I., Sukhanova, M.A., Reshetnyak, O.V., Nosov, A.M.: Steviol glycoside content in different organs of *Stevia rebaudiana* and its dynamics during ontogeny. - Biol. Plant. **47**: 261-264, 2003/4.

Bruss, M.L., Black, A.L.: Enzymatic microdetermination of glycogen. - Anal. Biochem. **84**: 309-312, 1978.

Ceunen, S., Geuns, J.M.C.: Influence of photoperiodism on the spatio-temporal accumulation of steviol glycosides in *Stevia rebaudiana* Bertoni. - In: Geuns, J.M.C. (ed.): Stevia: Six Months beyond Authorisation. Proceedings of the 6th EUSTAS Stevia Symposium. Pp. 5-40. Euprint, Heverlee 2012.

Ceunen, S., Werbrouck, S., Geuns, J.M.C.: Stimulation of steviol glycoside accumulation in *Stevia rebaudiana* by red LED light. - J. Plant Physiol. **169**: 749-752, 2012.

Chaturvedula, V.S.P., Clos, J.F., Rhea, J., Milanowski, D., Mocek, U., DuBois, G.E., Prakash, I.: Minor diterpenoid glycosides from the leaves of *Stevia rebaudiana*. - Phytochem. Lett. **4**: 209-212, 2011.

Corbesier, L., Lejeune, P., Bernier, G.: The role of carbohydrates in the induction of flowering in *Arabidopsis thaliana*: comparison between the wild type and a starchless mutant. - Planta **206**: 131-137, 1998.

De Guzman, R.A.R.: Autoecological role of steviol glycosides in *Stevia rebaudiana*. - PhD. Thesis, Central Queensland University, Rockhampton 2010.

Ermakov, E.I., Kochetov, A.A.: Specificities of the growth and development of *Stevia* plants under different controllable light conditions. - Russ. agr. Sci. **1**: 9-11, 1996.

Ferraresi, M.L.L., Ferraresi-Filho, O., Bracht, A.: [Photosynthetic incorporation of ^{14}C into components of *Stevia rebaudiana*.] - Rev. Unimar **7**: 133-146, 1985. [In Port.]

Fuente, A.L.O.: [Survey of pests and diseases of *Stevia rebaudiana* Bertoni.] - Rev. Ciênc. Tecnol. Dir. Invest. UNA **1**: 29-33, 2001. [In Span.]

Guleria, P., Kumar, V., Yadav, S.K.: Effect of sucrose on steviol glycoside biosynthesis pathway in *Stevia rebaudiana*. - Asian J. Plant Sci. **10**: 401-407, 2011.

Kinghorn, A.D. (ed.): *Stevia*, the Genus *Stevia*. - Taylor and Francis, London - New York 2002.

Ladygin, V.G., Bondarev, N.I., Semenova, G.A., Smolov, A.A., Reshetnyak, O.V., Nosov, A.M.: Chloroplast ultrastructure, photosynthetic apparatus activities and production of steviol glycosides in *Stevia rebaudiana in vivo* and *in vitro*. - Biol. Plant. **52**: 9-16, 2008.

Metivier, J., Viana, A.M.: The effect of long and short day length upon the growth of whole plants and the level of soluble proteins, sugars, and stevioside in leaves of *Stevia rebaudiana* Bert. - J. exp. Bot. **30**: 1211-1222, 1979.

Monteiro, W.R., Gifford, E.M.: Morphological aspects of the shoot apex of *Stevia rebaudiana* (Bert.) Bertoni (Asteraceae) during transition to flowering. - Rev. Bras. Bot. **11**: 1-10, 1988.

Nishiyama, P., Alvarez, M., Vieira, L.G.E.: Water soluble carbohydrates in leaves of *Stevia rebaudiana* and its relationship to the stevioside content. - Ciênc. Tecnol. Alim. **12**: 128-133, 1992.

Pathre, U.V., Sinha, A.K., Shirke, P.A., Ranade, S.A.: Diurnal and seasonal modulation of sucrose phosphate synthase activity in leaves of *Prosopis juliflora*. - Biol. Plant. **48**: 227-235, 2004.

Périlleux, C., Bernier, G.: Leaf carbohydrate status in *Lolium temulentum* during the induction of flowering. - New Phytol. **135**: 59-66, 1997.

Smith, A.M., Stitt, M.: Coordination of carbon supply and plant growth. - Plant Cell Environ. **30**: 1126-1149, 2007.

Teo, C.C., Tan, S.N., Yong, J.W.H., Ra, T., Peiling L., Ge, L.: Metabolomics analysis of major metabolites in medicinal herbs. - Anal. Methods **3**: 2898-2908, 2011.

Totté, N., Charon, L., Rohmer, M., Compernolle, F., Baboeuf, I., Geuns, J.M.C.: Biosynthesis of the diterpenoid steviol, an *ent*-kaurene derivative from *Stevia rebaudiana* Bertoni, via the methylerythritol phosphate pathway. - Tetrahedron Lett. **41**: 6407-6410, 2000.

Viana, A.M., Metivier, J.: Changes in the levels of total soluble proteins and sugars during leaf ontogeny in *Stevia rebaudiana* Bert. - Ann. Bot. **45**: 469-474, 1980.

Zeeman, S.C., Smith, S.M., Smith, A.M.: The breakdown of starch in leaves. - New Phytol. **163**: 247-261, 2004.