Chloroplast ultrastructure, photosynthetic apparatus activities and production of steviol glycosides in *Stevia rebaudiana in vivo* and *in vitro*

V.G. LADYGIN[1], N.I. BONDAREV[2]*, G.A. SEMENOVA[3], A.A. SMOLOV[1], O.V. RESHETNYAK[2] and A.M. NOSOV[2]

Institute of Basic Biological Problems[1], and Institute of Teoretical and Experimental Biophysics[3], Russ. Acad. Sci., 142290 Pushchino, Moscow Region, Russia
Timiryazev Institute of Plant Physiology, Russ. Acad. Sci., Botanicheskaya 35,127276 Moscow, Russia[2]

Abstract

The accumulation of steviol glycosides (SGs) in cells of *Stevia rebaudiana* Bertoni both *in vivo* and *in vitro* was related to the extent of the development of the membrane system of chloroplasts and the content of photosynthetic pigments. Chloroplasts of the *in vitro* plants, unlike those of the intact plants, had poorly developed membrane system. The callus cells grown in the light contained proplastids of almost round shape and their thylakoid system was represented by short thylakoids sometimes forming a little number of grana consisting of 2 - 3 thylakoids. In cells of the etiolated *in vitro* regenerants and the callus culture grown in the dark, only proplastids practically lacking the membrane system were observed. All the chloroplasts having developed thylakoids and forming at least a little number of grana were equipped with photochemically active reaction centers of photosystems 1 and 2. Leaves of *in vivo* plants accumulated greater amount of the pigments than leaves of the *in vitro* plants. In both the callus culture grown in the light and the etiolated *in vitro* regenerants, the content of the pigments was one order of magnitude lower than that in leaves of the intact plants. The callus tissue grown in the dark contained merely trace amounts of the pigments. Leaves of the intact and the *in vitro* plants did not exhibit any significant differences in photosynthetic O_2 evolution rate. However, photosynthetic O_2 evolution rate in the callus cells was much lower than that in the differentiated plant cells. The *in vitro* cell cultures containing merely proplastids did not practically produce SGs. However, after transferring these cultures in the light, both the formation of chloroplasts and the production of SGs in them were detected.

Additional key words: photosynthetic rate, photosynthetic pigments, rebaudiosides A and C, stevioside.

Introduction

It is well known that leaves of stevia (*Stevia rebaudiana* Bertoni) contain a number of steviol glycosides (SGs), sweet, low-energetic and nontoxic compounds (Kinghorn and Soejarto 1986, Lyakhovkin *et al.* 1993, Matsui *et al.* 1996). Major types of the SGs are stevioside, rebaudioside A and rebaudioside C (Lyakhovkin *et al.* 1993, Bondarev *et al.* 2001).

Earlier, we have found that in *Stevia* callus and suspension cultures grown in the dark, the SGs are produced only in minor amounts. At the same time, in the morphogenic callus grown in the light, the content of SGs was increased by one order of magnitude. In leaves of *Stevia* plants grown in greenhouse, the amount of the SGs was found to be more than by two orders of magnitude (Bondarev *et al.* 2001). In addition, the correlation between the plant development and the content of the SGs was revealed (Bondarev *et al.* 2002, 2003/4). Nevertheless, many peculiarities of both the formation and accumulation of the SGs in *Stevia* plant tissues are poorly understood.

The purpose of the present work was to reveal any features in ultrastructural chloroplast organization and activity of photosynthetic apparatus in plants of *Stevia* and its cultures *in vitro* in relation to the biosynthesis of diterpenoid steviol glycosides.

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Abbreviations: BA - 6-benzylaminopurine; HPLC - high performance liquid chromatography; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; PS - photosystem; RC - reaction centre; SGs - steviol glycosides.
* Corresponding author; fax: (+07) 495 977 9445, e-mail: nikbond@inbox.ru

Materials and methods

The experiments were performed using the following plant materials: *1*) plants of stevia (*Stevia rebaudiana* Bertoni) grown in greenhouse, 2) *in vitro* plants, 3) *in vitro* regenerants grown in the dark, 4) mixotrophic callus cultures grown in the light, 5) heterotrophic callus cultures grown in the dark. Sterile *Stevia* plants were obtained using apical buds of the intact plants (Bondarev 2001). The *in vitro* plants were grown in tubes on the Murashige and Skoog (1962; MS) medium containing 3 % (m/v) sucrose and 0.7 % agar but lacking of the plant growth regulators which appeared to be optimum for the accumulation of the SGs in leaves of the plants (Bondarev *et al.* 2003).

The formation of the callus was initiated on leaf blades of the diploid *in vitro* grown plants. The callus tissue was grown on the MS agar medium containing 1 mg dm^{-3} NAA and 0.5 mg dm^{-3} BA (Bondarev *et al.* 1997, 1998). The cultivation was conducted either in the light (40 μmol m^{-2} s^{-1}) or in the dark both at 25 ± 1 °C and at about 70 % room air humidity. In the experiments third pair of upper leaves of 5-week-old plants and *in vitro* cultures of *Stevia* were used. For each type of investigations three separate experiments in three replicates were performed.

The chlorophyll content was determined after extraction of the pigments with 80 % acetone (Arnon 1949). 1 g fresh mass of leaves was ground in small volume of acetone solution and then the extract obtained was diluted to a final volume of 3 cm^3. Absorbance at 662 nm (Chl *a*) and 645 nm (Chl *b*) was measured with spectrophotometer *Hitachi-557* (Japan).

The photosynthetic O_2 evolution rate measured during the first 3 min of exposure of the samples to the saturating irradiance which was equal to 200 μmol m^{-2} s^{-1}. The plant samples were placed into the amperometric cell of 5 cm^3 volume equipped by the Pt-Ag Clark type electrode and hermetically closed. Photosynthetic O_2 evolution rate was determined at 20 °C and CO_2 concentration of 0.03 %.

Chloroplast ultrastructure was observed on the ultrathin sections of the plant samples fixed for 1.2 - 2.0 h with 1.25 % glutaraldehyde prepared on phosphate buffer, pH 7.4, and then postfixed for 2 h at room temperature with 1 % OsO_4. Then the samples were dehydrated in an ethanol series (30, 50, 70 and 90 %) using 10 min treatment with each of the ethanol solutions, 10 min treatment with 100 % absolute ethanol and then 15 min treatment with 100 % acetone. The material obtained was embedded in the epoxide resin *Epon-812*. After 2 - 3 weeks of the resin polymerization at 37 °C, thin sections of the samples were prepared on the microtome *LKB-III* (*LKB*, Uppsala, Sweden), stained with uranylacetate for 1 h at 37 °C and contrasted with lead citrate solution according to the method of Reynolds (Reynolds 1963) for 25 min at room temperature. The ultrathin sections were examined in a *JEM-7A* (*JEOL*, Tokyo, Japan) electron microscope.

Photochemical activity of the reaction centre (RC) of the photosystems (PSs) was estimated according to the light-induced changes in the absorption of chlorophyll P_{700} (ΔA_{700}) belonging to the RC of PS 1 or in the variable fluorescence (ΔF_v) of the chlorophyll of PS 2 (Ladygin and Semenova 1999). Methods of measuring ΔF and P_{700} were described earlier (Ladygin *et al*. 1976). The experiments were conducted on suspensions of chloroplasts isolated from 1 g of leaf fresh mass with 3 cm^3 phosphate buffer (pH 7. 2).

The content and composition of the SGs in plant materials were determined by HPLC (*LKB*, Bromma, Sweden) as described earlier (Bondarev 2001, 2002).

Results and discussion

Content of the pigments: The chlorophyll and carotenoid contents were practically the same in leaves of the diploid and tetraploid intact plants (Table 1). In leaves of green plants grown *in vitro*, they were somewhat lower than that in intact plants, while in callus tissue grown in the light they were about one order lower as compared to those found in leaves of the intact plants and comparable with the corresponding value in callus cells of another plant species (Ladygin *et al*. 2003). Although a decreased content of chlorophyll in callus cells is usually

Table 1. Content of pigments in leaves of 5-week-old plants and different *in vitro* cultures of *Stevia rebaudiana* [mg g^{-1}(f.m.)]. Means ± SE. Three separate experiments in three replicates were performed.

Culture analyzed	Chl *a*	Chl *b*	Car	Chl *a*/*b*	Chl/Car
Diploid plants grown in greenhouse	1.65 ± 0.09	0.62 ± 0.03	0.36 ± 0.03	2.7	6.3
Tetraploid plants grown in greenhouse	1.77 ± 0.12	0.63 ± 0.04	0.39 ± 0.04	2.8	6.2
Leaves of *in vitro* plants	1.32 ± 0.11	0.51 ± 0.03	0.27 ± 0.03	2.6	6.8
Leaves of etiolated *in vitro* regenerants	0.13 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	4.3	5.3
Green callus	0.17 ± 0.02	0.04 ± 0.01	0.04 ± 0.01	4.2	5.2
Etiolated callus	trace	-	-	-	-

Table 2. Photosynthetic O_2 evolution rate in third pair of upper leaves of 5-week-old plants and different *in vitro* cultures of *Stevia rebaudiana*. Means ± SE. Three separate experiments in three replicates were performed.

Culture analyzed	Saturating irradiance [μmol m^{-2} s^{-1}]	O_2 evolution [μmol mg^{-1}(d.m.) s^{-1}]	O_2 evolution [μmol mg^{-1}(Chl) s^{-1}]
Diploid plants grown in greenhouse	230 ± 50	37.5 ± 6.9	2.3 ± 0.2
Tetraploid plants grown in greenhouse	254 ± 46	38.6 ± 3.4	2.2 ± 0.3
Leaves of *in vitro* plants	180 ± 20	24.3 ± 3.4	1.9 ± 0.4
Leaves of etiolated *in vitro* regenerants	-	trace	-
Green callus	150 ± 40	4.1 ± 0.9	5.3 ± 0.3
Etiolated callus	-	trace	-

attributed to the inhibiting action of sucrose, its presence in the nutrient medium did not practically affect chlorophyll content in leaves of *Stevia in vitro* (Table 1). In etiolated regenerants grown *in vitro*, the contents of chlorophylls and carotenoids were one order lower as compared to green plantlets. In addition, only trace amounts of the pigments were found in callus tissue grown in the dark.

Photosynthetic rate: The rate of O_2 evolution in both *Stevia* leaves and the callus cells achieved saturation at irradiances from 160 to 240 μmol m^{-2} s^{-1} (Table 2). In leaves of *in vitro* plants, the O_2 evolution was by 20 - 30 % lower than that in leaves of intact plants, while diploid and tetraploid plants did not practically differ in this parameter. However, O_2 evolution from callus cells (calculated per g dry mass) appeared to be about 6 - 9 times lower but about 2 - 3 times higher (calculated per mg chlorophyll) as compared to that of the plants grown both in *vivo* and in *vitro* (Table 2).

Chloroplast ultrastructure: The chloroplasts in mesophyll cells of *Stevia* leaves had both asymmetric and symmetric oblate shape and were situated along thick cell wall (Fig. 1*A*). Cells of leaves of diploid and tetraploid plants did not differ significantly in their ultrastructure, but the tetraploid cells were larger and contain about two-fold number of chloroplasts than the diploid ones (data not shown). These data provide strong evidence for the nucleus control of number of chloroplasts. Chloroplasts in leaves of both the diploid and tetraploid *Stevia* plants had well developed membrane system, with 7 - 15 thylakoids frequently forming grana (Fig. 1*B*). In some cases, the macrograna including up to 20 - 30 thylakoids were observed. All the thylakoids had longitudinal orientation with respect to a major axis of chloroplasts. These observations indicate the presence of all the pigment-protein complexes of the PS 1 and 2 in thylakoids as the putative factors determining the structure and spatial orientation of the membrane system of chloroplasts (Albertsson 2001, Semenova 2002, Ladygin 2003, Mustardy and Garab 2003). The chloroplasts of tetraploid plants accumulate higher amounts of starch than those of diploid plants. We suggest that different rates of metabolic and especially transport processes in cells of the tetraploid and diploid plants are responsible for this difference. One more notable finding of the present work was that all intrathylakoid space of *Stevia* chloroplasts in

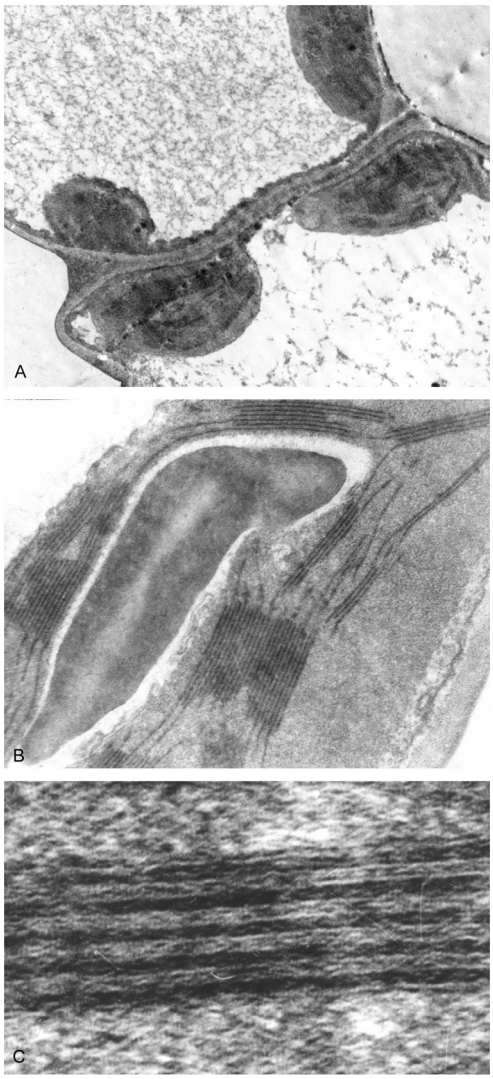

Fig. 1. Chloroplasts of leaf parenchyma cells of intact *Stevia rebaudiana* plants: *A* - chloroplasts situated along the cell wall; *B* - chloroplast ultrastructure of tetraploid plant; *C* - thylakoids filled up with electron dense material.

both the diploid and tetraploid plants were filled up by electron dense substance (Fig. 1*C*). Earlier electron dense inclusions inside the thylakoids were found in chloroplasts of some other plant species (Steveninck and Steveninck 1980a, Danilova and Kashina 1999). These authors conducted experiments on *Perilla ocymoides* and

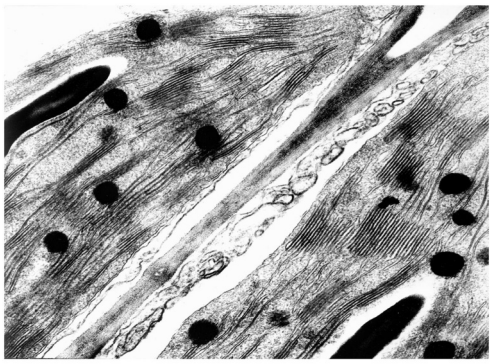

Fig. 2. Chloroplast ultrastructure in leaf cells of *Stevia in vitro* plants.

such a modified type of the thylakoids were dominant when short day plants were grown under continuous illumination. It is of interest to note in this connection that both *Stevia* and perilla are the short-day plants that display increased vegetation without transition to flowering stage when they are grown under long day conditions. Moreover, the content of the SGs in *Stevia* leaves grown for long day was found to be higher than that in the leaves of the same plants grown under short day. However, a chemical nature of these inclusions remains so far poorly understood and most likely may be different for different plant species. According to the results of our studies, such inclusions observed in the thylakoids of chloroplasts of *Stevia* cells are not the SGs, but can the diterpenoid precursors. However, in the thylakoids of *Nymphoides indica* chloroplasts, the electron dense inclusions represented phenols (Steveninck and Steveninck 1980b). It can be suggested that in chloroplasts of leaves of juvenile plants these substances might serve as an inhibitors of flowering and they could converse into inactive one, for example, glycosides, as may be required.

Stevia plants growing *in vitro* exhibited significant changes in chloroplast ultrastructure as well as in the size of the plants, their leaves and parenchyma cells, whereas the size of chloroplasts remained nearly unchanged. The inner membrane structure of chloroplasts in leaf cells of the *in vitro* grown plants appeared to be reasonably well developed as well (Fig. 2). Moreover, numerous grana were observed and the organelles kept their ability to form macrograna consisting of 20 and more thylakoids. Nevertheless, the *in vitro* grown plants showed some distinct features. These plants possessed only slight ability for starch accumulation that is likely due to lower photosynthetic rate related to growing the plants in closed vessels where fairly slow diffusion of gas takes place (Lucchesini *et al.* 2006). The membrane system of chloroplasts appeared to be less dense than that in the chloroplasts of plants grown in greenhouse. Chloroplasts in the *in vitro* grown plants had a large number of osmiophilic globules (10 and more per section of single chloroplast). This observation may serve as indicator of destructive processes in chloroplasts usually observed under various stress conditions (Ladygin and Semenova 1993, Staehelin 2003, Lucchesini *et al.* 2006).

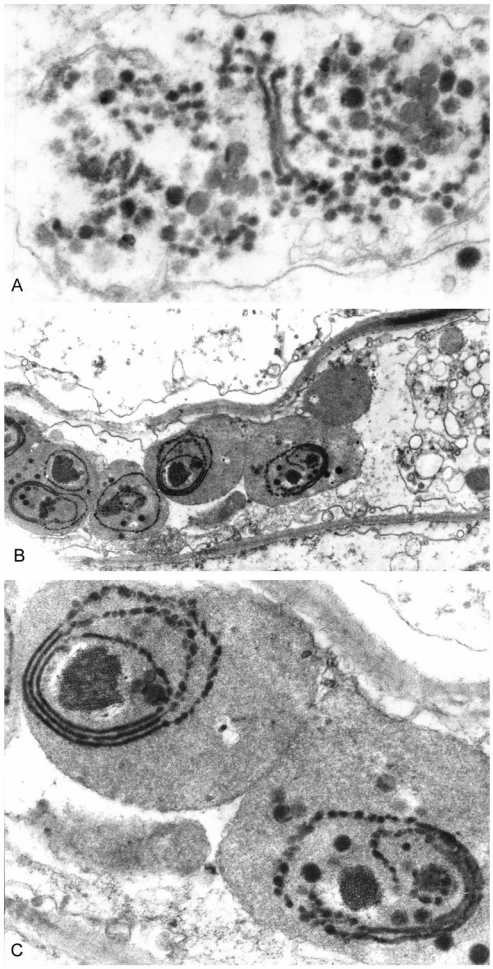

Fig. 3. Plastids of leaf cells of etiolated *Stevia in vitro* regenerants: *A* - plastids in cells of the lower leaf pair of *Stevia* etiolated regenerants; *B*,*C* - proplastids in cells of embryo leaves of the upper circle.

Chloroplast ultrastructure in cells of the *in vitro* regenerants grown in the dark was examined on three types of leaves: pair of the brown-yellow explant leaves (leaves of cuttings of the original green plantlets), lowest yellow leaves (buds of explants of the original green plantlets) and germ leaves belonging to upper circle. Chloroplasts from explant leaves contained a large number of thylakoids, and as a rule they are well developed and capable of forming grana. However, some chloroplasts contained poorly developed thylakoids, while the other appeared to be well structured only by

half or had no thylakoids at all and instead were filled up by matrix containing membrane vesicles of little size. In addition, single chloroplasts exhibited the presence of sufficiently large macrograna as well as numerous membrane vesicles and short thylakoids. A pronounced heterogeneity of the development of chloroplast membrane system is a characteristic feature of cutting leaves. This suggests changed chloroplast development and proceeding of destructive processes in these organelles after transferring the plants in the dark. Such interpretation is confirmed by the appearance of reasonably large lipid globules or osmiophilic drops inside chloroplasts. The lowest leaves of the *in vitro* etiolated plants had yellow color due to practically complete destruction of the membrane system of plastids. In such chloroplasts, a large number of osmiophilic globules were observed (Fig. 3*A*). We suggest that this effect is caused by destruction of thylakoids in the dark, loss of chlorophyll and, as a result, release of lipids from the membrane system of chloroplasts and formation of plastoglobules. Rudimentary leaves of the upper circles of the etiolated *in vitro* plants appeared to be very poorly developed. They had practically white color and were 3 - 6 mm and 1 mm in length and width, respectively. Their sizes were 4 - 8 times lower than those of leaves of the green *in vitro* plants. It is not surprising that leaf cells of the etiolated plants had much less size and clearly pronounced prolate shape (Fig. 3*B*). In contrast, etioplasts or proplastids in etiolated leaves had, as a rule, round shape, unlike disk-like organelles of the same type in green leaves of the *in vivo* and *in vitro* plants. In addition, proplastids in such cells were not located merely in the vicinity of the cell wall. Ultrastructure of plastids in etiolated leaves of the upper circles was found to markedly differ from that of chloroplasts in the lower explant leaves of the same plants. Thus, size of proplastids was less as compared to those of chloroplasts, and 70 - 90 % of their volume was filled up merely by matrix. The membrane system of proplastids was frequently not developed at all. Only in single cases, membrane system of plastids was represented by single thylakoids usually having ring-like shape and numerous single vesicles (Fig. 3*C*). As a rule, we observed only the first steps of membrane system formation, namely, small lipid-containing globules and ring structures formed by chain of membrane vesicles. It is likely that subsequent attachment to them in the light of protein complexes and the pigments results in the ordered membrane localization of the chlorophyll-protein complexes and in formation of the thylakoid membranes. However, as follows from our observations, in etiolated leaves of the *in vitro* plants grown in the dark, both the synthesis of the pigments and formation of the membrane system of chloroplasts did not occur.

Chloroplasts of mixotrophic callus cells accumulated only small amount of pigments and exhibited highly decreased photosynthetic capacity (Tables 1 and 2) in correspondence to those of other plants (Ladygin *et al*. 2003, Pakhlavouni *et al*. 2000). In callus cells chloroplasts had almost round shape and poorly developed membrane system. Some chloroplasts contained numerous thylakoids of small size forming in some regions contacts to one another and thereby grana consisting of 2 - 3 thylakoids (Fig. 4*A*). In the callus cells, we also observed the chloroplasts containing merely a large number of membrane vesicles, single short thylakoids and multiple large and small osmiophilic globules forming sometimes round-like chains (Fig. 4*B*). The matrix was present in the major part of plastid volume. By their structure such chloroplasts rather resembled proplastids of rudimentary leaves of the etiolated plants. In callus cells, we have found a certain substance the UV spectrum of which is similar to that of etiochlorin, the compound known as a derivative of chlorophyll (Schlyk 1965). This finding is consistent with the observed degradation of the membrane system of chloroplasts in *Stevia* callus cells.

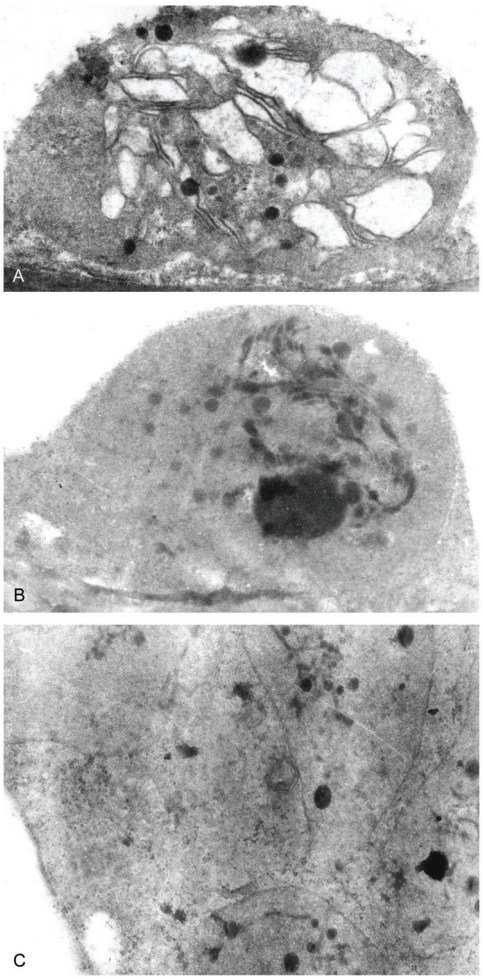

Fig. 4. Ultrastructure of plastids in cells of *Stevia* callus cutures: *A*, *B* - plastids of callus cells grown in the light; *C* - proplastids of callus cells cultivated in the dark.

The membrane system of plastids in the heterotrophic callus cells was further reduced. In this case, plastids rather resembled proplastids since they contained only

small number of membrane vesicles, single short thylakoids and osmiophilic globules, whereas the matrix filled up practically all the plastid volume (Fig. 4*C*). Plastids in the dark-grown callus cells had round or prolate shape. Taking into account almost complete absence of the proper membrane system in such plastids, it is most likely that they lack the capability to form the components of the PS 1 and 2, and, for this reason, do not possess any photosynthetic activity (Table 2).

Activity of photosystems: Photochemical activity of the RCs of both the PS 1 and 2, was the highest in chloroplasts of leaves of intact plants (Fig. 5*A*,*B*). Chloroplasts of the *in vitro* plants had two-fold lower activity of the RCs of both the PSs. The activity of the RCs in plastids of etiolated *in vitro* regenerants was 15 - 30 % of that of *in vitro* plants and was largely due to that of explant leaves. In the chloroplasts of callus cells in the light, the RCs activity was only 5 - 6 % of activity in the *in vitro* plants grown under the same conditions. Callus cells grown in the dark did not possess any photochemical activity.

Content of steviol glycosides: The highest content of the SGs was found in plants grown in greenhouse, largely in their leaves (Table 3). In the *in vitro* grown plants, the total content of the SGs in their leaves and stems was several times lower as compared to that in the greenhouse plants (Table 3). On the other hand, the plants did not practically differ by the qualitative composition of the SGs. In etiolated *in vitro* regenerants, the content of the SGs was found to be about ten times lower than that in the green *in vitro* plants. In the callus culture grown in the light, the SGs were accumulated up to 100 μg per g dry mass and in the etiolated heterotrophic callus cells only trace amounts of the SGs were found.

The results of the present work provide evidence for positive correlation between development and activity of the photosynthetic apparatus in *Stevia* leaves and their content of the SGs. The poorly developed membrane system of chloroplasts appears to be in accordance with reduced photosynthetic activity and low content of the SGs in *in vitro* grown plants. A complete absence of the production of the SGs in the heterotrophic callus cells may be interpreted on the same basis. As established,

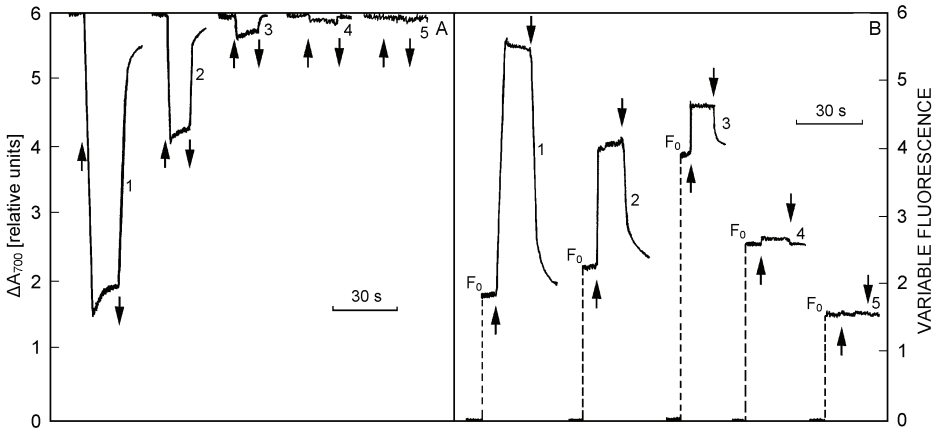

Fig. 5. Photochemical activity of the RCs of PS 1 and PS 2 in plastids of *Stevia* cells: *A* - light-induced changes in absorbance of chlorophyll belonging to the reaction centre of PS 1 (ΔA_{700}); *B* - changes in a variable fluorescence of chlorophyll (F_v) of PS 2. 1 - chloroplasts of leaf cells of intact plants; 2 - chloroplasts of leaf cells of *in vitro* plants; 3 - plastids of leaf cells of etiolated *in vitro* regenerants; 4 - plastids of callus cells grown in the light; 5 - proplastids of callus cells cultivated in the dark. The *arrows* up-directed (↑) and down-directed (↓) indicate switching on and switching off the light, respectively.

Table 3. Content of steviol glycosides [mg g^{-1}(d.m.)] in leaves of 5-week-old plants and different *in vitro* cultures of *Stevia rebaudiana*. Means ± SE. Three separate experiments in three replicates were performed. nd - not detected.

Culture analyzed		Stevioside	Rebaudioside A	Rebaudioside C	Total SGs
Diploid plants grown in greenhouse	leaves	25.40 ± 2.10	24.50 ± 3.40	6.30 ± 0.40	56.20 ± 5.50
	stems	4.50 ± 0.40	2.60 ± 0.20	0.40 ± 0.02	7.50 ± 0.60
Tetraploid plants grown in greenhouse	leaves	24.90 ± 3.50	12.00 ± 1.80	4.60 ± 0.70	41.50 ± 6.10
	stems	nd	nd	nd	nd
In vitro plants	leaves	3.30 ± 0.28	1.91 ± 0.91	0.70 ± 0.02	5.91 ± 0.31
	stems	0.80 ± 0.07	0.61 ± 0.03	0.11 ± 0.00	1.52 ± 0.07
Etiolated *in vitro* regenerants	shoots	0.28 ± 0.02	0.18 ± 0.01	0.07 ± 0.00	0.53 ± 0.03
Green morphogenic callus	cells	0.06 ± 0.01	0.02 ± 0.00	-	0.08 ± 0.01
	shoots	0.39 ± 0.03	0.11 ± 0.01	0.05 ± 0.00	0.55 ± 0.03
Etiolated callus	cells	trace	-	-	trace

these cells contain merely proplastids filled up by the matrix with a little number of membrane vesicles and lipid globules, and the pigments in them are practically absent. As a result, the biosynthesis and accumulation in them of the SGs did not likely occur. After transferring the cell cultures in the light, both the formation of the membrane system of chloroplasts containing the thylakoids and small grana and recovery of the biosynthesis of the SGs were observed.

All the above data suggest that the initial steps of synthesis of steviol glycosides occur in chloroplasts using alternative glyceralaldehyde-3-phosphate-pyruvate (1-deoxy-D-xyluloso-5-phosphate) pathway rather than mevalonate one operating in the cytoplasm. In recent years, strong evidence that namely the alternative pathway functions for production of some isoprenoids in higher plants was obtained (Schwender *et al.* 1996, Lichtenthaler *et al.* 1997, Lichtenthaler 1998, Paseshnichenko 1998, Rohmer 1999). Although the content of the SGs in *Stevia* callus tissue grown in the light was found to be higher than that in etiolated callus but significantly lower than that in plants, this does not contradict the above proposal. The callus cells grown in the light contain round chloroplasts having weakly developed membrane system and strongly resembling proplastids. It is most likely that prolonged period of intensive production of chloroplast isoprenoids (phytol, carotenoids and plastoquinone-9) in chloroplasts, the process essential for the photosynthesis as a whole, precedes an active formation of the SGs in these organelles during ontogenesis. These metabolites were shown to be capable of being synthesized in chloroplasts following an alternative pathway (Lichtenthaler 1998). However, the observed positive correlation between development and activity of the photosynthetic apparatus and production of the SGs in *Stevia* cultures suggests that synthesis of isoprenoids in *Stevia* chloroplasts is somewhat related to production in them of the SGs. Therefore, the production of both isoprenoids and the SGs in *Stevia* chloroplasts could be stimulated upon increasing the concentration of precursors of these compounds.

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