RESEARCH ARTICLE

Impact of cultivation factors in vitro on the growth and the biosynthesis of steviol glycosides in *Stevia rebaudiana* cell cultures

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Abstract The growth and the biosynthesis of steviol glycosides (SGs) in *Stevia rebaudiana* cell cultures subjected to the impact of different cultivation factors were investigated under in vitro conditions. The changes in composition and concentration of constituted components of the nutrient medium was found to exert a significant influence on the of cell culture growth and the biosynthesis of SGs. It was found that combination of growth regulators in the nutrient medium is more important factor for proliferation of isolated cells of *S. rebaudiana* while the level of SGs accumulation depends to greater extent on genotypical features of cell strain. Light was shown to activate the biosynthesis of SGs, providing evidence for the suggestion about chloroplasts participation in this activation. In morphogenic callus cultures, the content of SGs was about ten times higher, indicating that the level of the accumulation of the compounds in question depends on cell differentiation and specialization.

Keywords Cell culture · Steviol glycosides · Cultivation factors · In vitro · *Stevia rebaudiana*

Abbreviations

BAP	6-Benzylaminopurine
2,4-D	2,4-Dichlorophenoxyacetic acid
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige and Skoog
NAA	1-Naphthalene acetic acid
SGs	Steviol glycosides

Introduction

Stevia (*Stevia rebaudiana* Bertoni, *Asteraceae*) is short day plant, endemic to Paraguay (Chalapathi 1997; Brandle et al. 1998; Soejarto 2002). Leaves of stevia contain a number of diterpene tetracyclic steviol-glycosides (SG) with high sweetening capability (Kingham and Soejarto 1986). By their content, major SGs are stevioside and rebaudiosides A and C (Bondarev et al. 2001, 2004, 2007). SGs exhibit unique properties, such as low calorie, the absence of toxicity and mutagenicity (Tateo 1990; Lyakhovkin et al. 1993; Matsui et al. 1996; Geuns 2000). In this connection, SGs are extremely perspective compounds as sugar substitutes for people suffering diseases like carbohydrate metabolism disorders and, especially for diabetics. Besides, SGs possess hypoglycemic properties (Gregersen et al. 2004) and are also capable of lowering blood pressure (Hsieh et al. 2003). Glycosides of stevia are also promising for wide replacement of carbohydrates in food industry and are potential remedy for public health since human diet is over-saturated by carbohydrates leading to development of serious diseases.

S. rebaudiana cell cultures are excellent model systems to elucidate a number of features inherent in metabolism of

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diterpenoid glycosides (Bondarev et al. 2001). On the other hand, the results being obtained are capable of allowing us to effectively control an accumulation of low-caloric highly sweet compounds during growth of such cultures in bioreactors.

To date, it has been established that the content of SGs in leaves of intact *S. rebaudiana* plants constitutes about 3–8% (Bondarev et al. 2001, 2004, 2007, 2012) while in the cell cultures grown in the dark, glycosides are synthesized only in trace amounts (Bondarev et al. 2001).

It is known that components of the nutrient medium as well as light illumination are capable of exerting significant impact on the of growth and biosynthesis of substances of special exchange in plant cultures in vitro (Koppel and Butenko 1992; Butenko 1999; Bondarev et al. 2003) including *S. rebaudiana* cell cultures (Bondarev et al. 2001). Earlier it was found that essential components of the nutrient media for growth of cultivated stevia cells are mineral salts being contained in the MS medium and also plant growth regulators (Bondarev et al. 1997, 1998). Light is an essential cultivating factor as well influencing SGs biosynthesis since its intensity depends, as revealed, on development and the activity of chloroplasts (Ladygin et al. 2008). This circumstance is to be quite expected because SGs biosynthesis is initiated in plastids on alternative 1-deoxy-D-xyluloso-5-phosphate pathway of isoprenoids synthesis (Bondarev et al. 2014). In our earlier studies, an impact of nutrient medium components (Bondarev et al. 2003) and also of the parameters of light irradiation on development of *S. rebaudiana* shoots in vitro as well as SGs biosynthesis were investigated (Bondarev and Nosov 2004; Bondarev et al. 2008, 2010). At the same time, impact of these factors on the biosynthesis of glycosides in cultivated cells of stevia was studied only to slight extent (Bondarev et al. 2001; Rajasekaran et al. 2008; Janarthanam et al. 2010; Mathur and Shekhawat 2013).

Taking into account the data above, the purpose of the work presented was to reveal peculiarities of cell cultures growth of *S. rebaudiana* in vitro and of the biosynthesis of steviol glycosides when these cultures experience the action of different cultivation factors.

Materials and methods

Cell cultures

In the present work, both hetero- and mixotrophic cells of *S. rebaudiana* were used and these were grown using deep (suspension cultures) and surface (callus cultures) methods of cultivation. Callus formation was initiated using leaf blades of plants in vitro of diploid (0) and tetraploid (1) genotypes. Fixation and plant material preparation for

cytological analysis were performed according to conventional technique (Pausheva 1988). For preparation of samples related to chromosomes calculation, apical plant meristems were used. Cells of diploids (2n) and tetraploids (4n) had 22 and 44 chromosomes, respectively. Average fresh mass of explant was 4 ± 1 mg. Cell cultures were grown on liquid and agar Murashige and Scoog (MS) medium (Murashige and Skoog 1962) containing 1 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 0.5 mg l^{-1} 6-benzylaminopurine (BAP) which was used by us earlier for cultivation of isolated stevia cells (Bondarev et al. 1997, 1998). In the experiments with the plant growth regulators, callus was grown on the MS media containing optimal concentrations (2 mg l^{-1}) of auxin [indole-3-acetic acid (IAA) or NAA or indole-3-butyric acid (IBA)] and cytokinin (BAP or kinetin) for proliferation of isolated stevia cells. Average fresh mass of transplant was 35 ± 5 mg. Suspension culture was grown on shaker (90–100 rpm) in vessels with volume of 1 l containing 100 ml of liquid medium. Inoculum volume was 10 ml, dry mass was about 0.8 g l^{-1} . Cell cultivation was performed in the light with an intensity of 8 Wt m^{-2} and in the dark at 25 ± 1 °C and air humidity of about 70%. Parameters of cell culture growth (fresh mass and dry mass, viability, cell number and size) were determined according to standard techniques (Nosov 2011). In the experiments carried out, 2 weeks-old and 5 weeks-old cells were used using deep and surface their cultivations, respectively.

Analytical methods

Determination of composition and content of SGs in samples was performed by HPLC after their extraction and purification described in details earlier (Bondarev et al. 2001). This assay was conducted on chromatographic apparatus (LKB-Producter AB, Bromma, Sweden) and device Agilent 1100 (detection, UV-210 nm, volume of calibrated loop 10 μl). The samples were chromatographed on steel Ultra Pak column (TSK-OH-120, $4.6 \times 250 \text{ mm}^2$) with a particle size of 5 μm using an acetonitrile–water mixture (85:15 (v/v) at a flow rate of $0.5\text{--}1.0 \text{ ml min}^{-1}$. The content of the major SGs included stevioside, rebaudioside A and rebaudioside C was determined. Standard samples of the SGs were purchased from Arbuzov Institute of Organic and Physical Chemistry (branch of Russian Academy of Sciences, Kazan).

Statistical analysis

All results ($n > 3$) are expressed as the mean $\pm$ standard deviation. Statistical significance was analyzed using a Student *t* test. All *p* values below 0.05 were considered significant.

Results

Effect of mineral salts concentration in the nutrient medium

As followed from Fig. 1, two-fold elevating the concentration of mineral salts in the MS medium from half to standard one brought about significant increase in fresh and dry mass of the culture. The content of SGs at standard concentration of mineral salts was about ten times higher as compared to their half concentration and achieved almost 200 $\mu\text{g/g}$ dry mass (Fig. 2), and in both the variants, a major glycoside was stevioside. It needs to be noted that in the cultivated cells, composition and relationship of individual glycosides were kept to be characteristic for the donor plants (Bondarev et al. 2007).

Effect of illumination conditions

Growth of callus cultures on the light (8 Wt m^{-2}) was significantly intensified as compared to their growth in the dark, and this was expressed in 1.5-fold elevation in both fresh and dry cell biomass (Fig. 3). However, upon further elevating intensity of light flow to 30 Wt m^{-2} , sharp lowering (about two times) in parameters of culture growth was observed while at light intensity of 60 Wt m^{-2} , callus growth practically stopped.

In cells of callus tissue grown on the light, formation of many chloroplasts was noted, and callus exhibited mixotrophic properties and developed bright-green colour. Under these conditions, the biosynthesis of SGs was activated and their content in the case of light flow of 8 Wt m^{-2}

Fig. 1 Effect of mineral salts concentrations in the nutrient MS medium on accumulation of fresh and dry biomass of heterotrophic suspension stevia cell culture (strain 0). 1. $\frac{1}{2}$ of standard content; 2. standard content

Fig. 2 Effect of mineral salts concentrations in the nutrient MS medium on SGs accumulation in stevia cells upon their deep cultivation (strain 0). Symbols are the same as in Fig. 1. Standard deviations of the means did not exceed 5%

Fig. 3 Effect of light illumination conditions on accumulation of fresh and dry biomass by callus stevia cultures (strain 0). 1. dark; 2. light, 8 Wt m^{-2} ; 3. light, 30 Wt m^{-2} ; 4. light, 60 Wt m^{-2}

m^{-2} was elevated two and more times as compared to callus cultivation in the dark (Fig. 4). Here, it needs to note that in strain 1, predominant SG was rebaudioside A while in strain 0, it was stevioside.

Effect of the plant growth regulators

Efficiency of callus development is known to be determined by selection of the concentrations of auxin and cytokinin most suitable for the given culture. As found by us earlier, NAA appears to be more effective for cultivated *S. rebaudiana* cells growth as compared to 2,4-dichlorophenoxyacetic acid (2,4-D) (Bondarev et al.

Fig. 4 Effect of light illumination conditions on SGs content in callus stevia cell cultures (strains 0 and 1). Standard deviations of the means did not exceed 5%

1997, 1998). In this connection, the last auxin analogue was not used in present experiments. According to the data represented, IBA is more effective regulator of stevia callus tissue growth among auxins (Fig. 5). Parameters of cell culture growth on the media containing NAA were significantly worse. Most low growth indices were obtained on the media containing IAA.

In all variants of the experiments, composition of individual glycosides were practically not changed (Fig. 6). Total content of SGs in strain 1 was higher than in strain 0 about three times, and this elevation occurred largely due to enhancing rebaudiosides A and C biosynthesis as well to less extent stevioside. It should be noted that NAA had

Fig. 5 Effect of the plant growth regulators on accumulation of fresh and dry biomass by mixotrophic callus stevia cultures. 1. IBA/BAP (strain 0); 2. IBA/BAP (strain 1); 3. IAA/BAP (strain 1); 4. NAA/BAP (strain 1). Plant growth regulators at concentration 2 mg l^{-1}

Fig. 6 Effect of the plant growth regulators on SGs content in mixotrophic callus stevia cultures. Combinations of the plant growth regulators and their concentrations are the same as in Fig. 5. Standard deviations of the means did not exceed 5%

more pronounced effect on SGs biosynthesis as compared to that of IAA and IBA, with the strongest effect in relation to rebaudioside A synthesis.

Effect of the induction of cell culture differentiation

Under intensive light illumination (8 Wt m^{-2}), the morphogenic callus formed shoots, and in the course of this induction, the elevation of SGs took place (Fig. 7). In this callus, the content of glycosides was about two times higher than in non-morphogenic culture, while in its shoots, this value was ten times higher. The relationship of

Fig. 7 SGs content in cultures in vitro being distinguished by different extent of tissue differentiation (strain 0). 1. heterotrophic callus; 2. mixotrophic callus; 3. mixotrophic morphogenic callus; 4. shoots of morphogenic callus. Standard deviations of the means did not exceed 5%

steviol glycosides in these *S. rebaudiana* cultures in vitro was similar.

Discussion

The investigations described in present work allowed us to reveal a number of peculiarities in both *S. rebaudiana* cell cultures growth in vitro and the biosynthesis of steviol glycosides under the action of the cultivation factors.

The results obtained provide evidence that composition and relationship of SGs being practically unchanged in intact plants and shoots, in vitro upon variation of plant growing conditions (Bondarev et al. 2007) are capable of undergoing significant changes in the case of cell culture. However, it was found that in strain 1, in all experiments performed, the content of SGs was higher than in strain 0 when the first and the second ones from these were obtained from leaf blades of tetraploid and diploid genotypes, respectively. This argues in favor of the maintenance of genotypical peculiarities because the same regularity was found earlier in the content of SGs in organs of donor in vitro plants (Bondarev et al. 2007).

The revealed elevation of cultivated cells biomass in response to two-fold increase of the concentration of mineral salts in the nutrient MS medium may be associated with unlimited delivery of microelements, mainly nitrogen and phosphorus, as this was found for *Dioscorea deltoidea* (Nosov 1992) and *Ajuga reptans* (Anufrieva 1997).

A significantly lower content of SGs in the leaves when using half the concentration of mineral salts in the MS medium is due to the fact that the reduction of twice the macronutrients such as nitrogen and phosphorus leads to the cessation of the increase in the number of cells and, accordingly, biosynthesis of SGs. The accumulation of dry mass is due to cell growth, mainly due to the presence in the medium of carbohydrates. Similar results were obtained in the cultivation of *Dioscorea deltoidea* and *Ajuga reptans* (Nosov 1992; Anufrieva 1997).

It is important to note that in the case of studies of growth regulators effects, any positive correlation between cell culture growth intensity and the level of accumulation of SGs earlier found for *S. rebaudiana* shoots in vitro (Bondarev et al. 2003) is absent. It should be noted that for proliferation of isolated stevia cells, auxin/cytokinin combination was more important factor while the level of SGs accumulation depends more extent on genotypical origin of the culture.

Isolated stevia cells appeared to be far more sensitive to light irradiation than shoot cultures in vitro (Bondarev et al. 2008). Optimal intensity of light flow for callus cultures is in the range 8 Wt m^{-2} .

SGs production in mixotrophic callus lines as compared to heterotrophic cultures is significantly activated. This confirms the suggestion on participation of chloroplasts in the biosynthesis of these compounds (Bondarev et al. 2001, 2014; Ladygin et al. 2008). However, these organelles in cells of callus tissue have weakly developed membrane structure and by this reason low functional activity. In contrast, plant chloroplasts possess well developed membrane system represented by stromal and grana (from three to several tens of thylakoids in pile) thylakoids (Ladygin et al. 2008).

The content of SGs in morphogenic callus, especially in its shoots, was significantly higher than in non-differentiated cultures in vitro that argues in favor of dependence of their accumulation level on tissue differentiation extent and leaf cells specialization during its ontogenesis.

Thus, changes in composition and content of nutrient medium components (mineral salts, plant growth regulators) as well as light illumination conditions exerted significant impact on *S. rebaudiana* cell cultures growth and the biosynthesis SGs. In some variants of the experiments, these factors contributed to both cell biomass accumulation and ten-fold elevation of SGs content. However, the strongest effect on accumulation of these metabolites was caused by the differentiation and specialization of stevia cells cultivated in vitro.

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Authors contribution NB and TB carried out the in vitro experiments and the extraction of steviol glycosides, analyzed the data and wrote the manuscript. OR and MI developed the analytical method for determination of steviol glycosides and analyzed HPLC data. AN edited the version for publishing. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Anufrieva EN (1997) The growth and photosynthetic characteristics of cell cultures of *Serratula coronata* and *Ajuga reptans* as ecdysteroid producers. Cand Sci (Biol). Dissertation, Timiryazev Institute of Plant Physiology, Moscow
- Bondarev NI, Nosov AM (2004) Effect of light intensity on growth of *Stevia rebaudiana* cultures in vitro. In: Proceedings of the international conference «Topical issues of Botany and Plant Physiology», Ogarev Mordovia State University, Saransk, pp 40–41

- Bondarev NI, Nosov AM, Kornienko AV (1997) The influence of several cultural factors on the growth and efficiency of stevia callus and suspension cultures. *Biotechnol Russ* 7–8:30–37
- Bondarev NI, Nosov AM, Kornienko AV (1998) Effects of exogenous growth regulators on callusogenesis and growth of cultured cells of *Stevia rebaudiana* Bertoni. *Russ J Plant Physiol* 45:770–774
- Bondarev NI, Reshetnyak OV, Nosov AM (2001) Peculiarities of diterpenoid steviol glycoside production in in vitro cultures of *Stevia rebaudiana* Bertoni. *Plant Sci* 161(1):155–163
- Bondarev NI, Reshetnyak OV, Nosov AM (2003) Effects of nutrient medium composition on development of *Stevia rebaudiana* shoots cultivated in the roller bioreactor and their production of SGs. *Plant Sci* 165(4):845–850
- Bondarev NI, Sukhanova MA, Reshetnyak OV, Nosov AM (2004) Steviol glycoside content in different organs of *Stevia rebaudiana* Bertoni and its dynamics during ontogenesis. *Biol Plant* 47:261–264
- Bondarev NI, Reshetnyak OV, Nosov AM (2007) Growth features of various clones of *Stevia rebaudiana* Bertoni plants and accumulation of steviol glycosides in them in vivo and in vitro. *Biotechnol Russ* 1:22–28
- Bondarev NI, Reshetnyak OV, Nosov AM (2008) The effects of photoperiod and light intensity on the shoot development and steviol glycosides synthesis by *Stevia rebaudiana* Bertoni in vitro. *Izv Timiryazev Agric Acad* 4:102–107
- Bondarev NI, Reshetnyak OV, Nosov AM (2010) Effects of visible light spectral composition and UV-radiation on development of *Stevia rebaudiana* Bertoni plants in vitro and biosynthesis in them of steviol glycosides. *Scientific notes of Orel state university. Nat Tech Med Sci* 4:55–62
- Bondarev NI, Reshetnyak OV, Bondareva TA, Nosov AM (2012) Distribution and dynamic of diterpene glycosides contents in *Stevia rebaudiana* Bertoni intact plants. *Biotechnol Russ* 5:50–54
- Bondarev NI, Kurilov DV, Bondareva TA, Stomakhin AA, Nosov AM (2014) Study of electron-dense thylakoids in chloroplasts of *Stevia rebaudiana* Bertoni in relation to the biosynthesis of diterpenoids. *Biol Sect Cell Mol Biol* 69(1):70–75
- Brandle JE, Starratt AN, Gijzen M (1998) *Stevia rebaudiana*: its agricultural, biological and chemical properties. *Can J Plant Sci* 78:527–536
- Butenko RG (1999) Biology of higher plant cells in vitro and biotechnologies on their bases. FBC-Press, Moscow
- Chalapathi MK (1997) Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni). *Future Crop For India Crop Res* 14:347–350
- Geuns JMC (2000) Safety of stevia and stevioside. *Recent Res Dev Phytochem* 4:75–88
- Gregersen S, Jeppesen PB, Holst JJ, Hermansen K (2004) Antihyperglycemic effects of stevioside in type 2 diabetic subjects. *Metabolism* 53(1):73–76
- Hsieh M-H, Chan P, Sue Y-M, Liu J-C, Liang TH, Huang T-Y, Tomlinson B, Chow MSS, Kao P-F, Chen Y-J (2003) Efficacy and tolerability of oral stevioside in patients with mild essential hypertension: a two-year, randomized, placebo-controlled study. *Clin Ther* 25(11):2797–2808
- Janarthanam B, Gopalakrishnan M, Sekar T (2010) Secondary metabolite production in callus cultures of *Stevia rebaudiana* Bertoni. *Bangladesh J Sci Ind Res* 45(3):243–248
- Kinghorn AD, Soejarto DD (1986) Sweetening agents of plant origin. *Crit Rev Plant Sci* 4(2):79–120
- Koppel LA, Butenko RG (1992) Development of tomato apical meristems in vitro under the influence of light with different spectral composition. *Sov Plant Physiol* 39(2):223–231
- Ladygin V, Bondarev N, Semenova G, Smolov A, Reshetnyak O, Nosov A (2008) Chloroplast ultrastructure, photosynthetic apparatus activities and production of steviol glycosides in *Stevia rebaudiana* in vivo and in vitro. *Biol Plant* 52:9–16
- Lyakhovkin AG, Long TD, Titov DA, Anh MP (1993) Cultivation and utilization of stevia (*Stevia rebaudiana* Bertoni). Agricultural publishing house, Hanoi
- Mathur S, Shekhawat GS (2013) Establishment and characterization of *Stevia rebaudiana* (Bertoni) cell suspension culture: an in vitro approach production of stevioside. *Acta Physiol Plant* 35:931–939
- Matsui M, Matsui K, Kawasaki Y, Oda Y, Noguchi T, Kitagawa Y, Sawada M, Hayashi M, Nohmi T, Yoshihira K, Ishidate M, Sofuni T (1996) Evaluation the genotoxicity of stevioside and steviol using six in vitro and one in vivo mutagenicity assays. *Mutagenesis* 11(6):573–579
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15(13):473–497
- Nosov AM (1992) Steroid metabolism features in cultured *Dioscorea deltoidea* cells, as a basis for obtaining biotechnology of furostanol glycosides. Doctoral (Biol) Dissertation. Institute of the Applied Biochemistry and Machine-Building, Moscow
- Nosov AM (2011) Assessment methods and characteristics of growth rate of the higher plants cells. In: Kuznetsov VIV, Kusnetsov VV, Romanov GA (eds) *Molecular genetic and biochemical methods in modern plant biology*. BINOM, Laboratory of Knowledge, Moscow, pp 386–402
- Pausheva ZP (1988) Plant cytology practical training. Agropromizdat, Moscow
- Rajasekaran T, Ramakrishna A, Udaya Sankar K, Giridhar P, Ravishankar GA (2008) Analysis of predominant steviosides in *Stevia rebaudiana* Bertoni by liquid chromatography/electrospray ionization-mass spectrometry. *Food Biotechnol* 22(2):179–188
- Soejarto DD (2002) Ethnobotany of *Stevia* and *Stevia rebaudiana*. In: Kinghorn AD (ed) *Stevia: the genus Stevia*. Taylor & Francis, London, pp 40–67
- Tateo F (1990) Technological and toxicological problems connected with the formulation of low-calorie foods. *Riv Soc Ital Sci Aliment* 19(1–2):13–23

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