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Effects of Cultivation Parameters of *Stevia rebaudiana* Bertoni Callus Culture on Callus Proliferation

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ABSTRACT

Callus culture of *Stevia rebaudiana* Bertoni induced from apical buds of sterile plants was the focus of this investigation. The most intensive growth of *Stevia* callus (from 17 to 19 mg/week) occurred on modified Murashige and Skoog (MS) medium supplemented with 1-1.5 mg/l 2,4-dichlorphenoxy acetic acid (2,4-D) and 0.1 mg/l 6-benzylaminopurine (BAP). The effectiveness of callus growth is equal in both the light and dark. Adventitious roots could be obtained from callus masses on MS medium supplemented with 1 mg/l α-naphthylene acetic acid, while shoots formed from callus on MS medium supplemented with 0.1 mg/l 2,4-D + 0.5 mg/l BAP. Glycosides, which are the important medicinally-important secondary metabolites from *Stevia*, were organoleptically detected in root- and shoot-regenerating calluses. Organogenesis may be an indispensable requirement for glycoside synthesis.

Keywords: *Stevia rebaudiana*, *in vitro,* callus culture, growth rate, growth regulators, sweet glycoside
Abbreviations: 2,4-D, 2,4-dichlorphenoxiacetic acid; **6-BAP**, 6-benzylaminopurine; **MS**, Murashige and Skoog medium; **NAA**, α-naphthylene acetic acid; **PGR**, plant growth regulator; **SG**, steviol glycosides

INTRODUCTION

Sucrose derived from sugar-beet and sugar cane is still the main natural food sweetener. However its excessive consumption may have a negative impact on human health, especially for patients suffering from the diseases related to disturbances in sugar metabolism (Kingdorn and Soejarto 1986; Lee and Balick 2002). In Russia about eight million diabetics are registered, for whom sucrose is categorically forbidden. In this respect finding a suitable replacement for sucrose by other sweeteners which are harmless to people is difficult. Although sweet alcohols (xylitol, sorbitol, mannitol) do not increase the blood sugar content, acquired tolerance to them does occur. In addition, these traditional sugar substitutes have a high energetic value and are also undesirable for people with disturbed carbohydrate metabolism, obesity, atherosclerosis, pancreatite and other diseases (Crammer and Ikan 1986). Synthetic sweetener aspartame also has negative secondary effects. The absence of a valuable substitute for sugar has resulted in an intensive search for new sources of sweet, harmless substances, including those from plants (Lee and Balick 2002).

The perennial herbaceous bush *Stevia rebaudiana* Bertoni shows very positive perspectives in this field, containing a mix of glycosides, whose sweetness is 100-400 times superior to sucrose (Handro and Ferreira 1989; Meireles *et al*. 2006). The harmlessness of sweet glycosides isolated from *Stevia* was established for humans (Haebisch 1992; Geuns 2003) while the JECFA (Joint Expert Committee on Food Additives) in 2004 claimed that a temporary "acceptable daily intake" of 2 mg/kg of body weight is acceptable (for paradoxes between research results on safety of steviol glycosides in laboratory and clinical trials, see discussion in Meireles *et al*. 2006).

S. rebaudiana, of the family *Compositae* is a perennial bush, about one meter high, thin, but tough stems, branching on top. Leaves are opposite, inversely-lanceous, 55-65 mm long, 13-21 mm wide; inflorescences are a panicle with small-sized flowers, collected in small strings (Lyakhovkin *et al*. 1993).

S. rebaudiana is endemic and wild species in Paraguay and Southern Brasil. The agroecological features of this plant are well studied (Andolfi *et al*. 2002), and methods of *Stevia* cultivation as an annual culture are also developed (Meireles *et al*. 2006). *Stevia*'s natural growth is in a tropical climate of Latin America, characterized by excessive rain in summer, and their cultivation in regions with poor rainfall result in reduced yield (Brandle *et al*. 1998; Meireles *et al*. 2006).

In terms of light, *Stevia* is a short-day plant with critical flowering day length between 12 and 14 hours (Valio and Rocha 1977; Zaidan *et al*. 1980). This plant also has high heat requirements. During the vegetative phase the optimum mean diurnal temperature is 23-30°C for formation of a green crop. At temperatures lower than 13-15°C *Stevia* growth and development are notably inhibited, and below +8°C plants are irrevocably lost (Sumida 1980).

In the wild *Stevia* multiplies by seeds, but their reproduction requires specific tropical conditions since *Stevia* plants are adapted to unremitting water consumption in the vegetative phase and to heat. The difficulty of working with seed is that they are very small, air-dispersed and lose their germinating capacity quickly (Ching and Goettemoeller 1998). *Stevia* seeds do not ripen and mature even in Japan where subtropical climate prevails (Sumida 1980). Therefore a simpler and commonly used method is reproduction by green cuttings (Acuna *et al*. 1997; Truong and Valicek 1998). It is possible to prepare up to 200 green cuttings from a single adult plant (Murayama *et al*. 1980). A very efficient method has been established by Hwang (2006) using mature explants of *S. rebaudiana*. Adventinous shoots were induced from nodal explants of plants on MS medium containing 2 mg/l indole-3-acetic acid (IAA) and 0.5 mg/l kinetin (23 ± 2 shoots per explant).

The value of *S. rebaudiana* lies in the availability in leaves of substances with a high sweetening ability, whose

Received: 19 February, 2007. Accepted: 29 April, 2007.

Original Research Paper

physical, chemical, pharmacological and toxicological characteristics allow it to be used in human diets without secondary effects (Haebisch 1992; Geuns 2003). The first isolated component was stevioside, which has a sweetening ability approximately 300 times higher than sucrose (Adduci *et al*. 1987). Other sweet components were described later: rebaudioside A, B, C, D, E, dulcoside A and steviolbioside, the sweetening abilities ranging from 100 to 4,000 times higher than sucrose (Handro and Ferreira 1989). Stevioside and rebaudioside A and C exist in small amounts – 6.6%, 3.7% and 2.1% from dry weight of leaves, respectively (Kinghorn and Soejarto 1986). Eight sweet diterpenoid tetracyclic glycosides have been identified in total, with aglicon steviol as a general component (Makapugay *et al*. 1984; Swanson *et al*. 1992). Substances from *Stevia* leaves which give it its sweetness are broadly termed "steviosides" (steviol glycosides, SG). Studies of the SG contents of *S. rebaudiana* have shown that stevioside is present in all parts of the plant (except for the roots), but greater contents are found in leaf blades, less in inflorescences, and little in stems (Zaidan *et al.* 1980).

The total quantitative contents of diterpene glycosides in leaves oscillated greatly – from 6 to 16% on a dry weight basis; this is explained by timing of leaf collection and by the plant's developmental phase (Kolb *et al*. 2001). It is known that the glycoside content in the vegetative and reproductive organs of *Stevia* varied according to the phase of plant development (Bondarev *et al*. 2004). The highest amount of SG is inherent in the upper, actively growing shoot sections, whereas lower, senescent shoot sections have the lowest SG content. During ontogenesis, leaves and stems of *Stevia* gradually accumulate SG up to the budding phase and the onset of flowering; during the fruit development stage the amount of SG was found to revert to the initial level (Bondarev *et al*. 2004). The plants which have been growing in natural conditions have a higher stevioside content compared to plants grown in greenhouses (Zaidan *et al*. 1980). So, in *Stevia* plants which are grown in the glasshouse (at least in Russia) the highest total content of the SG was detected in leaves (about 34 mg/g d.m.), whereas their content in flowers and stems was 7-8– and 12-13–fold lower (about 5.3 mg/g d.m. and 2.9 mg/g d.m. respectively). The seeds and roots were found to have the lowest SG content – about 2.1 mg/g d.m. and less than 1 mg/g d.m., respectively (Bondarev *et al*. 2004).

As *Stevia* plants are very chilling-sensitive, their cultivation in open ground is inconvenient, and now the main emphasis of *Stevia* research is on *Stevia* cultivation *in vitro* (Hsing *et al*. 1983; Ferreira and Handro 1986, 1987; Bondarev *et al*. 2001; Sivaram and Mukundan 2003). All researchers used Murashige and Skoog (1962) basal medium with or without agar (solid or liquid media, respectively), supplemented with different growth regulators. This method can be applied to accelerate the mass production of *Stevia* (Truong and Valicek 1999); furthermore, this process allows the generation of genetically homogeneous and pathogen-free descendants – about 100,000 plants from one initial cutting per year. Tissue culture is a viable alternative to the usual methods of *Stevia* production, cultivation and selection and is an excellent way of consistently obtaining second metabolites in a season-dependent manner (Hsing *et al*. 1983; Ferreira and Handro 1986, 1987; Meireles *et al*. 2006). Cell and tissue culture *in vitro* have many advantages for *Stevia* culture: independence from climate; year-round production; ecologically clean production; capacity for process automation (Takayama and Akita 1994; Meireles *et al*. 2006). However in these conditions the synthesis of secondary metabolites, including glycosides is frequently affected (Swanson *et al*. 1992). In cell cultures the quantitative contents of SG is reduced by two orders compared with tube plants, and by three orders compared to plantation plants (Bondarev *et al.* 2001). Thus the spectrum of synthesized SG became poorer (Bondarev *et al*. 2004).

The purpose of this study was to optimize conditions for *Stevia rebandiana* callus cultivation with a view to maximizing the growth of tissue that contains glycosides. To achieve this, we established the optimum plant growth regulator (PGR) content in the medium (to increase callus), we determined the effect of light on culture growth, and finally explored the conditions that most promoted organogenesis from callus tissue. All of these ultimately to learn the effect of *in vitro* culture parameters on the availability of glycosides in *Stevia* callus.

MATERIALS AND METHODS

The material for this work was *S. rebaudlana* Bertoni callus culture obtained from the Institute of Plant Physiology RAS (Department of Cell Engineering and Biotechnology). This callus culture was induced from clone 0 created in the All-Russian Institute of Sugar-Beet (the Brazilian line has served as the basic material for this clone). Callus culture was induced from apical buds of sterile plants (sterilized by 0.03-0.04% (v/v) sulema (Moskow, Russia).

Effect of a medium hormonal composition on callus growth

For *Stevia* callus culture all basal nutrient media used Murashige and Skoog (MS) macro- and microsalts and vitamins (Murashige and Skoog 1962) with sucrose (30 g/l) and agar (ICN, USA) (0.9%), pH 5.5. Different combinations of PGRs were tested: 2,4-dichlorphenoxy acetic acid (2,4-D) from 1.0 up to 2.0 mg/l, α-naphthylene acetic acid (NAA) from 0.1 up to 1.0 mg/l and 6-benzylaminopurine (6-BAP) from 0.1 up to 1.0 mg/l.

Callus was cultured in the light (photon flux density 180 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and in the dark (in a thermostat) at 29°C for 4 weeks. Callus cultures were viewed weekly and characterized by evaluating the colour, consistency and growth rate (all measurements were visual). The increment in callus over four week culture in different conditions was determined by the difference between final and initial fresh weights (defined by weighing on analytical scales).

Organogenesis from callus culture

For assessment of their organogenic potential the pieces of calluses (mass of each about 50 mg) were transferred to cytokinin-free MS medium containing 1 mg/l NAA (for root formation) or 0.1 mg/l NAA + 0.5 mg/l 6-BAP (for shoot formation). Cultivation conditions and growth parameters were assessed as described above.

Statistical analyses

All experiments were repeated 5 times each, each experiment consisting of 5 calluses. For all measurements averages and standard errors were calculated by standard mathematical means using Microsoft Excel. The differences between the means were assessed by Tukey's method.

RESULTS AND DISCUSSION

The successful cultivation of plant tissues *in vitro* requires the selection of an optimal medium and often depends on the ratio of PGRs. In this work we used MS medium supplemented with different variations of auxins and cytokinins, and the effect of light vs. dark to establish efficient callus induction and proliferation, and shoot and root induction for *Stevia rebaudiana*, an important medicinal and nutrient plant.

Growth of *Stevia* calluses

The characteristic of calluses cultivated in the darkness is described in **Table 1**. In this experiment we used 12 variants of three PGR combinations (2,4-D, NAA, 6-BAP). The most intense growth of callus was detected on media supplemented with 1 mg/l 2,4-D + 0.1 mg/l 6-BAP as well as on media supplemented with 1.5 mg/l 2,4-D + 0.1 mg/l 6-BAP. Less intense callus growth was noted on media supplemented with 0.1-0.5 mg/l 2,4-D + 0.1-0.5 mg/l 6-BAP, and also on media containing NAA (instead of 2,4-D). Cal-

Table 1 The characteristics of *Stevia rebaudiana* callus cultivated in the dark in a thermostat at 29°C.

Medium	Plant growth regulators (mg/l)			Callus characteristics		
variant No.	2,4-D	NAA	6-BAP	Colour	Consistency	Proliferation response
	-	-	-	nc	nc	nc
1	1.0	-	-	yellow	friable	++
2	1.0	-	0.1	yellow	friable	++++
3	1.5	-	0.1	yellow	friable	++++
4	1.5	-	-	yellow	friable	+
5	2.0	-	-	yellow	friable	+
6	1.0	-	0.5	yellow	friable	++
7	1.5	-	0.5	yellow	friable	++
8	2.0	-	0.5	yellow	friable	++
9	2.0	-	1.0	yellow	friable	+
10	0.5	-	0.1	yellow	friable	+++
11	-	0.5	0.1	yellow	friable	++
12	-	1.0	0.1	yellow	friable	++

+ Very poor callus growth; ++ poor callus growth; +++ intensive callus growth; ++++ very intensive callus growth; nc = not conducted.

Table 2 The characteristics of *Stevia rebaudiana* callus cultivated in the light (intensity of illumination 180 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a thermostat at 29°C.

Medium	Plant growth regulator concentration (mg/l)		Callus characteristics		
variant No.	2,4-D	6-BAP	Colour	Consistency	Proliferation response
	0	0	nc	nc	nc
1	1.0	0.1	light green	friable	++++
2	1.5	0.1	light green	friable	+++
3	1.0	0.5	light green	friable	++
4	1.5	0.5	light green	friable	++
5	2.0	0.5	light green	friable	++
6	2.0	1.0	light green	friable	+

+ Very poor callus growth; ++ poor callus growth; +++ intensive callus growth; ++++ very intensive callus growth; nc = not conducted.

Table 3 Effect of a growth regulators ratio in MS media on increment of fresh weight in *Stevia rebaudiana* Bertoni callus tissue cultivated in darkness.

Medium	Plant growth regulator concentration (mg/l)			Fresh weight after 4 weeks	Weight increment	Weight increment
variant No.	2,4-D	NAA	6-BAP	(mg)	(mg)	(% of initial weight)
	-	-	-	nc	nc	nc
1	1.0	-	-	89 ± 3^{c}	39 ± 2^{bc}	78^{cd}
2	1.5	-	-	87 ± 7^{c}	37 ± 5^{bc}	74^{d}
3	2.0	-	-	79 ± 5^{c}	29 ± 3^{c}	58^{d}
4	0.5	-	0.1	102 ± 3^{b}	52 ± 7^{b}	104^{bc}
5	1.0	-	0.1	127 ± 8^{a}	77 ± 7^{a}	154^{a}
6	1.5	-	0.1	118 ± 9^{ab}	68 ± 5^{ab}	136^{ab}
7	1.0	-	0.5	92 ± 4^{bc}	42 ± 2^{b}	84^{cd}
8	1.5	-	0.5	95 ± 5^{bc}	45 ± 5^{b}	90^{c}
9	2.0	-	0.5	90 ± 5^{bc}	40 ± 3^{bc}	80^{cd}
10	2.0	-	1.0	81 ± 3^{c}	31 ± 3^{c}	62^{d}
11	0.1	-	0.5	108 ± 5^{ab}	58 ± 5^{ab}	116^{b}
12	-	0.5	0.1	93 ± 8^{bc}	43 ± 6^{bc}	86^{cd}
13	-	1.0	0.1	96 ± 7^{bc}	46 ± 4^{b}	92^{c}
14	-	1.0	-	104 ± 12^{abc}	54 ± 9^{ab}	108^{bc}

Superscripts in each column indicate statistical significance according to Tukey's test ($P<0.05$).

lus grew very poorly on media containing only auxins, and on media supplemented with high 2,4-D and 6-BAP concentrations (2 mg/l and 1 mg/l, respectively). In all growth conditions calluses were uniformly yellow in colour and with a friable consistency, irrespective of growth intensity.

The analysis of the effect of light on the growth of callus culture using six combinations of PGRs in medium is summarized in **Table 2**. Callus cultured in the light on MS supplemented with 1-1.5 mg/l 2,4-D + 0.1 mg/l 6-BAP showed the most intensive growth. Slowest callus growth occurred on MS supplemented with 2 mg/l 2,4-D and 1 mg/l 6-BAP. All calluses growing in the light was friable and light green.

Stevia callus cultures growing in the dark and light only differed in colour: light green in the light, yellow in the dark. As their consistency did not vary, and callus growth in both was practically identical, we propose that illumination is not essential for proliferating *Stevia* callus *in vitro*. The organoleptic determination showed that calluses growing in the light or dark contained only traces of stevioside irrespective of PGR concentration in the medium (data not shown).

We also wished to assess the optimal PGR ratio in the medium to increment *S. rebaudiana* callus biomass. Separate calluses following four weeks culture were weighed on analytical scales before subculturing (**Table 3**). Callus proliferation was most intense when 2,4-D and 6-BAP were used. Large and friable calluses were obtained in the dark, with maximum biomass on MS media supplemented with 1 or 1.5 mg/l 2,4-D + 0,1 mg/l 6-BAP. Callus fresh weight increased 154% and 136% relative to the initial weight, respectively after four weeks of culture. The smallest increase of callus fresh weight was on media containing high doses (2.0 mg/l) of 2,4-D (58-80%), and also on media containing 0.5-1.0 mg/l NAA (86-108%). Increasing 6-BAP concentration in MS media up to 0.5 mg/l or cytokinin-free media negatively affected the increase in callus fresh weight, showing only 80-116% and 58-108% increase, respectively.

Hsing *et al*. (1983) used modified MS medium supplemented with 2 mg/l NAA and 2 mg/l kinetin to induce callus. The cultures could be maintained at 26 ± 2°C with diffused light with a 16 h photoperiod. Ferreira and Handro (1987) induced callusogenesis on *Stevia* leaf disks on basic MS medium containing 0.2 mg/l benzyladenine (BA) and 0.8 mg/l 2,4-D. Then callus pieces were repeatedly sub-cultured on medium containing 0.5 mg/l BA and 0.5 mg/l 2,4-

Table 4 Organogenesis induction in *Stevia rebaudiana* Bertoni callus cultures.

Medium variant No.	Illumination	Plant growth regulator concentration (mg/l)			Callus characteristics			Organogenesis
		2,4-D	NAA	6-BAP	Colour	Consistence	Growth rate	
		-	-	-	nc	nc	nc	nc
1	dark	-	1.0	-	yellow	friable	+++	rhizogenesis
2	dark	0.1	-	0.5	yellow	friable	+++	shoot formation
3	180 micromol·m^{-2}·s^{-1}	-	0.1	0.5	light green	friable	++	shoot formation

+++ Intensive callus growth; ++ Poor callus growth; nc = not conducted.

D with or without the addition 1.0 mg/l gibberellic acid (GA_3). Different PGR ratios and different combinations of light (daylight, fluorescent lamps and incandescent lamp) or darkness, at 26°C were tested. Callus formation was noted in several treatments and was best when BA was combined with 2,4-D. Large and more friable callus was obtained in the dark, on media supplemented with GA_3 in combination with 0.2 mg/l 2,4-D and 0.8 mg/l BA. After 40 days of culture, fresh and dry callus weight increased by 45.6% and 25.0%, respectively (Ferreira and Handro 1987). Friable calluses with a high growth rate are the best material for initiating cell suspension cultures (Ferreira and Handro 1986).

Induction of organogenesis from *Stevia* callus tissue

To induce organ differentiation in *Stevia* callus tissue we changed the PGR concentration in the medium (**Table 4**). Callus lines cultured on medium spiked with auxin (1 mg/l NAA) without cytokinins (on light or darkness) exhibited weak rhizogenesis. Medium supplemented with 0.1 mg/l 2,4-D + 0.5 mg/l 6-BAP weakly induced shoots. Thus the consistence of calluses and colour did not vary. The link between callus type and shoot regeneration could not be detected as calluses from all previous treatments had very similar shoot regeneration.

Preliminary, organoleptic detection of glycosides showed that callus was mildly sweet (independent of the medium and plant growth regulator combination). Although speculative, we propose that only shoot- and/or root-forming cultures are capable of *de novo* biosynthesis of the aglicon particle stevioside and that both kinds of cultures (shoot- and root-forming) are capable of glycosilation responses. We confirm that the stevioside biosynthesis is a function of tissue differentiation (Swanson 1989). In studies in which SG content was quantified, Bondarev *et al.* (2001) showed that total SG content in *Stevia* leaves was 5900 µg/g d.w., 1500 µg/g d.w. in stems, 0 µg/g d.w. in callus, 15 µg/g d.w. in suspension culture, 83µg/g d.w. in morphogenic callus, and 552 µg/g d.w. in shoots derived from morphogenic callus.

Stevia callus proliferation: time-scale perspective

To determine whether *Stevia* callus culture proliferation depended on cultivation duration or not, we analyzed callus with very intense growth (Line 1; growing on MS medium supplemented with 1 mg/l 2,4-D and 0.1 mg/l 6-BAP), and callus with less intense growth (Line 2; cultivated on MS medium supplemented with 0.5 mg/l 2,4-D and 0.1 mg/l 6-BAP).

The increase in callus fresh weight was observed over four weeks. The initial fresh weight of callus pieces was identical (50 mg). In Line 1 callus the weight increased to 55 mg in one week, 75 mg in two weeks, 97 mg in 3 weeks and 127 mg in four. In Line 2 the increment in biomass after one week was more than in Line 1, and had reached 10 mg; the increase in biomass after two weeks of growth was also marked (increment 31 mg), but the callus failed to proliferate; final callus weight of Line 2 was only 102 mg after four weeks.

CONCLUSIONS

The culture of *Stevia rebaudiana* Bertoni callus requires media with defined PGR combinations, creating thus optimum conditions for proliferation and shoot formation. *Stevia* callus culture can be used to obtain sweet glycosides. For this purpose it is necessary to develop a two-step indirect organogenic pathway, primarily through the production and proliferation of callus –whose growth and proliferation is influenced by light, temperature and the genetic background of callus lines – and finally through the implementation of root- and/or shoot-inducing/forming media. Although not quantified (except for organoleptic assessment) we hypothesize that the switch from callogenic to organogenic pathway would increase the level of glycosides in callus tissue.

ACKNOWLEDGEMENTS

The authors wish to thank the technical assistance of K. Shima.

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