Examination of Steviol Glucosides Production by Hairy Root and Shoot Cultures of Stevia rebaudiana

Takeshi Yamazaki, Hector E. Flores,
Koichiro Shimomura, and Kunitoshi Yoshihira

J. Nat. Prod., **1991**, 54 (4), 986-992• DOI:
10.1021/np50076a010 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 54, No. 4, pp. 986-992, Jul-Aug 1991

EXAMINATION OF STEVIOL GLUCOSIDES PRODUCTION BY HAIRY ROOT AND SHOOT CULTURES OF *STEVIA REBAUDIANA*

TAKESHI YAMAZAKI,[1] HECTOR E. FLORES,*

Department of Plant Pathology and Biotechnology Institute, Pennsylvania State University, University Park, Pennsylvania 16802

KOICHIRO SHIMOMURA,

Tsukuba Medicinal Plant Research Station, National Institute of Hygienic Sciences, 1 Hachimandai, Tsukuba, Ibaraki 305, Japan

and KUNITOSHI YOSHIHIRA

Division of Food Additivies, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan

ABSTRACT.—"Hairy root" cultures of *Stevia rebaudiana*, whose leaves contain the sweet diterpene glycoside stevioside among other steviol glucosides, were induced upon infection with *Agrobacterium rhizogenes* strain 15834. Steviol glucosides were not produced by the hairy root cultures in the dark. Addition of cytokinins (kinetin and benzyladenine) to solid medium resulted in a morphological change of hairy root cultures in the light into callus. Stevioside was not detected in the cultures. The primary shoot tip cultures from the in vitro cultured plantlets produced stevioside in liquid medium in the light. Our results suggest that the organ for the synthesis site of steviol glucosides is leaves, not roots in the whole plants. The shoot tip culture of *S. rebaudiana* in liquid medium may be useful to produce stevioside.

The leaves of *Stevia rebaudiana* Bertoni (Compositae) contain the diterpene glucosides stevioside and rebaudioside A, among others, which are used as natural sweeteners (1). Micropropagation of *S. rebaudiana* (2,3) and the formation of stevioside in callus culture (4) have been reported. However, the formation of stevioside in organ cultures in vitro has not been reported. It is not yet known which plant organ is the site of synthesis of stevioside and/or its precursors containing steviol.

The aglycone of the sweet diterpene glucosides, steviol (13-hydroxy-*ent*-kaurenoic acid), is biosynthesized from *ent*-kaurenoic acid, which is known to be an intermediate in gibberellin biosynthesis. It is thought that steviol is synthesized through the same pathway as gibberellin and is substituted with glucose (5).

We have established "hairy root" cultures of *S. rebaudiana* upon infection with *Agrobacterium rhizogenes* and studied the synthesis of steviol glucosides in these systems as a production system of sweet compounds. No steviol glucoside was detected in the hairy roots cultured either on solid or in liquid 1/2 Murashige and Skoog's (MS) and White media without phytohormone in the dark. We then examined whether a morphological change of the hairy root cultures into calli or shoots in the light induces or enhances the formation of stevioside. In addition, we examined the effects of phytohormones on the various cultures and the production of steviol glucosides.

RESULTS AND DISCUSSION

DISTRIBUTION OF STEVIOL GLUCOSIDES IN *S. REBAUDIANA* PLANTLETS IN VITRO.—In the cultured plantlets, stevioside and rebaudioside A were detected in the leaves (stevioside 12.9 mg/g fresh wt, rebaudioside A 95 μg/g fresh wt) and the stems (stevioside 2.1 mg/g fresh wt, rebaudioside A 5μg/g fresh wt) but not in the roots.

[1]Present address: Division of Food Additives, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan.

TABLE 1. Effects of Benzyladenine on Light-grown Hairy Root Cultures of *Stevia rebaudiana*.[a]

	Benzyladenine (μM)								
	0	0.1	0.5	1	3	5	10	30	100
Root Formation[b]	+++	+++	+++	+++	+/++	+/−	−	−	−
Callus Formation[c]	−	−/+	+	+/++	+/++	++	+++	+++	++/+++
Steviolbioside-like Steviol Glucosides (μg/g fresh wt)									
in Clone #3	3	ND[d]	2	2	ND	ND	ND	ND	ND
in Clone #16	8	5	6	6	6	5	ND	ND	ND
in Clone #38	13	11	ND	11	ND	ND	ND	ND	ND

[a]Hairy roots (1 g fresh wt/plate) of clones #3, #16, and #38 were cultured on solid 1/2 Murashige-Skoog medium containing benzyladenine in 18 h light/6 h dark for 11 weeks.

[b]Root Formation: −, not formed; +, poor; ++, moderate; +++, confluent.

[c]Callus Formation: −, not formed; +, swelling of roots; ++, blocks of calli; +++, large calli.

[d]ND: not detected.

Steviolbioside, the precursor of stevioside, was detected in all the three organs (275 μg/g fresh wt in leaves, 265 μg/g fresh wt in stems, and 17 μg/g fresh wt in roots).

EFFECTS OF HIGH SUCROSE CONCENTRATION ON HAIRY ROOT CULTURES IN LIQUID MEDIUM.—No steviol glucoside was detected in the hairy roots (6 clones) cultures in liquid 1/2 MS and White media containing 2% sucrose without phytohormone in the dark for a month.

It has been reported that osmotic stress affects secondary metabolism in tissue cultures from some species of plants (6). We examined the effects of sucrose concentration on the hairy root cultures that were cultured in liquid medium containing 2–14% sucrose in the dark for 4 weeks and harvested for analysis. High concentrations of sucrose (5–11%) resulted in increased fresh wt of the hairy root cultures grown in 1/2 MS medium. The cultures in the 8% sucrose medium reached a 3.3 times higher fresh wt than the cultures in the 2% sucrose medium. The cultures in 14% sucrose had one-half the fresh wt of the cultures in 2% sucrose medium. Sucrose concentration had no effect on the growth of the hairy root cultures in White medium. It appears that osmotic stress was not able to induce the synthesis of steviol glucosides, because no steviol glucoside was produced in the hairy root cultures in 1/2 MS and White media containing any concentration of sucrose.

EFFECTS OF PHYTOHORMONES ON HAIRY ROOT CULTURES ON SOLID MEDIUM IN THE LIGHT.—No steviol glucoside was detected in the hairy roots (16 clones) cultured on solid 1/2 MS and White media in the dark for 2 months. It has been reported that "green hairy roots" from some plant species grown in the light produce higher levels of useful secondary metabolites characteristic of aerial parts than hairy roots grown in the dark (7–9). Photosynthetic/photoautotrophic root cultures may thus be made to produce secondary metabolites. We examined the cultures of *S. rebaudiana* hairy roots on solid 1/2 MS and White media in the light. No steviol glucoside was detected in the green hairy root cultures.

We examined whether a morphological change of the hairy root cultures into calli or shoots in the light induces the formation of stevioside. The hairy roots were cultured on solid 1/2 MS medium containing 0–100 μM benzyladenine (BA) in the light for 11 weeks and harvested for analysis. Addition of BA (≥1 μM BA) to 1/2 MS medium resulted in a morphological change of the hairy root cultures into calli (Table 1), decreased fresh wt, and decreased chlorophyll levels (Figure 1). No shoot nor bud formation occurred in any cultures. A trace amount of "steviolbioside-like compound" was detected by hplc, though no steviol glucoside was detected to tlc. We were not able to identify the "steviolbioside-like compound," because the amount of it was too little to measure the absorption spectra of the peaks on chromatograms that had the retention time corresponding to steviolbioside. The ratio of the absorbance of steviol glucosides at 220 nm to that at 200 nm (reference: air) was 5%. When the small peaks on the chromatograms at 200 nm had a retention time corresponding to steviolbioside and had no or negligible peaks on the chromatograms at 220 nm, we considered the peaks "steviolbioside-like compound." No other steviol glucosides were detected by hplc. These results show that BA did not induce the synthesis of steviol glucosides. Addition of kinetin (≥30 μM kinetin) also resulted in a similar morphological change and had no effect on the induction of steviol glucoside synthesis. In both experiments, the roots showing small or moderate amounts of callus induced by exogenous cytokinins showed a trace level of "steviolbioside-like compound," but from the viewpoint of the production method of steviol glucosides, the amount is negligible. The complete morphological change of the hairy roots into callus resulted in decreased activity in growth and biosynthesis. The biosynthesis of stevioside was not induced in any light-grown hairy root cultures.

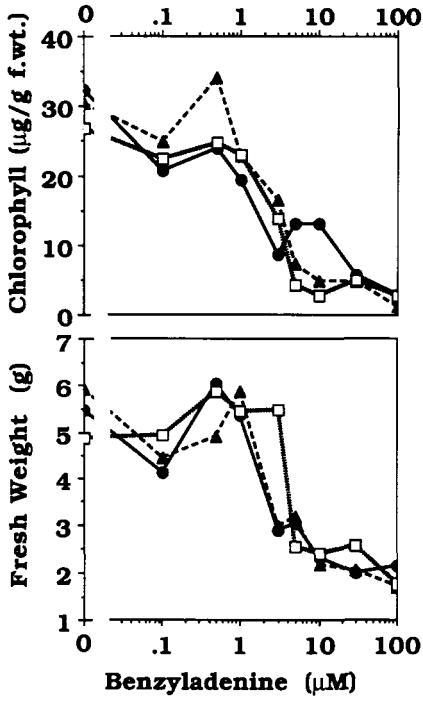

FIGURE 1. Effects of benzyladenine on *Stevia rebaudiana* hairy root cultures. The hairy roots (1 g fresh wt/plate) of clones #3 (●), #16 (▲), and #38 (□) were cultured on solid 1/2 MS medium containing benzyladenine in 18 h light/6 h dark for 11 weeks and harvested for analysis. The chlorophyll content was measured by the spectrophotometrical method of Harborne (10).

INDUCTION OF *S. REBAUDIANA* CALLUS CULTURES.—We investigated the formation of stevioside in callus cultures induced on solid MS medium containing 10 μM α-naphthaleneacetic acid (NAA) and 1 μM BA in the light as reported by Lee *et al.* (4). They detected stevioside in the extracts from callus cultures by *n*-BuOH partition and tlc analysis. We found that a large amount of sucrose in the extracts from the callus or root cultures interfered with the development of compounds, including stevioside, on tlc plates even after *n*-BuOH partition. In addition, some unknown compounds in the extracts showed R_f values similar to those of steviol glucosides and grayish-green color (characteristic of sugars and glucosides) with the vanillin-sulfuric acid reagent (10). In order to remove a large amount of sucrose and the unknown compounds, silanized Si gel cc was used, followed by tlc and hplc analyses. We were not able to reproduce the stevioside production by callus cultures reported by Lee *et al.* (4). It appeared that tlc analysis of the extracts purified only by *n*-BuOH partition may lead to erroneous conclusions.

PRODUCTION OF STEVIOSIDE IN SHOOT TIP CULTURES.—It has been reported that shoot cultures from some species of plants grow vigorously in liquid medium in the

light and produce high levels of secondary metabolites (11,12), and that low concentration of cytokinin had effects on shoot formation and proliferation in liquid medium (12). We investigated the effects of kinetin on the production of steviol glucosides in primary shoot tip cultures in liquid medium.

S. rebaudiana shoot tips from the sterile plantlets were grown in 1/2 MS medium in the light for 4 weeks. No roots appeared. The shoot tips grew vigorously both in the absence and in the presence of kinetin. Stevioside was detected in these cultures both in the absence and in the presence of kinetin as shown in Table 2. One μM kinetin enhanced the growth of shoots and the synthesis of stevioside. A small amount of steviolbioside (15–20 μg/g fresh wt) was detected in all the cultures. No rebaudioside A was detected in any cultures.

TABLE 2. Effects of Phytohormones on *Stevia rebaudiana* Shoot Culture in Liquid Medium in the Light.[a]

Kinetin (μM)	Fresh wt[b] (g)	Stevioside[b]	
		(μg/g fresh wt)	(μg/culture)
0	4.3	190	814
1	5.8	369	2223
3	5.7	228	1313
10	5.0	128	650

[a]The shoot tips (1 node, 5 mg) of *S. rebaudiana* plantlets were cultured in liquid 1/2 MS medium containing phytohormones described in the table in continuous light for 4 weeks and harvested for analysis.

[b]The values are the averages obtained from duplicate cultures.

ORGAN CULTURE FOR PRODUCTION OF STEVIOL GLUCOSIDES.—We examined the possibility of the production of the sweetener by *S. rebaudiana* organ cultures, especially the hairy root cultures or the whole plantlets reproduced from the hairy roots. The root cultures of *S. rebaudiana* were not able to produce stevioside. On the other hand, the shoot cultures in liquid medium in the light were able to produce steviol glucosides. Our results suggest that roots of *S. rebaudiana* plants are not the main site of synthesis of stevioside and that steviol glucosides are synthesized in leaves. The root cultures may be of no use in producing steviol glucosides. We did not measure the amount of steviol, the aglycone of the sweet glucosides, in the cultures, because steviol is not sweet. There is, however, the possiblity that steviol is produced in the roots or in the callus cultures. We were not able to get the whole plantlets reproduced from the hairy roots. *S. rebaudiana* may be one of the plants in which reproduction of whole plants from hairy roots is difficult.

EXPERIMENTAL

CULTURE MEDIA.—1/2 Murashige and Skoog's (1/2 MS) medium (13) contained one half of the macroelemental salt formula and 2% sucrose (if not otherwise noted). White medium (14) contained 2% sucrose (if not otherwise noted). Solid medium contained 0.2% Gelrite™ (Kelco).

INDUCTION OF *S. REBAUDIANA* HAIRY ROOT CULTURES.—Sterile *S. rebaudiana* plantlets were maintained on hormone-free solid 1/2 MS medium at 25° in 18 h light/6 h dark. Small pieces of the stem internodes were infected with *A. rhizogenes* strain 15834 grown on YEB agar medium (15). The inoculated internodes were cultured on solid 1/2 MS medium for a week and then on solid 1/2 MS medium containing 0.5 g/liter of cefotaxime sodium salt for 6–7 weeks in the dark. Segments of the hairy roots were cut off and subcultured every month on solid 1/2 MS medium containing cefotaxime sodium. After 3 passages, the antibiotic was omitted. Sixteen clones were developed and subcultured every 2 months on solid 1/2 MS

medium in the dark. Moreover, 6 clones from the original 16 clones were transferred and subcultured every month in liquid 1/2 MS medium in the dark.

CULTURE OF *S. REBAUDIANA* HAIRY ROOTS.—Hairy roots (1 g fresh wt/dish) were cultured on solid 1/2 MS medium containing 1–300 μM kinetin or 0.1–100 μM BA at 25° in 18 h light (photon flux density 100 μmol/m^2 per sec) /6 h dark for 11 weeks until the most vigorous cultures became almost confluent in the dishes, and they were harvested for analysis. Hairy roots (0.1 g fresh wt/flask) were also cultured in liquid 1/2 MS and White media containing 2–14% sucrose without phytohormone in the dark for 4 weeks, and they were harvested for analysis.

CULTURE OF *S. REBAUDIANA* SHOOT TIPS IN LIQUID MEDIUM.—Shoot tips (1 node, 1 cm, 5 mg) of the sterile plantlets were cultured in liquid 1/2 MS medium containing various doses of kinetin on a gyratory shaker (120 rpm) at 25° in continuous light (photon flux density 100 μmol/m^2 per sec) for 4 weeks, and they were harvested for analysis.

ANALYSIS OF STEVIOL GLUCOSIDES.—*Steviol glucosides.*—Steviolbioside (purity 99%) and the mixture of steviolbioside and rebaudioside B were prepared from stevioside (purity 99%; given kindly by Dr. Tanaka, Hiroshima University, Japan) and Pure Marumilon™ (contents stevioside 58%, rebaudioside A 18%; Maruzen Pharmaceutical Company, Japan), respectively, by alkaline hydrolysis by the method of Ahmed *et al.* (16).

Extraction and purification.—The culture (2–5 g fresh wt) was ground with MeOH (10 ml/g fresh wt) and kept at room temperature for more than 1 day. After filtration, the filtrate was evaporated to dryness. H_2O (3 ml) and *n*-BuOH (3 ml) were added to the residue, and the solution was mixed well. The *n*-BuOH layer was removed. The partition was repeated between the aqueous layer and another 1 ml of *n*-BuOH. The *n*-BuOH fractions were evaporated to dryness. The residue was dissolved in H_2O (10 ml) and applied to a silanized Si gel column (3 g Si gel/column) by the method of Fujinuma *et al.* (17). The column was rinsed with H_2O (50 ml) followed by 20% MeOH (50 ml). Steviol glucosides were eluted with 50 ml of 80% MeOH. The 80% MeOH fraction was evaporated to dryness.

Tlc analysis.—The samples were dissolved in 75% MeOH or in MeCN-H_2O (80:20) and applied to Si gel plates. The plates were developed with different solvent systems: (A) EtOAc-iPrOH-H_2O (11:4:2); (B) EtOAc-HOAc-H_2O (8:3:2); or (C) $CHCl_3$-MeOH-H_2O (15:10:2). The R_f values of steviolbioside, rebaudioside B, stevioside, and rebaudioside A were 0.34, 0.24, 0.19, and 0.12 (solvent A), respectively; 0.49, 0.39, 0.32, and 0.21 (solvent B), respectively; 0.46, 0.40, 0.36, and 0.30 (solvent C), respectively. The bands of steviol glucosides were located with the vanillin/H_2SO_4 reagent and heating at 110° (18). The detection limit was 20–40 μg/fresh wt.

Hplc analysis (quantitative analysis).—After tlc analysis, the samples were analyzed on a TSK-Gel Amide-80 column (4.6 mm i.d. × 25 cm, Tosoh, Japan) with an hplc instrument equipped with an M990 photodiode array detector (Millipore Ltd., USA), or with an SPD-M6A photodiode array uv-vis detector and an SPD-6AV uv-vis spectrophotometric detector (Shimadzu Corporation, Japan) at room temperature. The samples were eluted with a linear gradient of MeCN-H_2O (85:15)→(65:35) for 15 min at a flow rate of 1 ml/min, maintaining the final solvent proportion for 5 min. The retention times of steviolbioside, rebaudioside B, stevioside, and rebaudioside A were 9.5 min, 13.4 min, 14.9 min, and 17.2 min, respectively. The steviol glucosides have a broad absorption maximum at 197–199 nm in the mobile phase of hplc. The ratio of the absorbance of steviol glucosides at 220 nm to that at 200 nm (reference: air) was 5%. We measured contour chromatograms at a range of 190–230 nm, and in addition, we compared the chromatograms of absorbance at 200 nm and those at 220 nm. Stevioside in the extracts from the shoot tip cultures was confirmed by uv absorbance spectra with the photodiode array detector. The amounts of steviol glucosides were calculated by measuring the absorbance at 200 nm. The steviol glucosides had the same calibration curve when the concentration was expressed in molar amounts.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the National Science Foundation (EET-8713078) (USA), and in part by Special Cooperation Funds for Promoting Science and Technology (Basic Research Core System) from Science and Technology Agency, Japan.

LITERATURE CITED

1. K.C. Phillips, in: "Developments in Sweeteners." Ed. by T.H. Grenby, Elsevier Applied Science Publishers, London, 1987, Vol. 3, pp. 1–65.
2. Y. Tamura, S. Nakamura, H. Fukui, and M. Tabata, *Plant Cell Rep.*, **3**, 183 (1984).
3. C.G. Ferreira and W. Handro, *Planta Med.*, **54**, 157 (1988).

4. K.R. Lee, J.R. Park, B.S. Choi, J.S. Han, S.L. Oh, and Y. Yamada, *Korean J. Food Sci. Technol.*, **14**, 179 (1982) (in Korean); *Chem. Abstr.*, **97**, 515 (1982).
5. C.A. West, in: "Biosynthesis of Isoprenoid Compounds." Ed. by J.W. Porter and S.L. Spurgeon, John Wiley and Sons, New York, 1981, Vol. 1, pp. 390–409.
6. H. Suzuki, T. Matsumoto, and Y. Mikami, *Agric. Biol. Chem.*, **48**, 603 (1984).
7. H.E. Flores, M.W. Hoy, and J.J. Pickard, *Trends Biotechnol.*, **5**, 64 (1987).
8. H.E. Flores, J.J. Pickard, and M.W. Hoy, in: "Chemistry and Biology of Naturally-occurring Acetylenes and Related Compounds (NOARC)." Ed. by J. Lam, H. Breteler, T. Arnason, and L. Hansen, Elsevier, Amsterdam, 1988, pp. 233–254.
9. K. Yoshimatsu, M. Satake, K. Shimomura, J. Sawada, and T. Terao, *J. Nat. Prod.*, (1991) in press.
10. J.B. Harborne, "Phytochemical Methods. A Guide of Modern Techniques of Plant Analysis," Chapman and Hall, London, 1981, pp. 214–221.
11. M. Sauerwein, H.E. Flores, T. Yamazaki, and K. Shimomura, *Plant Cell Rep.*, (1991) in press.
12. K. Yoshimatsu and K. Shimomura, *Plant Cell Rep.*, (1991) in press.
13. T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
14. P.R. White, "The Cultivation of Animal and Plant Cells," 2nd ed., Ronald Press, New York, 1963.
15. G. Vervlliet, M. Holsters, H. Teuchy, M. van Montagu, and J. Schell, *J. Gen. Virol.*, **26**, 33 (1975).
16. M.S. Ahmed, R.H. Dobberstein, and N.R. Farnsworth, *J. Chromatogr.*, **192**, 387 (1980).
17. K. Fujinuma, K. Saito, M. Nakazato, Y. Kikuchi, A. Ibe, and T. Nishima, *J. Assoc. Off. Anal. Chem.*, **69**, 799 (1986).
18. H. Wagner, S. Bladt, and E.M. Zgainski, "Plant Drug Analysis. A Thin Layer Chromatography Photo-Atlas," translated by T.A. Scott, Springer-Verlag, Berlin, 1984.

Received 6 November 1990