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ACCUMULATION OF STEVIOSIDE AND REBAUDIOSIDE A IN CALLUS CULTURE OF *STEVIA REBAUDIANA* BERTONI[1]

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Abstract

Accumulation of stevioside and rebaudioside A in the callus tissue derived from leaves of *Stevia rebaudiana* Bertoni was investigated. Stevioside content in callus was twice as much as that of leaves and four times as that of flowers. The growth intervals of callus after inoculation were found to be an important factor that would affect the contents of these two sweetners. Application of casein hydrolysate to the medium made the contents of stevioside and rebaudioside A of calli double to that of control.

Introduction

Because of the sweetening agents of its leaves, the perennial composite *Stevia rebaudiana* Bertoni has been attracted much attention for half a century. Bridel and Lavieille (1930) isolated a pure crystalline glucoside (stevioside) from leaves and found it to be 300 times as sweet as sucrose. They also found the enzymatic hydrolysis of stevioside to 3 moles of D-glucose and one mole of an acidic tasteless aglycone (steviol). The structure of the aglycone was later clarified by Mosettig *et al.* (1963) while the configurations and linkage positions of D-glucose were comfirmed by Wood and Fletcher (1956). Kaneda *et al.* (1977) isolated rebaudioside A, found it to be a combination of a steviol with 4 moles of D-glucose, and reported taste properties superior to stevioside.

Tissue culture of this sweetening plant has been established (Handro *et al.*, 1977; Yang and Chang, 1979; Yang *et al.*, 1981). Accumulation of stevioside and rebaudioside A in cultured tissues, however, has not been documented so far. This

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investigation was carried out in order to ascertain if the callus tissues derived from *S. rebaudiana* do retain the ability for synthesis of these sweeteners, and if so, to investigate the amounts of stevioside and rebaudioside A accumulated in calli. Leaves and flowers sampled from field-grown plants of *S. rebaudiana* were used in comparing with the callus samples.

Materials and Methods

The initiation and maintenance of callus from excised leaves of *S. rebaudiana* has been documented (Yang and Chang, 1979; Yang *et al.*, 1981). The callus tissues were grown as static cultures on a modified basal medium (Murashige and Skoog, 1962) supplemented with 2 mg/l α-naphthaleneacetic acid and 2 mg/l kinetin. The cultures were maintained at 26±2°C under diffuse light with a photoperiod of 16 hr per day. The callus sampled for analysis has been subcultured every 2 months and maintained in these conditions for 2 years. Cultures were harvested, freeze-dried for analysis. Leaves and flowers of *S. rebaudiana* were collected from field-grown mature plants, air-dried, and ground for sweetener analysis.

The sweeteners in samples were extracted according to the method of Hashimoto *et al.* (1978). One gram of ground sample and 0.3 g of calcium carbonate were blended in 6 ml distilled water for 15 h, then heated at 50°C for 4 h. This was followed by addition of 18 ml of acetonitrile to the cooled mixture and then left at room temperature for 5 h. Extracts for analysis were obtained by filtration through nuclepore filter (0.4 μm).

High performance liquid chromatography (HPLC) was used for qualitative and quantitative analyses of stevioside and rebaudioside A. A high pressure pump (Constimetric IIG, Laboratory Data Control, Riviera Beach, Florida, U. S. A.) equipped with 20 μl loop-loading sample injector (model 7120, Rheodyne, Berkeley, California, U. S. A.) and a reflect index detector (model 1107, Laboratory Data Control, Reviera Beach, Florida, U. S. A.) were used as the chromatographic system. A Pedersen (Walnut Creek, California, U. S. A.) model 37-MR strip chart recorder was used to plot chromatograms. Assay samples were separated isocratically using a Carbohydrate Analysis Column (30 cm × 4 mm I. D., Waters, Milford, Massachusetts, U. S. A.). The column was eluted with acetonitrile-water (4:1) at a flow rate of 1.5 ml/min. The acetonitrile-water solution was filtered through a 0.4 μm nuclepore filter and degassed prior to use.

Authentic samples of stevioside and rebaudioside A used as standards were kindly provided by Professor O. Tanaka of Hiroshima University (Hiroshima, Japan). Quantitation was based upon the peak height ratios of the standards.

Results and Discussion

Accumulation of Sweetener in Callus Tissue

In one set of our experiments, stevioside contents of leaves and flowers from the field-grown plants and the leaf-derived callus cultured for 70 days were examined. Based on dry weight the stevioside content in leaves was 8.46%, flowers, 3.66% and calli, 16.24%. Namely, the stevioside content in callus was about twice as much as that of leaves and four times as that of flowers. The stevioside in leaves of local cultivated accessions was 5.17–8.73%, and in flowers, 2.90–4.70% (Chen *et al.*, 1979). Therefore, our test showed that the callus tissue derived from *S. rebaudiana* retained the chemical totipotency as far as stevioside was concerned.

Contents of Sweeteners in Callus Tissues at Different Growth Intervals after Inoculation

One month after inoculation, callus tissues were sampled at different intervals, and the contents of sweeteners in calli were analyzed. Table 1 shows that for both stevioside and rebaudioside A, the contents reached maxima at around 40 days after inoculation and then decreased. For instance, stevioside content was 36.4% at 38th day and dropped to 18.2% at 74th day. Rebaudioside A content was 19.1% at 38th day and dropped to 8.2% at 74th day. Namely, the content of both stevioside and rebaudioside A at 74th day was only one half of that at 38th day. Therefore, the different growth intervals of callus tissues of *S. rebaudiana* after inoculation showed a great influence on the contents of stevioside and rebaudioside A.

Effect of Casein Hydrolysate on the Content of Sweeteners

Attempts on promoting accumulation of sweeteners in callus by adding a

Table 1. *Contents of stevioside and rebaudioside A of callus tissues at different intervals after inoculation*

Days after Inoculation	% Dry Wt.	
	Stevioside	Rebaudioside A
31	32.4	17.2
38	36.4	19.1
45	33.0	16.4
52	23.6	10.6
67	17.0	7.3
74	18.2	8.2

Table 2. *Effects of casin hydrolysate (CH) and growth conditions (dark or light) on callus proliferation and accumulation of stevioside and rebaudioside A**

Growth Condition	CH Treatment (g/l)	Callus Dry Wt. (mg.)	% Dry Wt.	
			Stevioside	Rebaudioside A
Dark	0	721 b**	18.45	12.30
	0.5	424 c	16.20	11.95
	2	128 d	30.76	23.55
Light	0	998 a	11.48	10.95
	0.5	768 b	16.88	11.50
	2	155 d	30.38	28.80

* Each value is the mean of 15 replications sampled after 79 days.
** Different alphabets represent significant at 5% level by Duncan's multiple range test.

variety of chemicals to the medium were conducted. Among the chemicals tested, casein hydrolysate (CH, Difco Bacto vitamine-free casamino acid, Detroit, Mi., U. S. A.) (Table 2) greatly promoted the accumulation of stevioside and rebaudioside A but retarded the growth of callus. The control callus was yellowish green in color while the callus grown in the medium supplemented with CH was brown or dark brown. The proliferation of control callus was significantly faster than that of the CH-treated callus. The higher the concentration of CH applied to the medium, the lower the callus proliferation resulted. However, the contents of both sweeteners showed a reverse trend. In the medium supplemented with 2 gm/l CH, the contents of sweeteners of callus tissue were twice as much as that of control which meant approximately four times to that of leaves sampled from field-grown accessories. The situations were the same for calli grown in dark or illuminated.

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甜菊癒合組織中含有 stevioside 及 rebaudioside A

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由甜菊 (*Stevia rebaudiana* Bertoni) 葉片誘導及繼代培養之癒合組織在含 2 mg/l α-naphthaleneacetic acid 及 2 mg/l kinetin 的 MS 培養基中含有多量之 stevioside (16–36%) 及 rebaudioside A (7–19%)。於培養基中添加 0.05–0.2% 之 casein hydrolysate，促進此兩種甜精提高兩倍。