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Marsolais et al.

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[54] **STEVIA PLANT NAMED 'RSIT 94-751'**

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[21] Appl. No.: **657,463**

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[51] **Int. Cl.**[6] **A01H 5/00**

[52] **U.S. Cl.** **Plt./100**

[58] **Field of Search** Plt./100

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[57] **ABSTRACT**

A new and distinctive variety of stevia characterized by: (1) a high ratio of rebaudioside A to stevioside; (2) a high ratio of rebaudioside A to rebaudioside C; (3) a high ratio of rebaudioside A to dulcoside A; (4) a high percentage of steviol glycosides consists of rebaudioside A; (5) a high concentration of rebaudioside A; and (6) a high sweetener concentration (i.e., the sum of stevioside, rebaudioside A, rebaudioside C, and dulcoside).

2 Drawing Sheets

BACKGROUND OF THE INVENTION

The present invention relates to a new and distinct variety of stevia (*Stevia rebaudiana* Bertoni), referred to by the varietal name 'RSIT 94-751'.

Eight steviol glycosides with sweetening properties have been identified in leaf tissues of stevia. The four major glycosides are: stevioside, rebaudioside A, rebaudioside C, and dulcoside A. The chemical structures of these compounds are shown in FIG. 1. Each of these steviol glycosides has unique sensory properties, and they can be used singly or in combination to provide a sweetener that has sensory properties tailored to a specific use. Stevia sweeteners are non-caloric, making them suitable for diabetics and weight-conscious consumers, and pH-stable and heat-stable, making them useful in a wide range of bottled beverages, confectioneries, baked goods, and dairy and canned products. For reviews of the use of stevia as a source of sweeteners, see, e.g., Handro and Ferreira, 1989, Lee 1982, and Phillips, 1987.

We set out to develop stevia varieties having high concentrations of individual steviol glycosides that could be extracted and recombined in ratios suitable for specific product uses. Landrace stevia has a combination of steviol glycosides that is not optimal for all product applications. Stevia varieties have been described that have high ratios of rebaudioside A to stevioside, but these varieties had relatively low sweetener concentrations (Morita Kagaku Kogyo, 1984a, 1984b, 1985, 1986, Nakazato, 1987, 1988, Stevia Co. Inc., 1985).

We discovered our new variety in a cultivated area at the Agriculture and Agri-Food Canada Research Station near Delhi, Ontario, Canada ("Delhi Research Station"). The following unique combination of characteristics is outstanding in 'RSIT 94-751' and distinguishes it from its parents and all other stevia varieties of which we are aware: (1) a high ratio of rebaudioside A to stevioside; (2) a high ratio of rebaudioside A to rebaudioside C; (3) a high ratio of rebaudioside A to dulcoside A; (4) a high percentage of steviol glycosides consists of rebaudioside A; (5) a high concentration of rebaudioside A on a leaf dry weight basis; and (6) a high sweetener concentration (i.e., the sum of stevioside, rebaudioside A, rebaudioside C, and dulcoside A) on a leaf dry weight basis.

This variety has not been observed under all possible environmental conditions. The following observations, measurements and comparisons describe plants grown under conditions that are similar to those generally used in commercial practice.

Asexual reproduction of this new variety by shoot-tip and stem cuttings was performed at the Delhi Research Station and showed that the foregoing characteristics are established and transmitted through succeeding asexual propagations.

BRIEF DESCRIPTIONS OF THE PHOTOGRAPHS

The accompanying photographs show typical specimens of this new variety, depicted in color as nearly true as is reasonably possible in a color photograph of this character. The photographs were taken at the Delhi Research Station in February 1996.

FIG. 1 shows the chemical structure of four major steviol glycoside sweeteners in stevia, stevioside, rebaudioside A, rebaudioside C, and dulcoside A.

FIG. 2 is a view of a plant of the present invention.

FIG. 3 is an enlarged view of leaves of the plant of FIG. 2.

DETAILED DESCRIPTION

In 1989, seed of a landrace stevia variety from China was increased in a greenhouse at the Delhi Research Station by inter-pollination. In the summer of 1990, approximately 1000 plants were grown. Fifteen plants were selected from this population and designated SR1 through SR15. Crowns of these plants were dug out of the field in the fall of 1990 and grown in a greenhouse in the fall and winter of 1990-1991. These plants were inter-crossed and half sib seed collected from each parent plant.

In the summer of 1991, these half sib families were evaluated in a genetic heritability trial (Brandle and Rosa, 1992). One of the entries in this trial was the SR13 half sib population.

In the summer of 1994, SR13 and several other half sib populations were evaluated. 'RSIT 94-751' was selected from the SR13 half sib population on the basis of its agronomic traits and steviol glycoside profile, based on replicated trials conducted at the Delhi Research Station.

Table 1 shows the leaf yield (kg/ha), height (cm), and lodging of 'RSIT 94-751' as compared with SR13, the maternal parental population from which 'RSIT 94-751' was selected, and 'Brazil', an open-pollinated landrace variety of stevia and the most widely grown Stevia variety in Brazil.

TABLE 1

Agronomic traits of 'RSIT 94-751', SR13, and 'Brazil'

Variety	Leaf Yield (kg/ha)	Height (cm)	Lodging (0–9)[1]
94-751	2214	65.3	6.0
SR13	1766	51.0	2.5
Brazil	2227	68.8	4.8

[1]Lodging is based on a scale of 0–9 where 0 = no lodging and 9 = all the plants lodged flat on the ground at harvest. A rating of 6 indicates that most of the plants were leaning at an angle at harvest.

Table 2 shows steviol glycoside profiles in leaves of 'RSIT 94-751', SR13, and 'Brazil'. In particular, the following were examined (on a dry weight basis): percent dulcoside A (% dulc.), percent stevioside (% stev.), percent rebaudioside C (% reb.C), percent rebaudioside A (% reb.A), and percent total glycosides (% total=% dulc.+stev.+% reb.C+% reb.A).

TABLE 2

Steviol glycoside profiles of 'RSIT 94-751', SR13, and 'Brazil'

Variety	Year	% dulc.	% stev.	% reb. C	% reb. A	% Total
94-751	1994	0.22	4.68	1.3	11.4	17.6
94-751	1995	0.05	4.88	1.33	11.82	18.08
SR13	1995	0.35	9.25	0.59	3.39	13.58
Brazil	1995	0.25	7.61	0.54	3.00	11.40

'RSIT 94-751' was dug out of the field in the fall of 1994 and vegetatively propagated at the Delhi Research Station by shoot-tip and stem cuttings. The stability of the steviol glycoside and agronomic traits of 'RSIT 94-751' was verified in replicated trials conducted in 1995.

Taxonomic Description Of 'RSIT 94-751'

'RSIT 94-751' is a suffrutescent, erect perennial (FIG. 2). Field-grown plants may attain a height of 1.1 meters, but more commonly have a height of 6—8 dm. The stems are round, pubescent, and have internodes of medium length. The leaves are simple, opposite, sessile, exstipulate, and oblanceolate. The leaf apices are obtuse and the leaf margins are crenate above the middle and entire on a cuneately narrow base. The leaves are three-nerved and conspicuously veiny. The leaf surface is puberulent with short glandless hairs. The largest cauline leaves are up to 9 cm long 4 cm wide and are often proliferous in the axils (FIG. 3).

The inflorescence is loosely paniculate with the heads appearing opposite the bracts in irregular sympodial cymes. The flower corollas have a pale purple throat and white limb. The seed are nearly uniform achenes, 15—17 aristate (Robinson, 1930).

Method of Determining Steviol Glycosides and Concentrations

Fresh leaf samples were harvested from field-grown stevia plants and stored in a freezer prior to extraction. Samples

were freeze dried, ground to pass through a 40-mesh screen, and again freeze dried before extraction. Ground, freeze-dried plant material (300 mg) was weighed into a 15 ml polypropylene centrifuge tube (Fisher). Ten ml of 1:1 (v/v) acetonitrile-water (acetonitrile from Caledon Laboratories Ltd., Georgetown, Ontario, 190 HPLC grade) was added to each sample. Sample tubes were suspended in an ultrasonic bath at maximum ultrasonication (#9) for 15 min (Lab-line Inst. model #9333, Melrose Park, Ill.) with occasional stirring and rotation to ensure optimal extraction. Sample tubes were then centrifuged at 1500 rpm for 15 min (International Centrifuge, Model V Size 2, International Equipment, Boston, Mass.). The extraction solvent was transferred to a volumetric flask with a Pasteur pipette. For routine analyses, duplicate extractions were utilized and the solvent transferred to a 25 ml volumetric flask filled to volume with acetonitrile. Samples were filtered through a 0.45 micron filter (Acrodisc 13, Gelman Sciences, Ann Arbor, Mich.).

Analyses were performed on a liquid chromatograph (Hewlett-Packard 1090) equipped with a three-channel solvent delivery system, auto sampler, and diode array detector interfaced with a chem station. Stevioside, rebaudioside A, rebaudioside C, and dulcoside A standards were obtained from FWB Chemical Consulting Ltd., Calgary, Alberta, Canada. Stevioside was also obtained from Sigma Chemical Co., St. Louis, Mo. Due to the wide variation in plant material analyzed, co-eluting peaks occasionally were apparent in the chromatogram. Often these plant constituents were eliminated by filtration through a Waters NH2 Sep-Pak cartridge (Part No. WATO 20535) prior to analysis. For routine analyses, only stevioside was used as a standard. The response factor found for stevioside was used for the other three steviol glycosides after correcting for differences in molecular weight. In order to monitor this assumption during the sample determinations, a standard sample of rebaudioside A was analyzed after each calibration.

The chromatographic column was a Waters cartridge carbohydrate column (Part No. WAT 044355) having an inside diameter of 250×4.6 mm. The guard column was a carbohydrate sentry guard column (Part No. WAT 046895). The "A" solvent was H_2O (pH 5.25 with AcOH), the "B" solvent was acetonitrile, and the "C" solvent was acetonitrile. The flow rate was 1.5 ml/min. The time table for use of solvents A, B, and C is found in Table 3.

TABLE 3

Time table for use of Solvent A (H_2O, pH 5.25 with AcOH), Solvent B (acetonitrile), and Solvent C (acetonitrile)

Time (min.)	% Solvent A	% Solvent B	% Solvent C
0	13	43	44
12	17.5	41	41.5
26	24	38	39
26.1	13	43	44

The post time was six minutes and the elution times for the steviol glycosides were 14.5 minutes for dulcoside A, 18.8 minutes for stevioside, 20.4 minutes for rebaudioside C, and 23.5 minutes for rebaudioside A. Retention times of the components of interest decreased with extensive use of the column. It was usually sufficient to simply reduce the amount of water in the initial chromatographic conditions to restore the retention times. Individual columns have been used for more than one thousand samples. Guard columns

typically were replaced after about 300 samples or when system pressure became excessive. Periodically the column was washed with water to remove other plant constituents which accumulated on the column. Steviol glycosides were identified by retention time and concentrations were determined by an external standards method.

Steviol glycosides may be produced from leaves or other parts of 'RSIT 94-751' and formulated into a sweetener for use in the food industry or by consumers by any conventional method. See, e.g., U.S. Pat. No. 4,892,938. Purified extract solutions may be directly added to a foodstuff or beverage, for example, in liquid form or converted to a crystalline form. The sweetness intensity of steviol glycosides is usually about 150 to 300 times that of sucrose, depending on the concentration of the steviol glycosides used. If desired, stevia extracts can be suitably diluted with a conventional diluent, e.g., a tasteless soluble starch, to achieve a sweetness intensity comparable to that of sugar (i.e., sucrose). For convenient use by the consumer, an unit of a stevia sweetener, e.g., an amount having a total sweetness approximately that of a standard lump of sugar, can be packaged for commercial distribution in small paper bags or in other forms known in the art. Steviol glycosides from 'RSIT 94-751' can be used singly or in combination with steviol glycosides from other stevia varieties or with other conventional sweeteners to provide a sweetener composition that has sensory properties tailored to a specific use.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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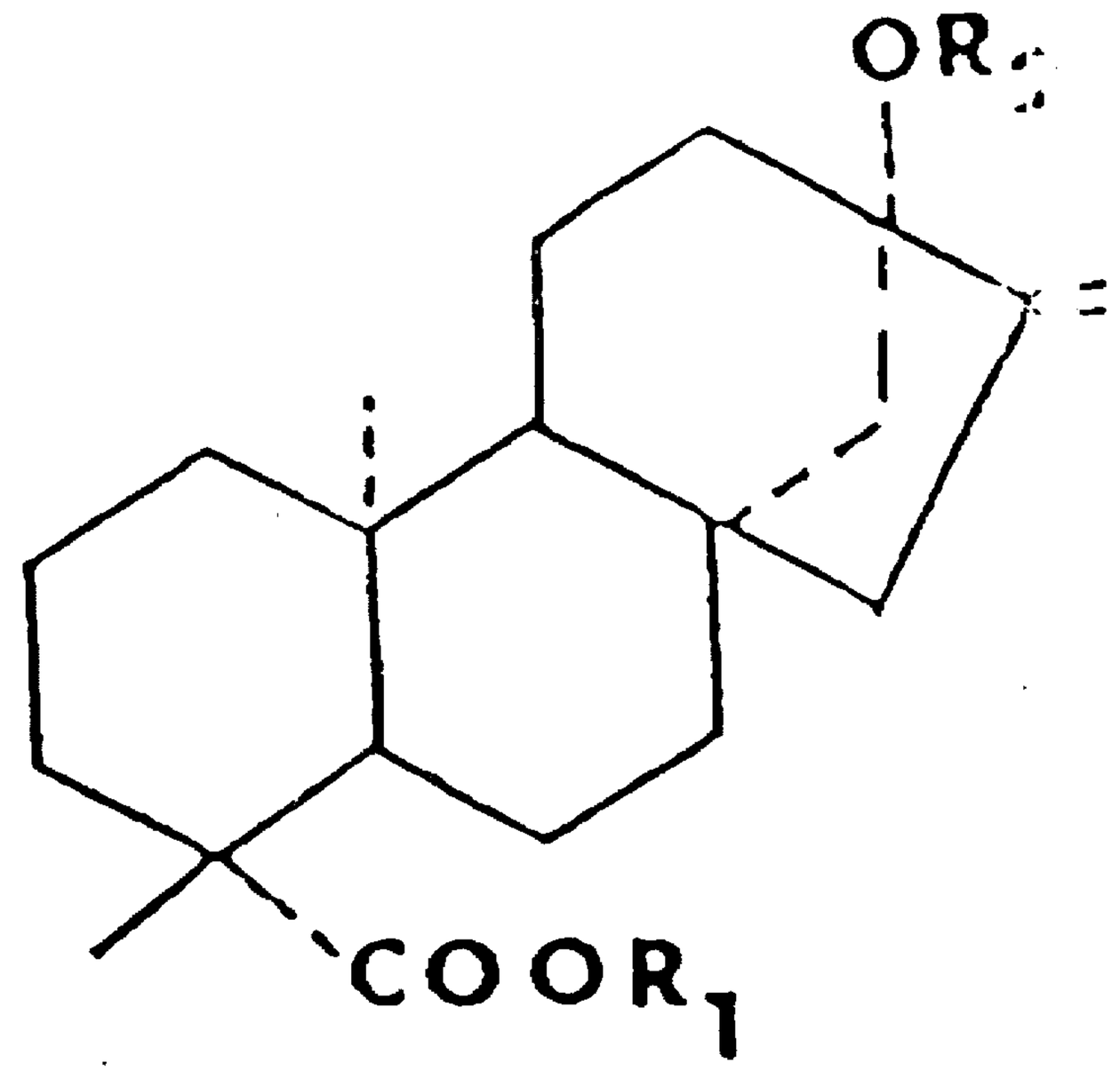
We claim:

1. A new and distinctive variety of Stevia plant, substantially as herein shown and described.

* * * * *

STRUCTURES



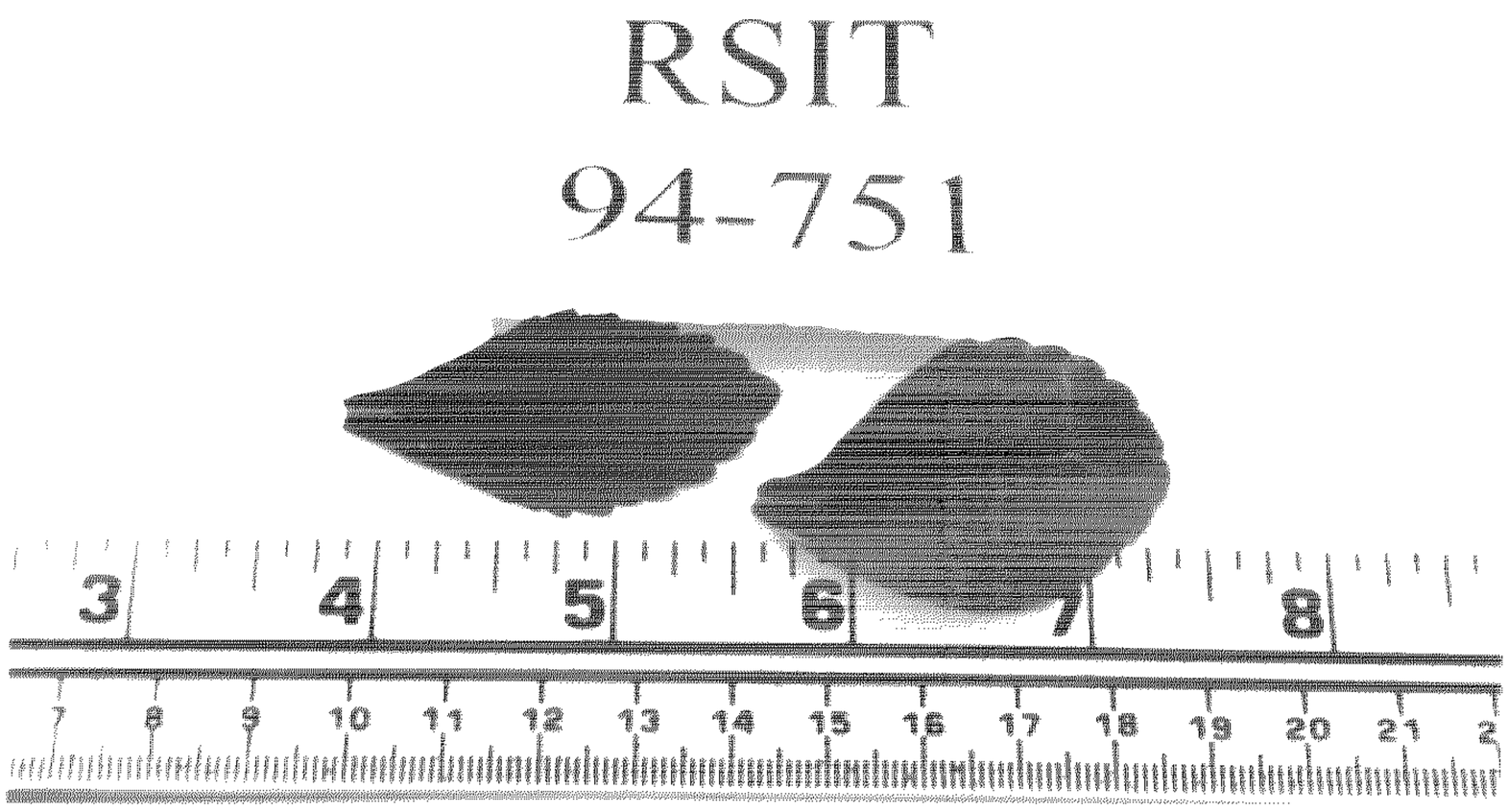
	R_1
Stevioside	glc ^{2}-glc $_{3}$.glc
Rebaudioside A	glc ^{3}-glc $_{2}$.rham
Rebaudioside C	glc ^{3}-glc $_{2}$.rham
Dulcoside A	glc ^{2}-rham

glc ; glucose
rham ; rhamnose

FIG. 1

FIG.2

FIG. 3



UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

Plant 10,564

PATENT NO. :

DATED : August 18, 1998

INVENTOR(S) : Albert Anthony Marsolais; James Brandle; Elizabeth Ann Sys

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

The drawing sheet, consisting of Fig. 1, should be deleted to be replaced with the drawing sheet consisting of Fig. 1, as shown on the attached page.

Signed and Sealed this

Thirtieth Day of November, 1999

Attest:

Attesting Officer

Q. TODD DICKINSON

Acting Commissioner of Patents and Trademarks

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

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DATED : August 18, 1998

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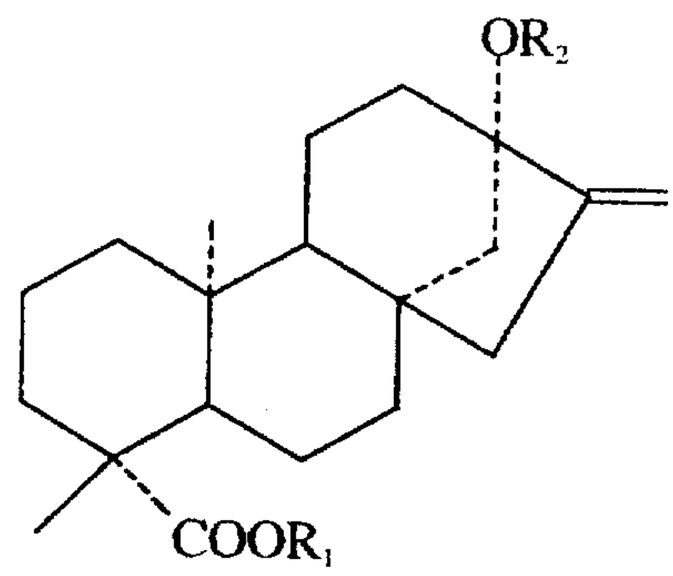
STRUCTURES



	R_1	R_2
Stevioside	glc ^{2}–glc	glc
Rebaudioside A	glc ^{2}(–glc, ^{3}–glc)	glc
Rebaudioside C	glc ^{2}(–rham, ^{3}–glc)	glc
Dulcoside A	glc ^{2}–rham	glc

glc ; glucose
rham ; rhamnose

FIG. 1