INTERNATIONAL JOURNAL OF DRUG DISCOVERY AND HERBAL RESEARCH (IJDDHR)
3(3): July-September: (2013), 662-667
ISSN: 2231-6078

Research Article

Estimation of Some Secondary Metabolites Through Difiltration Method From *In-Vitro* Regenerated *Stevia Rebaudiana* Bertoni

Seema Tiwari[1], Reena Chaurasiya[1], Ramdeo Saket[4], Rashmi Arnold[2], Santosh Agnihotri[2] and Arti Saxena[3]

[1] **Study centre for Biochemistry, A.P.S.U., Rewa M.P.**
[2]**Dept. of Botany, Model Science College, Rewa M.P.**
[3] **Dept. of Zoology, Model Science College, Rewa M.P.**
[4]**Dept, of Biotechnology APSU, Rewa, MP.**

Abstract

Natural sweeteners that can substitute sucrose have caught great attention due to the growing incidence of obesity and diabetes. Much attention has been placed on Stevioside, a sweet glycoside extracted from *Stevia rebaudiana* Bertoni. Due to the sweetness and supposed therapeutic properties of its leaf. The great interest in Stevia as a non-calorific, natural sweetener has fueled many studies on it - including toxicological ones. For hundreds of years, indigenous peoples of Brazil and Paraguay have used the leaves of Stevia as a sweetener. The Guarani Indians of Paraguay call it *kaa jheé* and have used it to sweeten their yerba mate tea for centuries. They have also used Stevia to sweeten other teas and foods and have used it medicinally as a cardiotonic, for obesity, hypertension, and heartburn, and to help lower uric acid levels. In addition to being a sweetener, Stevia is considered to be hypoglycemic, hypotensive, diuretic and tonic. Stevioside is a diterpenoid glycoside, comprising of an aglycone (Steviol) and three molecules of glucose. Present investigation shows the isolation of some secondary metabolites including stevioside from *Stevia rebaudiana* Bertoni.

Key words: Stevioside, Rebaudioside, *Stevia rebaudiana* Bertoni, Bioactive principal compounds etc.

Introduction

In the last couple of decades, growing concern about health and life quality has encouraged people to exercise, eat healthy food and decrease the consumption of food rich in sugar, salt and fat. Omission of added sucrose in foods increases the relative proportion of polymeric carbohydrates that may have beneficial effect for a balanced food intake as well as for human health[1]. More recently, the main concept has become to use food as a means of promoting health and welfare, while reducing the risk of disease[2] .The food industry has responded to this demand and as a consequence, there has been a fast growing increase in diet foods and beverages available to consumers in many markets of the world[3]. With increased consumer interest in reducing sugar intake, food products made with sweeteners rather than the sugar have become popular. Sweeteners are alternative substances to sugars, which give food a sweet taste and are used to partially or totally replace sucrose [4].

*** Corresponding Author**
Email : seemat452@gmail.com

The discovery of great number of sweeteners during the last decade has triggered the development of sugar free products, particularly for diabetic, obese people and for dietetic purpose[5].

Several highly sweet plant constituents are used commercially as sucrose substitutes in one or more countries. The plant secondary metabolites of most widespread interest in this regard are Steviol glycosides i.e. Stevioside and Rebaudioside A, constituents of the *Stevia rebaudiana Bertoni*. These two products, made from *Stevia rebaudiana are* widely available in Japan, with Stevioside approved as a sweetener in Brazil and having limited use pin Korea too[6]. *Stevia* (*Stevia rebaudiana* Bert.) is a small, herbaceous, semi-bushy, perennial shrub of Compositae family originated from Paraguay. It grows well at the temperature ranging between 15 - 30°C. It is one of 154 members of the genus Stevia, which produces stevioside, a diterpenoid glycoside isolated from plant leaves[7].

Stevia plants grow better having temperature range of 0-40^{0}C. So these conditions allow Stevia, growing annually in areas having diverse environment. However, it is cultivated on commercial basis by seed, cutting or division of mother plants in green house during winter every year. The seeds of Stevia show a very low germination percentage also. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition[8]. Vegetative Propagation too, is limited by lowering number of individuals that can be obtained from single plant[9]. Propagation through stem cutting requires enough stocks of stem cutting and high labor inputs. Due to above mentioned difficulties tissue culture is the only alternative method to prepare sufficient amount of plants within short time duration for mass propagation of Stevia plants. Plant tissue culture of plant through shoot tip or axillary bud culture may help to rare medicinal plants by recovery of genetically stable and of plants[10]. The success of *in vitro* culture depends mainly on the growth conditions of the source true to type progeny. It is now evident that plant tissue culture is an essential component of Plant Biotechnology which offers novel approaches to the production, propagation, conservation and manipulation material medium composition[11,12]and culture conditions and on the genotypes of donor plants[13].

Bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Secondary metabolites are produced within the plants besides the

primary biosynthetic and metabolic routes for compounds associated with plant growth and development, and are regarded as products of biochemical "side tracks" in the plant cells and not needed for the daily functioning of the plant. Several of them are found to hold various types of important functions in the living plants such as protection, attraction or signaling. Most species of plants seem to be capable of producing such compounds. Bioactive compounds in plants are compounds produced by plants having pharmacological or toxicological effects in man and animals. The typical bioactive compounds in plants are produced as secondary metabolites. Secondary metabolites are produced within the plants besides the primary biosynthetic and metabolic routes of compounds aimed at plant growth and development, such as carbohydrates, amino acids, proteins and lipids. For example, flavonoids can protect against free radicals generated during photosynthesis[14, 15].

Materials and Methods

In-vitro regeneration

Healthy *Stevia rebaudiana* plants were obtained from Jayanti Kunj, Rewa M.P. and were raised in pots containing Soil and Manure (1:1) under green house condition at Department of Environmental Biology, A.P.S.U. Rewa, M.P. Leaf segment were cut from processed for aseptic culture. Explants were washed thoroughly under running tap water for 20 min, washed with a solution of Tween 20 (2 drops in 100 ml of water) for 1 min, and again washed with sterile distilled water. The cleaned explants were surface sterilized with mercuric chloride $HgCl_2$ (0.1%) for 4 minutes under aseptic conditions and washed six times with sterile distilled water to remove traces of $HgCl_2$.

Effect of BAP, Kn and NAA on MS medium for regeneration of shoot from nodal explants of *Stevia rebaudiana* Bertoni

The surface sterilized leaf explants were cultured on MS (Murashige and Skoog) basal media containing various concentrations of BAP, Kn and NAA [16]. Leaf explants were cut from petiolar end and placed on sterile medium with dorsal surface in contact with the medium supplemented with BAP and NAA. After 4 weeks the shoot buds induced on leaf explants were excised from base along with some portion of mother explants and placed on proliferation medium supplemented with different conc. of BAP, Kn and NAA combinations adding 30 g /l sugar and 0.7% agar. The pH was adjusted to 5.7 with 0.1 NaOH before autoclaving at a temperature of 120^0 C for 20 minutes. Cultures were established in different culture vessels and it was observed that shoots regenerated in flask showed normal morphology and were well elongated. After 2–4 weeks there was significant increase in the number of shoots. All the cultures were incubated in culture room, at a temperature of 25±2°C, 16 h photoperiod and light intensity of 25 µmol/(m^{-2} s^{-1}) provided by white fluorescent tubes. Subcultures were done after every 21 days interval. Nodal segments from the proliferated shoots were subculture again for further multiple shoot induction. A total of 16 concentrations i.e. BA alone (0.5, 1.0, 1.5 and 2.0mg/l), Kn alone (0.5,2, 1.0, 1.5 and 2.0), BA+Kn (1.0+1.0, 10+1.5, 1.5+0.5, 1.5+1.0) and BA+NAA(1.0+0.5, 1.0+1.5, 1.5+2.0, 1.5+2.5) to know the % of explants showing shoot proliferation, No. of total shoots per culture and Average length of shoots (in cm).

Effect of IAA, IBA and NAA in MS medium for root formation

Microcuttings (longer than 2 cm) taken from the *in-vitro* proliferated shoots were implanted on half-MS medium containing different concentration (0.5, 1.0, 1.5 and 2.0 mg/l) of IAA, NAA and IBA for rooting. *In-vitro* rooted shoots were kept under normal growth room conditions for 4 weeks until the induced roots became partially brown. The shoots were then taken out from the growth room, kept under room temperature for 15 days and then taken out from the culture tubes carefully and the medium attached to the roots was gently washed out with running tap water. The plantlets were transplanted to small polythene bags containing garden soil: compost (2:1) treated with 0.1% Agrason (fungicide). The transplanted plantlets were kept under shade for 15 days and then were transferred to normal environmental conditions. Through this process of acclimatization almost 70% survival was achieved.

Diafiltration extraction

The Diafiltration mode involves using extra extraction solvent to further process the retentate by washing out additional permeable compounds i.e. the desired sweet compounds (when using an ultrafiltration membrane to remove higher molecular weight substances), or washing out undesirable impurities (when using a nanofiltration or reverse osmosis membrane, to remove lower molecular weight substances.

Procedure

- Providing an extraction column, the column being vertically disposed and having a top opening for receiving plant material and extraction solvent, and a bottom opening for discharging extract.
- Adding substantially dry Stevia plant material to the column to form a bed.
- Adding an aqueous extraction solvent at a temperature of 0 to 25° C. to the column, to provide a controlled plant material: solvent ratio in the range of 0.02 :1 to 0.1:1 w/w.
- Removing from the column an extract including the sweet compounds.

- Passing the extract through an ultrafiltration membrane having a pore size defined by a molecular weight cut-off (mwco) of 2 to 3 kDa, at a trans-membrane pressure of 200 to 700 kPa.
- Passing the permeate including the sweet compounds, at a controlled temperature in the range of 50 to 85° C. through a high temperature nanofiltration membrane having a pore size defined by a molecular weight cut-off(mwco) of 200 to 600 Da, at a trans membrane pressure of 600 to 1300 kPa, and recovering the retentate including the sweet compounds.

Equipment

The UF system used was SEPA CF (Osmonics) with effective membrane area of 0.0155 $m^{2.}$ The microfiltration system used was zirconia microfiltration system with effective membrane area of 0.0055 $m^{2.}$ The nanofiltration system module was a NF spiral membrane module (40×40 module, membrane area of 7m^2). The temperature of the feed was controlled by a heat exchanger. Each membrane unit consists of a cylindrical stainless housing. The supplier of the membrane was U.S. Filter. The membrane pore sizes were 0.035, 0.080 and 0.2 µm respectively. Nitrogen blankets were applied for the feed tank or permeate tank in order to prevent air oxidization of feed/permeate.

Sample preparation

Two mL of aqueous sample, 15 mL of acetonitrile, 2.5 mL of buffer solution (5 g of sodium acetate, 0.5 g of Glacial acetic acid and water were added up to 1 L) and water were added into a 25 mL flask. On standing overnight and centrifuged, there were two phases observable at this stage, however only the top liquid phase was used for HPLC analysis. The pigment in the solution was analyzed by measuring the optical absorbencies at 420 nm (A420) and 670 nm (A670), the wavelengths of pigments maximum absorbencies, using a spectrophotometer (Spectronic Genesys 2).

Membrane characterization

Prior to the feed tests, all the UF membranes were characterized by 200 ppm solution of PEG of 3 kDa molecular weight. Operating pressure was 276 kPa and temperature was 22-24° C. The NF membranes were characterized by 200 ppm solution of PEG of 400 Da molecular weight at an operating pressure of 585 kPa and a temperature of 20, 40, 60 and 70° C.

Membrane cleaning

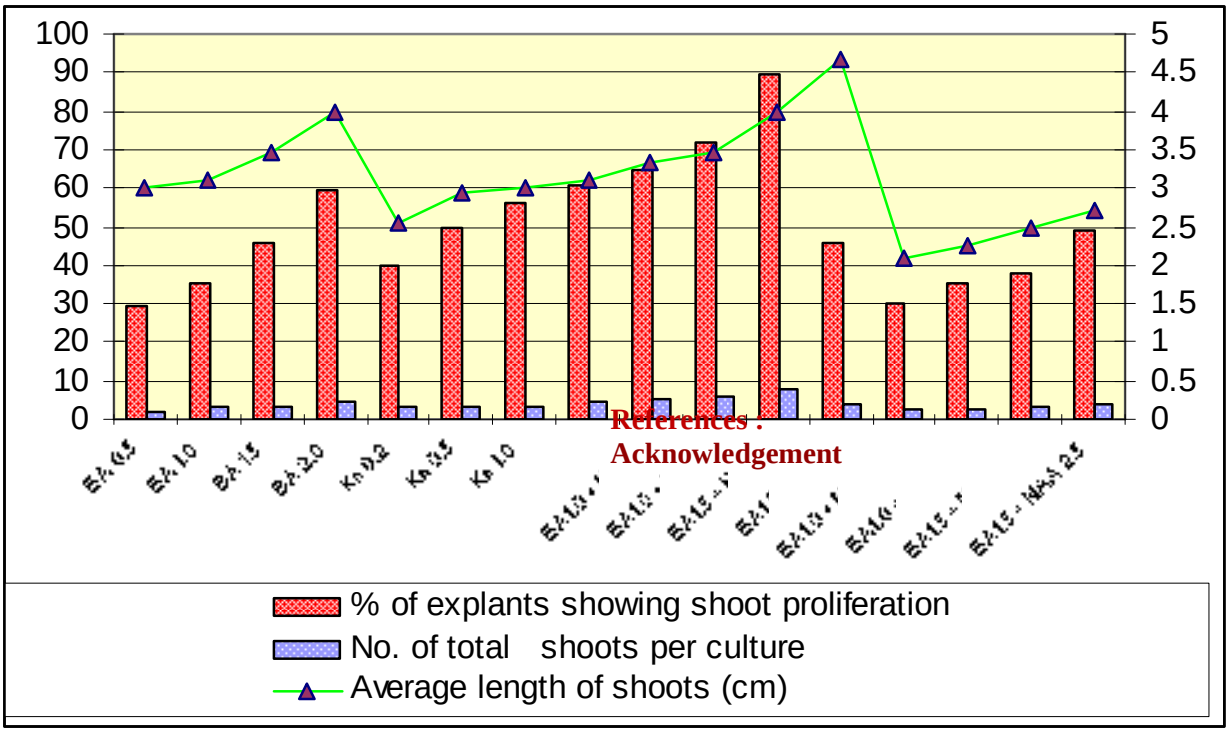
The membranes were cleaned in place after each test using a recycling technique. In this technique the membranes were first flushed with tap water at room temperature. The membranes were then washed with 0.2 N NaOH for 30 minutes. Finally, the system was thoroughly flushed with room temperature distilled water. In some circumstances, membranes were soaked in 1.5% Ultrasil™ 53 solution overnight. (The powdered enzymatic detergent membrane cleaner, Ultrasil™ 53 was chosen because it is authorized by the U.S. Department of Agriculture for use in federally inspected meat and poultry plants as a cleaner for reverse osmosis and ultrafiltration membranes.) Thoroughness of the cleaning was tested by measuring the pure water permeate rate after 30 minutes. The system was deemed clean when this was not significantly different from the pure water permeation rate of an unexposed membrane.

Results and Discussion

Nodal explants were incubated on MS medium fortified with different concentrations of BA (0.5, 1.0, 1.5 and 2.0 mg/1) and Kn (0.2, 0.5, 1.0 and 1.5 mg /l) alone or BA with Kn or with NAA. After six weeks multiple shoots emerged directly from axillary nodes of the cultured explants. The response was best at 1.5 mg/l BA + 0.5 mg/1 Kn combination to the highest percentage of explants (89.46%) showing shoot proliferation, highest number of total shoots (7.59±0.54) and highest average length of longest shoots (4.00±0.52) were recorded (table 4.9). The response was poor and considerable amount of callus was formed with BA and NAA combination (Graph-1). Microcuttings (longer than 2 cm) taken from the *in-vitro* proliferated shoots were implanted on MS medium containing different concentrations (0.5, 1.0, 1.5 and 2.0 mg/l) of IAA, NAA and IBA for rooting. Maximum root induction was observed in medium fortified with 1.5 mg/l that is 7.6±0.5 number of total roots and 5.5±0.4, average length of roots. Root induction gradually decreased with increasing concentrations of auxin (Graph-2).

The leaves of *in-vitro* propagated *Stevia rebaudiana* Bertoni were used as material for the isolation of sweetening compounds i.e. stevioside, rebaudioside etc. It has also been found that if the leaves are used in a small substantially uniform size of 10 to 40 mm, most preferably about 20 mm then extraction of the sweet compounds of interest is enhanced. The used Stevia leaves also provide a good filter bed for the removal of larger debris and reduce the chances of plugging the column. The amount of extraction solvent is also reduced. However, we have found that if the leaves are ground to a powder, the column becomes plugged. We have also found that for low temperature operation i.e. at the lower end of the operating range i.e. 0 to 10° C., temperature control is improved by the addition to the column of ice chips, along with the dried leaves.

Graph-1: Effects of different combined concentrations of cytokinin and auxins in MS medium on *Stevia rebaudiana* Bertoni



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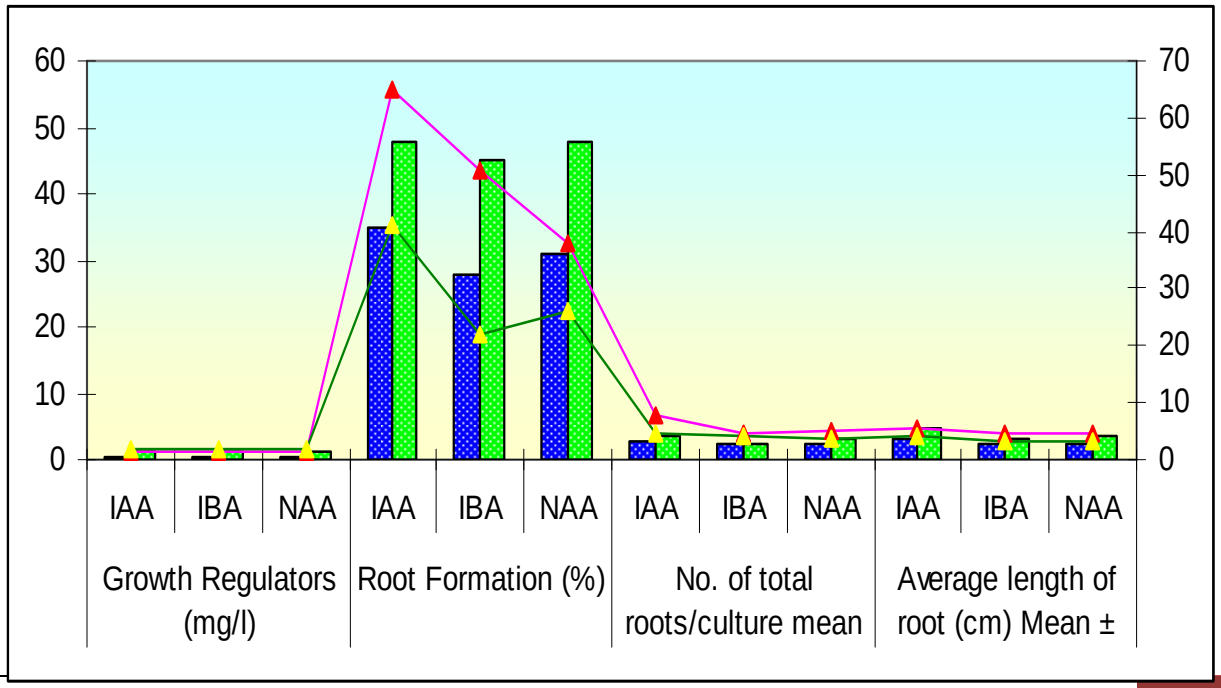
Acknowledgement

Graph-2: Effect of IAA, IBA and NAA in MS medium for root formation



ISSN: 2231-6078

For enhanced selectivity of separation of the desirable sweet compounds, the temperature of the extraction solvent is preferably in the range of 2 to 6°C, most preferably about 4°C. At these lower temperatures extraction of the undesirable higher molecular weight bitter compounds is lower than at the higher temperatures. The amount of the solvent and the leaf: solvent ratio is also controlled to enhance selectivity. We have found that with less leaf material, higher amounts of undesirable compounds are eluted. Also, if too much leaf material is added, the yield of the desirable sweet compounds decreases. Accordingly, a balance is needed. A leaf: solvent ratio of 0.03 to 0.10, by weight, has been found to be useful. The preferred water: leaf ratio is about 0.05: 1.

The solvent flow rate/residence time is also significant. A flow rate in the range of 24-30 ml/minute has been found to be useful for the column dimensions used in the examples. This provides a retention time of 10 to 20 minutes. Further, extraction of the sweet compounds is also enhanced by lowering the pH of the extraction water to the acid range i.e. about 2-4, preferably about 2. Also, at this pH, the solubility of some of the higher molecular weight undesirables such as proteins are lower, so they are excluded from the extract containing the sweet compounds. This is done by adding to the solvent a source of the phosphate ion e.g. from phosphoric acid. Others ions such as sulphate and calcium could also be used. The addition of phosphate also enhances the value of the retained leaf material as animal fodder. The aqueous extraction solvent is preferably water, and where sources of good quality water are not available, the use of reverse osmosis (RO) water or distilled water is recommended. Similar results were found by Tang and Wei, 1983 for difiltration extraction method for recovery of the sweet compounds.

Table-1: Effect of solvent pH on the Stevioside extraction

Stevioside (mg/l)	pH	Optical abs. of pigment at 420 nm
8000	2.0	9.0
8100	7.0	8.0
7900	9.0	5.9

Table-2: Effect of solvent temperature on the Stevioside extraction

Temperature Concentration (^{0}C)	Optical abs. of pigment at 420 nm	Stevioside (mg/l)	RC (mg/l)	RA (mg/l)
2-4	59.6	3.5	25	250
5-8	75.6	4.6	43	200

Table-3: Recoveries of Stevioside RC and RA with membrane filtration

Pore size (µm)	Recoveries % (µm)	Stevioside & RC (mg/l)	RA (mg/l)
78.3	77.4	77.8	8.0
74.8	81.8	79.0	3.5

Conclusion

Sugar (primarily sucrose) has been a part of the daily diet for literally hundreds of years, but research is now suggesting that sugar intake can be detrimental to our health. In particular, excessive consumption of simple sugars with high glycemic index values have shown to cause overeating and weight gain, as well as elevated postprandial hyperglycemia can result after consuming too much sugar. This has also been linked to being prone to disease. This leads to development of advanced glycation end products, inflammation and increased mortality rates. *Stevia rebaudiana* Bertoni is a plant having a recent focus as a worldwide supplement of sugar The leaf of this plant is the main attraction for its economic and commercial uses because of its valuable quality of extreme sweetness particularly with the presence of the main active constituents Stevioside and rebaudioside.

Acknowledgement

We express our heartily thanks to the Plant Biodiversity and Conservation Biology Dept., N.B.R.I. Lucknow, India for cooperating us in completing research experiments.

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