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Studies on rapid micropropagation of *Stevia rebaudiana* Bertoni: A natural sweetener

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Abstract

Stevia rebaudiana is and an important non-caloric sweetening herb contains diterpene glycosides need to be explored for its commercialization. The evolving commercial importance of secondary metabolites in recent years has resulted in a great demand in the Pharma industry.Stevioside is a diterpene glycoside present in *Stevia rebaudiana* Bertoni. 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 of plants. The success of *in vitro* culture depends mainly on the growth conditions of the source material medium composition and culture conditions and on the genotypes of donor plants.

Key-Words: *Stevia rebaudiana* Bertoni, glycosides, stevioside and stock solutions

Introduction

Stevia rebaudiana Bertoni, belongs to the Asteraceae family is a natural sweetener perennial herb commonly known as “Sweet Weed”, “Sweet Leaf”, “Sweet Herbs” and “Honey Leaf”. The leaves of this plant are estimated to be 300 times sweeter than sucrose obtained from sugar beat, sugarcane etc., with a zero calorie value and the sweetness is due to glycosides of which the most abundant is stevioside. *Stevia rebaudiana* (Bertoni) was discovered by Europeans in Paraguay in 1888 by Dr. Moises Santiago Bertoni. He later botanically described and named the plant (in 1905) in honor of Paraguayan chemist Dr. Rebaudi[1].

The natural habitat of Stevia is semi-humid subtropical on the Tropic of Capricorn (22 – 23^{o} S latitude), 200 – 400 meters above sea level, with 1,500 – 1,800 mm of rain and temperature extremes of minus 6^{o} C to plus 43^{o}C. It naturally grows in low lying areas on poor sandy acidic soils adjacent to swamps, and so is adapted to and requires constantly wet feet or shallow water tables[2]. Stevia is a genus of about 200 species of herbs in the sun flower family. It grows up to 1 m tall and has leaves 2-3 cm long. The leaves of Stevia are the source of diterpene glycosides, viz. stevioside and rebaudioside[3].

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Natural sweeteners that can substitute for 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, *Stevia rebaudiana* Bertoni has attracted economic and scientific[4].

The plant *Stevia rebaudiana* Bertoni (compositae) has been widely cultivated in the world for the sweet diterpene glycosides that are mainly contained in its leaves. In Egypt, the gap between sugar production (1.757 million tons) and consumption (2.6 million tons) represents a serious problem, since it was estimated to be 0.843 millioin tons. Nowadays, attention is concentrated upon using Stevia in food industries, in order to close the gap between the production and consumption. Stevia cultivation in different places of the world; it is expected that in the Egyptian agricultural environment; one feddan of Stevia may produce up to 400 kg of Stevia sugar, annually. Taking the sweetening powder of the *Stevia* sugar into consideration; these 400 Kg of Stevia sugar are equivalent to about 80,000 sweetening units[5]. Note that one feddan of “Sugar cane” produces about 5,000 sweetening units and one feddan of “Sugar beet” produces about 3,500

sweetening units. A sweetening unit is equivalent to the sweetness of one kilogram of sucrose[6].

Material and Methods

Plant and media preparations

Different parts of the plant were used as explants for the *in-vitro* regeneration of *Stevia rebaudiana* Bertoni. The seeds of the plant were obtained from J.N.K.V.V. Jabalpur, M.P. For *in-vitro* regeneration of *Stevia rebaudiana* Bertoni Murashige and Skoog's medium was used with various concentrations of plant growth regulators[7].

Preparation of stock solutions

For convenience and to reduce time taken for weighing individual ingredients each time, concentrated stock solutions of selected components of the medium were prepared and stored in refrigerator. At the time of media preparation they were brought to room temperature and mixed proportionately to get the desired media.

After mixing all salts and organic components the pH was adjusted to 5.7 by adding 1 M NaOH. Then 5.6 g agar was mixed to the media (by using microwave oven) for making it semi-solid. Total amount of media was then subdivided into four conical flasks each containing 250 ml. Then the hormone, 2, 4-D was added at 2, 3, 4 and 5 mg/L. Each 250 ml media was then poured into 18 test tubes. Thus a total of 72 test tubes were filled with media. In the same way other 36 test tubes were prepared and finally 108 test tubes were used in the experiment. The test tubes were autoclaved 121°C temperature and 15 psi for 20 min.

Stock solution of macro nutrients

To make 1 liter of macro nutrients stock solution, the salt mentioned in table, were dissolved one after another in 600 ml of double distilled water and then the volume was made to 1 L, after filtering this solution was stored in refrigerator at 4^0 C.

Stock solution of micro nutrients

To make 1 liter of micro nutrients stock solution, the salts were dissolved sequentially as shown in table in 800 ml of distilled water. Final volume was adjusted to 1L.

Stock solution of irons

7.45 gm OF Na_2EDTA was dissolved in boiling water. Thereafter, 5.57 gm of ferrous sulphate was added gradually. This was kept on magnetic stirrer for at least one hour in hot condition until the colour of the solution changed to golden yellow. Finally, the volume was made up to 1L and stored in another colored bottle refrigerator.

Stock solution of vitamins

To make 50 ml of vitamin stock solution, 25 mg of nicotinic acid was first dissolved in 25 ml of boiling distilled water and after cooling the other two vitamins pyridoxine HCL (25 mg) and thiamine HCL (5 mg) were added. The final volume was made to 50 ml. this solution was stored in refrigerator at 0^0C for 10 days.

Stock solution of amino acids

100 mg glycine was dissolved in 50 ml of distilled water and stored at 0^0C for a maximum period of 15 days.

Inoculation technique

General aseptic techniques concerning in vitro culture of the explants were followed in the present experiment. After surface sterilization the explants were cut into very small pieces (about 1 cm) and inoculated onto shoot induction and proliferation media. When the shoot was fully growth (for 1 month) the shoots were taken out from the test tube a laminar flow hood and were cut (about) 1c.m. Then the micro cuttings were sub cultured on root induction medium. All inoculations and aseptic manipulations were carried out in a laminar air flow cabinet. Before use the working surface of the cabinet was cleaned by swabbing with 90% ethyl alcohol and UV light (for 20 minutes) to reduce the chances of contamination. The instruments like scalpels, forceps, needles etc were sterilized by an alcoholic dip followed by flaming inside the laminar air flow cabinet. Other requirements like Petri dishes, bottles, conical flasks, cotton, distilled water etc were sterilized by steam sterilization method. Before the onset of inoculations hands were washed thoroughly by soap and then swabbing with 70% ethyl alcohol. Cutting and transfer of the explants were carried out taking all possible care to ensure contaminations free inoculations.

Transplantation

The rooted plantlets were first transferred to plastic pots containing garden soil, sand and peat moss (1:1:1), covered with transparent polythene bags and placed in acclimatization room at 28±2°C with 70-90% relative humidity, after five days temperature was increased from 28 to 32°C. After two weeks, transparent bags were removed from pots for proper hardening. After four weeks, the plants were then shifted in greenhouse and in field under low light intensity. The data for various growth attributes were recorded such as % explants regeneration, multiple shoot formation, shoot length, % plantlets rooted, number of roots per plantlet, and survival of plants during acclimatization and in the field was recorded.

Results and Discussion

Multiplication and elongation of *Stevia rebaudiana* Bertoni

After the initiation of explant plantlet is a generated. In this experiment for the multiplication of plantlet, it is transferred to the multiplication MS medium supplemented with BAP (Cytokinin) at different concentrations viz. 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/l and 0.00 mg/l were used as control.

Effect of BAP (alone) on multiplication

The concentrations of BAP used found to be not significant differences for all parameters. Frequencies of bud break and bud elongation and mean number of regenerated shoots and shoot length were observed at 5mg/l BAP i.e. frequency of bud break is 82% and bud elongation is 88% while mean shoot number is (14.83) and mean shoot length is (4.51). While at concentration lower then 5.0 mg/l treatment resulted decline in all parameters.

Effect of Kn (alone) on multiplication

After bud break of explant plantlet is a generated. In this experiment for the multiplication of plantlet, it is transferred to the multiplication MS medium supplemented with Kn (Cytokinin) at different concentrations viz. 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mg/l and 0.00 mg/l were used as control. The concentrations of Kn used found to be not significant differences for all parameters. Frequencies of bud break and bud elongation and mean number of regenerated shoots and shoot length were observed at 5mg/l Kn. i.e. frequency of bud break is 72% and bud elongation is 88% while mean shoot number is (9.25) and mean shoot length is (5.51). While at concentration lower then 5.0 mg/l treatment resulted decline in all parameters.

Effect of IBA (Auxins)

After multiplication of plants the plantlets clumps were dissected and plantlets were transferred in to rooting medium. The one way factorial design was carried to study the effect of IBA (auxins) and their interaction on frequency of Root no. (FRN) and frequency of Root length (FRL) at different concentrations.

IBA is a plant growth regulator play important role in the rooting of plants. No significant differences in rooting were observed by various concentration of IBA i.e. 0.5, 1.0, 1.5, 2.0, 2.5 mg/l. Frequencies of Root No. observed in 2.0 mg/l IBA (3.62) and frequencies of Root length observed in 2.0 mg/l IBA (3.71). While at concentration lower then 2.0mg/l treatment resulted decline in all parameters.

Conclusion

Stevia sweetener extractives have been suggested to exert beneficial effects on human health, including antihypertensive, antihyperglycemic and anti-human rotavirus activities. In this study experiments were conducted to standardize the explant source and culture media for multiple proliferation of shoot and result in mass propagation of homogenous elite plantlets of *S. rebaudiana*. The result of the experiment and other earlier research report clearly support the possibility of propagating *S. rebaudiana* by adopting *in vitro* techniques.

References

1. Sakaguchi M. and Kan T. Japanese research on *Stevia rebaudiana*. Cl cult. 34, 235-248, (1982).
2. Antonie A. Stevia: A plant for sweetness; U.S. Pharmacist- a Johnson publication, (Campbell University School of Pharmacy). Vol 25, (2000).
3. Ma L. and Yan S. Identification of *Stevia rebaudiana* Bertoni Proteins by Sodium Dedecyl Sulphate Polyacrylamide Gel Electropherisis. Asian Journal of Crop Science. 1(1), 63-65, (2009).
4. Suanarunsawat T., Klongpanichapak S., Rungseesantivanon S. and Chaiyabutr N. Glycemic effect of stevioside and and *Stevia rebaudiana* in strep tozotocin induced diabetic rats. Eastern Journal of Medicine. 9, 51-56, (2004)
5. Chalapathi M.V. and Thimmegowda S. Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni) A future crop of India. Crop Research Hisar, 14 (2), 347-350, (1997).
6. Thorpe T.A. Physiology and Biochemistry of shoot Bud Formation in vitro. In: advances in Developmental Biology and Biotechnology of Higher Plants. Soh, W.Y., J.R. Liu and A.Komamine (Eds.), the Korean Society of *Plant Tissue Culture, Korea*, pp: 210-224, (1993).
7. Murashige, T. and Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. 15, 473-497, (1962).

Table 1: Effect of cytokinin (BAP) on multiplication of *Stevia rebaudiana* Bertoni

BAP(mg/L)	FBB	FBE	MNS	MSL
0.00	24.12	41.24	01.88±0.522	01.92±0.0752
1.0	46.02	71.66	07.25±0.4522	02.53±0.0651
2.0	58.56	82.56	09.58±0.5149	02.74±0.0669
3.0	68.82	84.52	10.83±0.5773	03.15±0.0674
4.0	72.68	86.42	12.83±0.5772	03.77±0.0754
5.0	82.24	88.16	14.83±0.5772	04.51±0.0718

Table 2: Effect of cytokinin (Kn) on multiplication of *Stevia rebaudiana* Bertoni

Kn(mg/L)	FBB	FBE	MNS	MSL
0.00	24.16	38.46	2.16±0.572	1.91±0.0256
1.0	39.76	56.32	3.17±0.5773	3.53±0.0651
2.0	48.52	70.02	4.580.5149	2.34±0.066
3.0	68.56	82.24	4.91±0.6685	4.15±0.0674
4.0	70.78	84.56	7.08±0.5149	3.70±0.0754
5.0	72.24	88.94	9.25±0.4522	5.51±0.0718

Table 3: Effect of Auxins (IBA) on Rooting of *Stevia rebaudiana* Bertoni

S. No.	IBA	FRN	FRL
1	0.5	3.47±0.55	3.29±0.083
2	1.0	3.51±0.59	3.48±0.054
3	1.5	3.38±0.54	3.55±0.07
4	2.0	3.62±0.57	3.71±0.12
5	2.5	3.24±0.43	3.65±0.078

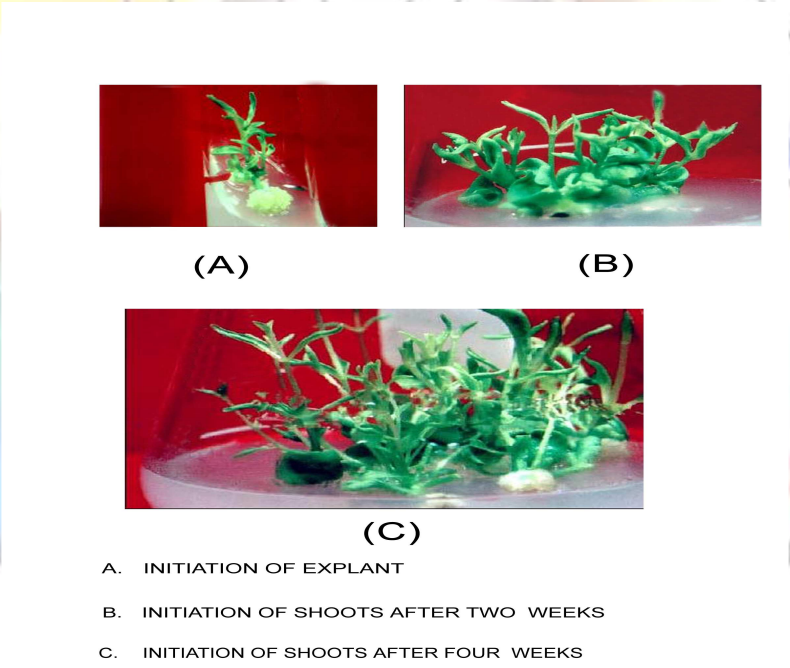

Fig. 1: *Stevia rebaudiana* Bertoni Culture: Intiations

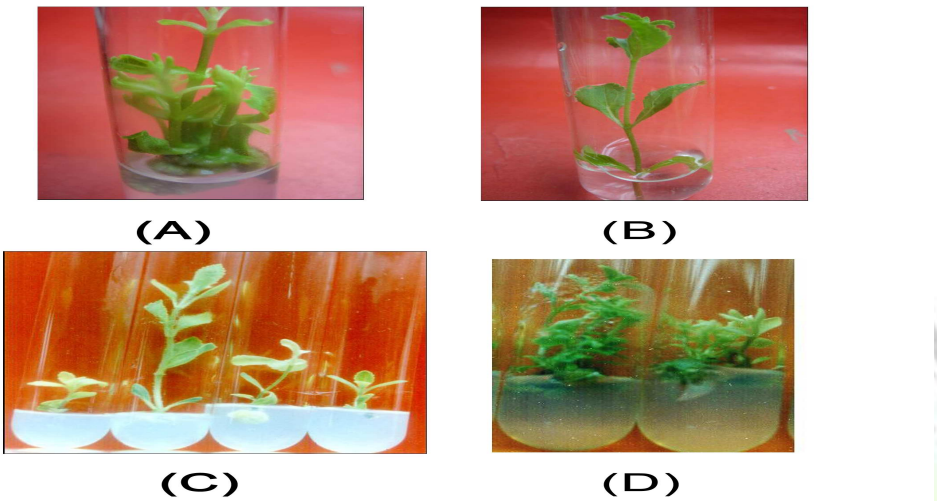

Fig. 2: *Stevia rebaudiana* Bertoni: Shoot multiplication

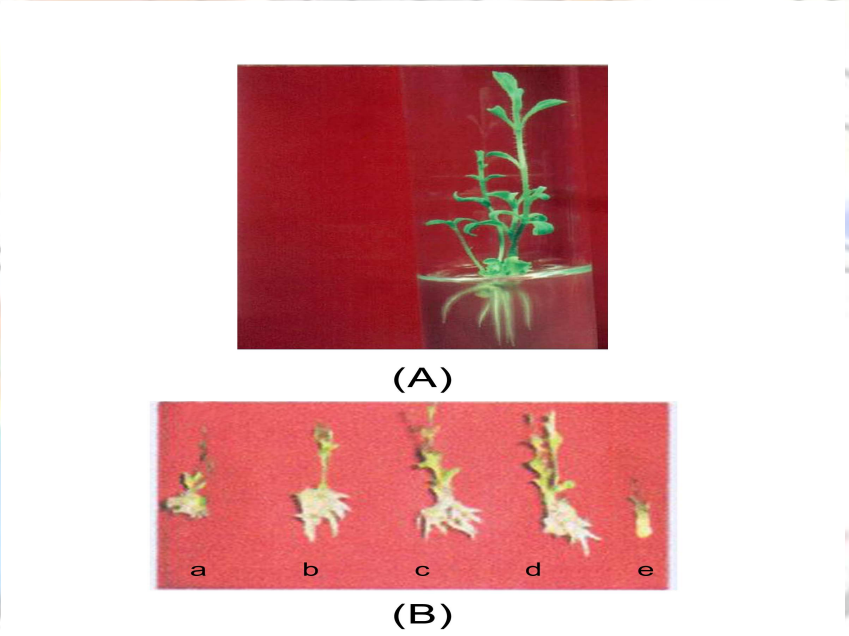

Fig. 3: *Stevia rebaudiana* Bertoni: Root formation