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IN VITRO CLONAL PROPAGATION AND BIOCHEMICAL ANALYSIS OF FIELD ESTABLISHED *STEVIA REBAUDIANA* BERTONI

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Abstract

An experiment was conducted to optimize growth medium and growth conditions for *In vitro* propagation of *Stevia rebaudiana* Bertoni through nodular stem sections. Varying concentrations of different growth regulators BAP, Kn, and IAA were applied through growth medium to assess their effects on shoots development while NAA and IBA were applied to assess their role in root formation. Although all plant growth regulators promoted shoot formation, maximum shoot formation was observed by supplementing 2.0 mgL^{-1} BAP. In contrast 0.5 mgL^{-1} NAA caused the maximum root formation in nodular stem sections of *S. rebaudiana*. Furthermore, survival rate of regenerated plants were 92 and 83% during hardening and shifting to green house, respectively. Various biochemical attributes (carbohydrates, proteins) in the leaves were also measured and compared with leaf extract of Egyptian cultivar to assess its quality.

Abbreviations: BAP Benzyl amino purine; **2,4-D** 2,4-Dichlorophenoxyacetic acid; **MS** Murashige and Skoog (1962) basal medium; **NAA** *a*-naphthalene acetic acid; **IBA** Indole-3-butyric acid; **IAA** Indole acetic acid

Introduction

Stevia rebaudiana Bertoni is a perennial herb of Asteraceae family, indigenous from higher elevations of northern Paraguay (Soejarto *et al*., 1983; Lewis 1992), but it could be found growing in semi-arid habitat ranging from grassland to scrub forest to mountain terrain. The plant has gained wide access to Pacific Rim countries, where in recent decades it is being cultivated domestically, used in its raw leaf form and is now commercially processed into sweetener. After first report of commercial cultivation in Paraguay in 1964 (Lewis, 1992), it has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada (Brandle & Rosa, 1992; Fors, 1995).

Stevia plants grow better having temperature range of 0-40°C. So these conditions allow *Stevia,* growing annually in areas having diverse environment. However, its cultivation on commercial basis is by seed, cutting or division of mother plants in green house during winter every year. The poor seed germination is one of the factors limiting large-scale cultivation while propagation through stem cutting requires enough stocks of stem cuttings and high labour inputs (Carneiro *et al*., 1997). A suitable alternative method to prepare sufficient amount of plants within short time duration is the use of *In vitro* cultures. The micropropagation of plants through shoot tip or axillary bud culture allow recovery of genetically stable and true to type progeny (George & Sherrington, 1984). *Stevia* can form multiple shoots from nodal explants, which are convenient type of culture for culturing on large scale. There are few reports of *In vitro* clonal propagation of *Stevia* using leaf, nodal, internodal segments and shoot tips (Yukiyoshi *et al*., 1984; Salim *et al*., 2006).

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A *Stevia* leaf produces a variety of high potency natural-source, low calorie (non-sucrose) sweeteners with 300 times sweeter than sucrose (Soejarto *et al*., 1983). These sweet compound pass through the digestive process without chemically breaking down, making safe to control sugar level (Strauss, 1995). The refined extracts of Stevia leaves are officially used as low calorie sweetener in processed foods, artificial diets and pharmaceuticals in Japan, Brazil, China and Korea (Brandle & Rosa, 1992; Kim & Kinghorn, 2002; Mizutani & Tanaka, 2002). It is recommended for diabetes and has been extensively tested on animals and has been used by humans with no side effects.

Pakistan has very diverse weather conditions from very cold (below 0°C) in winter to very hot (above 48°C) in summer season. There is no report of *Stevia In vitro* propagation or cultivation in Pakistan. The present investigation was undertaken to develop a protocol for rapid *In vitro* clonal propagation and to establish the plantlets in local environment of Pakistan and to assess the quality and quantity of sugars in leaf extracts.

Material and Methods

***In vitro* propagation of *Stevia rebaudiana*:** The *In vitro* clonal propagation of *Stevia rebaudiana* was carried out at Plant Biotechnology Laboratory, Qarshi Research International (Pvt.) Ltd. Hattar, Haripur, Pakistan. The seeds were surface sterilized with 1% Sodium hypochlorite for 10 minutes, washed with sterile distilled water for three times, inoculated on simple MS medium (Murashige & Skoog, 1962) and placed under light in growth room at 25±2°C. A plant bearing four nodal segments with leaves, divided into 0.5 cm pieces of nodal segments was used as explants.

For *In vitro* shoot multiplication, the nodular stem sections were inoculated on MS medium supplemented with 3.0% sucrose and 0.5, 1.0, 2.0, 3.0 and 4.0 mgL^{-1} of BAP and Kn separately and in combinations with 0.25 and 0.5 mgL^{-1} of IAA. While for root induction, excised microshoots (3.0-3.5 cm size) were cultured on MS medium supplemented with 2.0% sucrose and 0.25, 0.5, 1.0 and 1.5 mgL^{-1} of NAA and IBA separately. The pH of media was adjusted to 5.8 and solidified with 1.0% agar. All cultures were incubated in growth room at 25±2°C with photoperiod of 16h and 2500 lux white fluorescent light. 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 % explant 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.

Biochemical analysis of leaf extract: The biochemical analyses were conducted at Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan. The leaves were collected from two months old-field grown plants of *Stevia rebaudiana* growing at Qarshi Research International (Pvt) Ltd. Hattar, Haripur, Pakistan. For comparative study dry *Stevia* plants were kindly gifted by Agriculture Research Center, Giza, Egypt. The leaves were dried in oven at 72°C for 24 h. The leaf extract was prepared following method described by Dahot (1993). The total protein was determined followed by method described by Lowry *et al*., (1951) using serum albumin as the standard. The total carbohydrates were measured by Montgomery (1961) method while reducing sugars were estimated by Miller (1959) method using glucose as standard.

Free sugars from leaf extract were identified by applying 50 µL sample and standards on silica G-60 coated thin layer chromatographic (TLC) plates and developed with butanol: acetic acid: water (4:1:5 v/v), the spots were visualized by spraying with aniline pthalate. Free amino acids from leaf extract were identified by applying 50 µL sample and standards on TLC plates and were developed two-dimensionally using butanol: acetic acid: water (4:1:1 v/v) and phenol: water (4:1 v/v) solvents. After drying, the spots were visualized by spraying with ninhydrin.

Results and Discussion

***In vitro* shoot proliferation and multiplication:** The axillary buds in nodal zones proliferated with in 4-5 days of culture on cytokinin-supplemented MS medium. When the explants were cultured on 0.5 to 4.0 mgL^{-1} BAP containing media; 62-82% of them produced shoots. The axillary shoots proliferated and elongated 2.3-3.56 cm within three weeks of culture. In these experiments, MS medium containing 2.0 mgL^{-1} BAP showed the best response of multiple shoot formation with 8.33 ± 0.21 microshoots per explants (Table 1). When the explants were cultured on Kn based media, only 56-74% were proliferated to shoots and only 3.40 ± 0.24 microshoots per explant were produced. Similarly, no specific increase in multiple shoot formation was observed on different concentrations of BAP and Kn in combination with 0.2 and 0.5 mgL^{-1} of IAA. In all treatments, the highest shoot length 3.73 ± 0.14 cm per microshoot was observed on MS medium containing 2.0 Kn and 0.25 mgL^{-1} IAA after 15 days of inoculation. Further more, MS medium containing 1.0 and 2.0 mgL^{-1} BAP also showed the maximum callusing (+++) at base of explants (Table 1).

The nature and concentrations of cytokinin used in this study enhanced proliferation of axillary shoots derived from nodal segments of *In vitro* raised shoots of *Stevia*. Of all concentrations, the optimal concentrations of different plant growth regulators were 2.0 mgL^{-1} BAP, 0.25 mgL^{-1} IAA for BAP and IAA respectively were more effective in inducing proliferation of axillary shoots while Kn was considerably less effective. Other researchers also reported the influence of cytokinin on shoot multiplications in different plant species. Bondarev, (2001) reported that micro-cuttings with apical or axil buds were most effective explants for large-scale production, when cultivated on the hormone-free medium, and this allowed producing the plants by several thousands for 3-4 months. The addition of little concentrations of 6-BAP or α-NAA and 6-BAP to the nutrient medium was established to induce adventitious shoot formation by increasing a propagation coefficient. Sivaram & Mukundan (2003) reported the efficient regeneration of shoot apex, nodal, and leaf explants of *Stevia rebaudiana* Bertoni when cultured on MS medium supplemented with 8.87 BAP and 5.71 µ*M* IAA.

Root induction from microshoots: *In vitro* raised microshoots (2-3 cm) were excised from parent culture and grown on MS medium without or with growth regulators. Initiation of roots from microshoot was very slow in medium without IBA and NAA. The percentage of shoots that formed roots and number of roots/shoot significantly varied at different concentrations of NAA and IBA. The optimal rooting 81% in microshoots was observed on MS medium containing 0.5 mgL^{-1} NAA with 2% sucrose within two weeks of microshoot transfer (Table 2). Further more, the number of roots per microshoot was optimal 6.20 ± 0.2 on MS medium containing 0.5 mgL^{-1} NAA but the average root length per shoot was higher 3.23 ± 0.27 cm on 0.25 mgL^{-1} NAA containing MS medium (Table 2).

Table 1. Effect of different concentrations of BAP, Kn and IAA on multiple shoot formation and shoot length.

MS media + Growth regulators (mgL^{-1})		IAA	% of explants with multiple shoots	No. of shoots per explant (Mean + S.E)	Average shoot length (Mean + S.E)	Callusing at base
BAP	Kn					
0.5	--	--	79	4.33 ± 0.33	3.56 ± 0.16	+
1.0	--	--	82	5.25 ± 0.25	3.34 ± 0.23	+++
2.0	--	--	78	8.33 ± 0.21	3.44 ± 0.11	+++
3.0	--	--	72	4.75 ± 0.48	3.36 ± 0.12	++
4.0	--	--	62	2.50 ± 0.5	2.51 ± 0.16	--
--	0.5	--	72	3.00 ± 0.41	3.44 ± 0.11	--
--	1.0	--	69	3.25 ± 0.25	2.56 ± 0.21	+
--	2.0	--	74	3.40 ± 0.24	3.04 ± 0.1	++
--	3.0	--	62	2.80 ± 0.2	3.07 ± 0.12	+
--	4.0	--	56	2.60 ± 0.24	2.35 ± 0.14	--
0.5	--	0.25	72	4.20 ± 0.37	3.16 ± 0.14	--
1.0	--	0.25	68	4.80 ± 0.2	3.26 ± 0.15	+
2.0	--	0.25	73	7.38 ± 0.26	3.43 ± 0.11	++
--	0.5	0.25	76	3.78 ± 0.22	2.62 ± 0.19	--
--	1.0	0.25	71	4.17 ± 0.17	2.44 ± 0.13	--
--	2.0	0.25	66	3.89 ± 0.26	3.73 ± 0.14	+
1.0	--	0.5	72	5.33 ± 0.42	3.39 ± 0.15	+
2.0	--	0.5	74	7.14 ± 0.14	3.37 ± 0.14	++
--	1.0	0.5	70	2.88 ± 0.23	2.87 ± 0.17	+
--	2.0	0.5	69	3.00 ± 0.17	3.01 ± 0.23	+
--	--	--	72	1.67 ± 0.21	3.29 ± 0.25	--

Table 2. Effect of different concentrations of NAA and IBA on root formation, average root length and days of rooting.

MS media + Growth regulators (mgL^{-1})	Percentage of microshoots rooted	Number of roots per shoot (Mean + S.E)	Average root length (Mean + S.E)	Days to rooting
NAA				
0.25	73	4.00 ± 0.41	3.23 ± 0.27	7
0.5	81	6.20 ± 0.2	2.89 ± 0.35	7
1.0	72	3.83 ± 0.17	1.96 ± 0.21	9
1.5	66	2.86 ± 0.14	1.88 ± 0.26	10+
IBA				
0.25	72	3.00 ± 0.32	3.14 ± 0.3	7
0.5	74	2.86 ± 0.34	2.67 ± 0.27	8
1.0	71	1.50 ± 0.22	1.29 ± 0.26	12
1.5	64	1.14 ± 0.14	1.20 ± 0.28	13+
No hormone	43	1.11 ± 0.1	2.76 ± 0.38	15+

The effect of NAA and IBA on root induction was positive, however, auxin's concentration had a positive effect on the number of roots/shoot and it was higher in the case of NAA than IBA. Steven *et al.*, (1992) reported that 1.0 mgL^{-1} NAA in medium showed maximum rooting in regenerated shoots of *S. rebaudiana*. The potential of IBA in root induction bas been reported in many species (Epstein *et al*., 1993). In root induction experiment, 0.5 mgL^{-1} NAA was found better as maximum roots proliferated, that results are better than reported by Steven *et al.*, (1992).

Table 3. Approximate composition of one-gram dry leaves sample of *Stevia rebaudiana*.

Carbohydrates/Proteins	Sample 1 (mg/g)	Sample 2 (mg/g)
Total carbohydrates	306	311
Reducing sugars	27.28	27.41
Total proteins	33.2	32.93

Sample 1: Leaves sample collected from field-established plants in local environment of Pakistan
Sample 2: Leaves sample kindly gifted by ARC, Giza, Egypt.

Acclimatization and field establishment: Rooted plantlets were shifted to plastic pots containing sterilized soil, sand and peat moss at 1:1:1 ratio, covered with transparent polythene bags and placed in acclimatization room at 28°C with 70-90 humidity. The temperature was gradually increased to 30, 32 and 36°C after every week, while the transparent bags were partially removed for 4 to 6 hours from pots after two weeks and than completely removed after three weeks for proper hardening. A total number of 92% plants survived through this process. When the plants were shifted in green house and field under shade and bright light conditions with average temperature 36°C; 83, 79 and 53%, plants survived in green house, under shade and bright light conditions at 48±2°C during hot summer and 0±2°C during cold winter (data not shown). Megeji *et al*., (2005) studied the *Stevia* introduction in new area and revealed that growing pattern and stevioside yield of *S. rebaudiana* cultivation was successful under agro climatic conditions of Palampur, North Indian region.

The acclimatization of plantlets by gradual increase in temperature and use of polythene bags to control humidity improved the survival of plants during hardening process. Many researchers used different parameters to increase the survival rates of plants. Storing the plantlets at low temperature is believed to improve survival of plants *In vivo* and enhance their normal development in the field, while *In vitro* hardening of explants is also an alternative that could abridge transplanting shock and speed up the whole propagation procedure (Kramarenko, 1999). In many species, the reduced survival rate of the explants during acclimatization is affiliated to the utilization of high concentrations of IBA or NAA in the rooting medium (Al- Maarri *et al*., 1994).

Biochemical analysis: The approximate composition of carbohydrate and protein contents of leaf extracts of *Stevia* was found almost similar to leaf extracts of *Stevia* growing in ARC, Giza, Egypt. The soluble total sugar contents were 306 mg/gram in sample 1 with reducing sugars and total proteins 27.28 and 33.2 mg/gram of dry weight (Table 3). The free amino acids in leaf extracts of *Stevia* were detected through thin layer chromatographic analysis. Out of 20, eight amino acids were identified as glutamic acid, aspartic acid, lysine, serine, isoleucine, alanine, proline and tyrosine in both samples. Two unknown spots were also visualized; these may be peptides or small non-protein amino acids such as β-alanine. For sugars, 6 spots were separated through thin layer chromatography; 5 were identified as mannose, galactose, maltose, glucose and fructose, while one spot was identified as unknown in leaf extracts of both samples.

In conclusion, a protocol for *In vitro* clonal propagation of *S. rebaudiana* has been optimized and the *In vitro* raised plantlets have been established in local environment of Pakistan. The initial study of few biochemical attributes showed no significant difference. This study will be helpful to establish and cultivate *S. rebaudiana* for commercial scale production in different local environments of Pakistan.

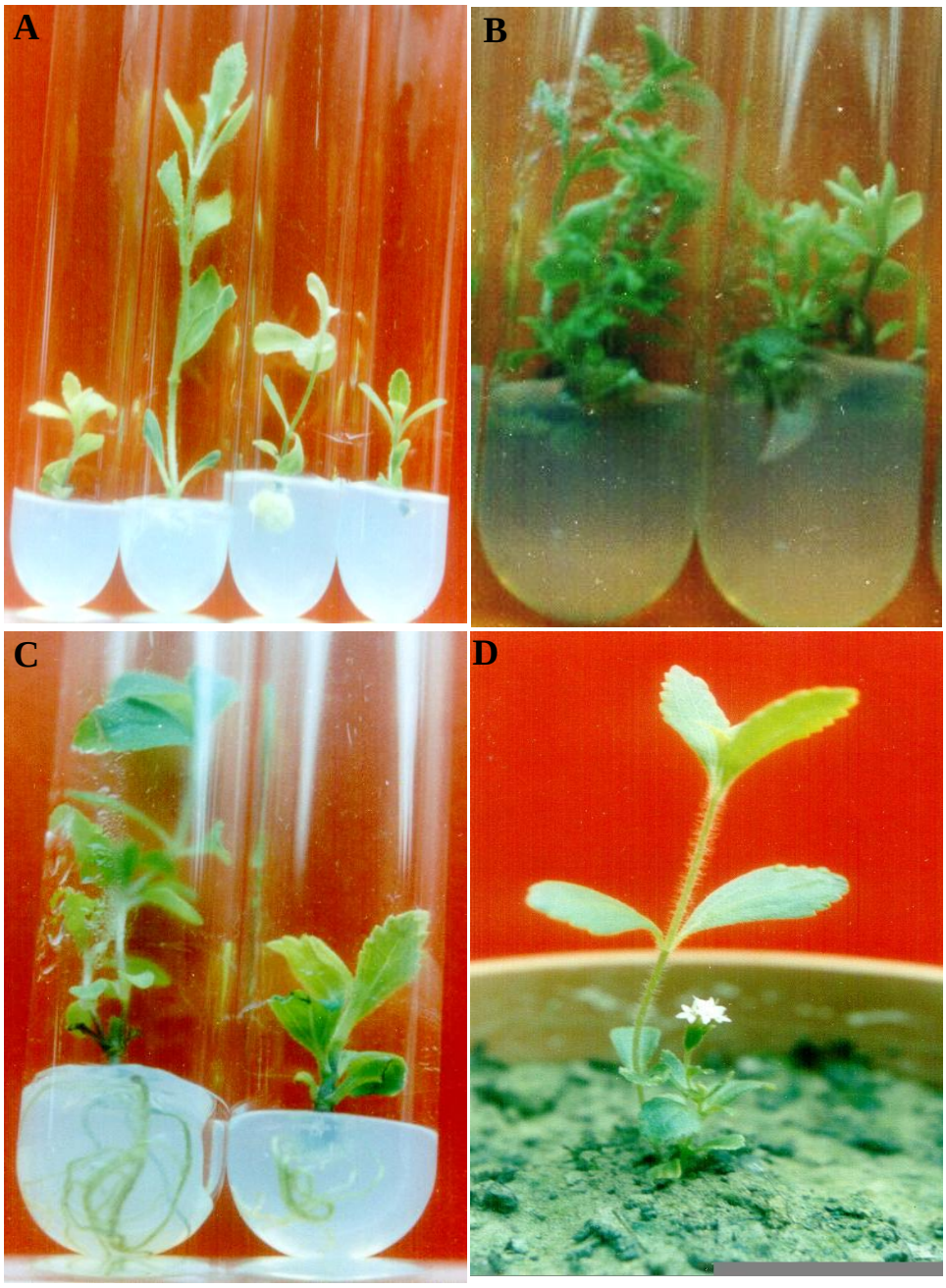

Fig. 1 (A). Explant proliferation and regeneration of *Stevia* on MS medium with different hormonal concentrations, (B). Multiple shoot formation on BAP containing MS medium, (C). Root formation on NAA and IBA containing MS medium, (D). Plantlet established in pot containing garden soil, sand and peat moss (1:1:1 ratio)

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