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Micropropagation of an Elite Medicinal Plant: *Stevia rebaudiana* Bert.

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ABSTRACT

To sustain the supply of quality propagules, the present study was carried out to develop a novel protocol for accelerated *in vitro* mass multiplication in stevia (*Stevia rebaudiana* Bert.) through multiple shoot induction using shoot tips, nodal segments and axillary bud explants. The earliest bud induction was recorded from the shoot tip explants within 6 days of culture in Murashige and Skoog (MS) medium, in comparison to the other two explants. MS basal medium supplemented with sucrose (30 g L^{-1}), agar (7 g L^{-1}) and kinetin (2 mg L^{-1}) performed best in multiple shoot proliferation, resulting more than 11 multiple shoots from a single shoot tip explant within 35 days of culture. For root induction and elongation, MS medium devoid of plant growth regulator performed most dynamically, where MS medium plus indole-3-acetic acid and 6-benzylaminopurine showed an adverse effect, promoting undesirable callus growth at root zone. *In vitro* generated propagules were successfully acclimatized in a balanced mixture of sand, soil and farm yard manure (1:1:1 v/v). Peroxidase assay along with ISSR fingerprinting confirmed the genetic clonality of *in vitro* generated propagules.

Key words: Direct organogenesis, genetic clonality, *in vitro*, peroxidase, shoot tip

INTRODUCTION

Stevia (*Stevia rebaudiana* Bert.), the popular family member of Asteraceae, is a sweet, medicinal herb of Paraguay, consisting a non-caloric natural sugar, alternative to artificially produced sugar substitutes. The stevia leaf is 300 times sweeter than sugar (sucrose) obtained from sugar beet, sugarcane *etc.*, with a zero-calorie value (Richman *et al.*, 1999). The stevia leaves are the significant resource of diterpene glycosides, like rubsoside, steviolbioside, dulcoside, rebaudiosides and stevioside (Starratt *et al.*, 2002). Amid these compounds, stevioside ranks top in dramatically accelerated use in health concerns related to dental cares, diabetes and obesity. The sweetness is due to stevioside, the most abundant glycoside (Kinghorn, 1992). The sweet compounds pass through the digestive process without chemically breaking down, making stevia safe for diabetic and obese people. Recently, stevia attained a better awareness owing to its superior sweetness and the curative values for restraining the accretion of fat and lowering blood pressure in human (Chalapathi *et al.*, 1997). Additionally, the diterpene glycosides of stevia are being used in array of foods products and beverages. The extract from stevia leaves formally have been accepted as food supplement in a number of countries like Brazil, Korea and Japan (Mizutani and Tanaka, 2002). The stevia leaves are treated as prospective source in the area of

pharmacology and pharmacognosy (Anbajhagan *et al.*, 2010). In Asia, Japan was the first to commercialize the stevioside as a sweetener in medicine industries. Later, the cultivation of stevia has been extended to quite a lot of countries in Asia, along with USA, Canada and several countries of Europe (Brandle *et al.*, 1998). Though in recent times, stevia is being popularized in a number of countries, but large-scale propagation techniques are yet to be standardized to restore the quality and quantity of plants (Dhananjay and Deshpande, 2005). Propagation through seeds is very difficult due to self-incompatibility which results in sterile seeds. Hence *in vitro* propagation appears as an alternative technique for rapid multiplication of stevia within a short span of time (Das *et al.*, 2005). Further *in vitro* propagation through direct organogenesis provides a rapid reliable system for a production of large number of genetically uniform disease free plantlets, irrespective of the season. Although, earlier attempts have been made for the propagation of stevia through tissue culture (Bespalhokk *et al.*, 1997; Kornilova and Kalashnikova, 1997; Ahmed *et al.*, 2007; Debnath *et al.*, 2008), but a considerable effort is still required to make it more practicable.

The present study has been undertaken to develop a novel protocol for *in vitro* accelerated mass multiplication with an efficient acclimatization procedure and to assess the clonal fidelity of *in vitro* generated propagules through biochemical and molecular markers.

MATERIALS AND METHODS

Collection of explants and surface sterilization: The present experiment was carried out in the Tissue Culture Laboratory and departmental green house of Biotechnology department, Bidhan Chandra Krishi Viswavidyalaya, W.B., India for the period of 2006-09. Young, actively growing shoot tips, nodal segments and axillary buds were collected from two months old stevia plants, being aseptically maintained in the greenhouse of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, India in the year 2006. The collected explants of 2-3 cm were surface sterilized by treating with mercuric chloride solution (0.1% w/v dissolved in 50% v/v alcohol) for 2 min and then rinsed 5-6 times with sterile water. Excess water adhering to the explants was soaked on sterilized blotting paper. The exposed ends of the explants were trimmed to 1.5 cm before inoculation. The whole process was performed at a strict aseptic condition under a laminar air flow.

Culture conditions: MS (Murashige and Skoog, 1962) basal medium with 30 g L^{-1} sucrose was used after solidifying with 7 g L^{-1} agar. Different plant growth regulators (PGRs) like 6-benzylamino purine (BAP), Indole-3-butyric acid (IBA), Indole-3-acetic acid (IAA) and 6-furfurylaminopurine (KIN) were added at various concentrations to MS before the pH of the medium was adjusted to 5.6. Media were autoclaved at 1.06 kg cm^{-2} and 121°C for 20 min. Cultures at all growth stages were incubated under artificial conditions: 23±2°C, 60% RH and a 16-hr photoperiod under a photosynthetic photon flux density of 20 μmol/m^2/sec.

***In vitro* propagation:** For shoot bud induction and standardization of explants, MS basal media devoid of any PGR was tested (Table 1). Explants were inoculated in the media, dipping approximately up to 0.5 cm for good contact. The cultures were incubated following the above mentioned artificial growth conditions, until fresh multiple greenish buds appeared. The best suited explant was identified in terms of performance over response, days to bud induction and; number and length of shoot buds. Next, the induced buds were separated and cultured for multiple shoot proliferation in MS, fortified with varying Levels of IAA, BAP and KIN that are used (Table 2). The best resulting media formulation was identified in the respect of response, number and length of shoots.

Table 1: Response of different explants on shoot multiplication in MS basal medium

Explants	Percent response	Days to bud break	Shoot length (cm)	No. of shoots	Remarks
Shoot tip	85.14±0.7a	6.47±0.5c	11.13±0.2a	8.33±0.5a	+++
Axillary bud	71.09±0.4b	8.80±0.5b	8.53±0.1b	6.28±0.5b	++
Nodal segment	57.85±0.8c	10.33±0.9a	8.36±0.04c	5.19±0.0c	+
Mean	71.360	8.539	9.123	6.603	
CD (5%)	1.824	0.701	0.261	0.289	
SE(±)	0.592	0.227	0.085	0.094	

+++: Prolific, ++: Good, +: Fair. Data represent Mean±SD of 20 replicants per treatment in seven repeated experiments. Means within columns separated by Duncan's multiple range test p = 0.05; Duncan (1955)

Table 2: Effect of growth regulators on shoot multiplication

PGRs (mg L^{-1})							
IAA	BAP	KIN	Percent response	Days to bud induction	Shoot length (cm)	No. of shoots	Remark
0.25	1	-	50.42±3.5f	12.75±0.1a	5.15±0.0f	2.33±0.5e	Thin shoots, roots, callus
-	2	-	63.00.±1.5d	11.00±0.0c	6.70±0.0d	5.00±0.0c	Thin shoots with callus
-	3	-	53.00±1.5e	12.00±0.0b	6.00±0.0e	4.33±0.4d	Thin shoots with callus
-	-	1	75.00±0.2b	9.50±0.5d	8.95±0.1c	5.33±0.5 c	Healthy shoots
-	-	2	85.00±0.7a	6.47±0.5f	11.13±0.2a	11.33±0.5a	Healthy shoots
-	-	3	78.00±0.1c	7.75±0.1e	10.26±0.2b	7.33±0.4b	Thin shoots
Mean			67.403	9.917	8.061	5.774	
C.D. (5%)			4.723	0.635	0.408	0.579	
SE(±)			1.567	0.211	0.135	0.219	

Data represent Mean±SD of 20 replicants per treatment in seven repeated experiments. Means within columns separated by Duncan's multiple range test p = 0.05; Duncan (1955)

Table 3: Response of growth regulators for root induction and elongation

PGRs (mg L^{-1})						
IAA	BAP	Percent response	Days to root induction	Length root (cm)	No. of roots	Remarks
0.10	0.25	52.00±0.3c	10.75±0.1c	6.73±0.0c	5.66±0.5d	Thin, hairy root growth with profuse callus
0.25	0.25	47.14±1.5b	11.75±0.1b	6.83±0.0b	6.00±0.4d	Thin, hairy root growth with callus
0.50	0.25	39.00±0.9d	12.75±0.2a	6.63±0.0d	4.33±0.4c	Thin, hairy root growth with small callus
-	-	82.14±2.3a	9.25± 0.1d	9.16±0.0a	15.58±0.5a	Significant root growth, healthy, long, profuse root
Mean		55.070	11.125	7.653	8.583	
CD (5%)		2.513	0.705	0.076	0.926	
SE(±)		0.786	0.221	0.025	0.301	

Data represents Mean±SD of 20 replicants per treatment in four repeated experiments. Means within columns separated by Duncan's multiple range test p = 0.05; Duncan (1955)

The *in vitro* grown healthy stevia shoots were transferred for root development. MS basal media was supplemented with different levels of IAA or IBA where only MS mediu, (excluding PGR) served as a control (Table 3). The proliferated multiple shoots were separated and transferred subsequently to the rooting media. The efficiency of IAA and IBA over simple MS medium was assessed in terms of root induction response, days to root induction, number, length and health of *in vitro* roots.

Acclimatization: The well-rooted stevia plantlets were removed gently from the culture vessels and washed thoroughly with distilled water to remove all the traces of the adhering agar. The

regenerated plantlets were planted in small plastic cups filled with sterile sand and transferred to a poly house, accompanied with intermittent water spraying, for a period of ten days. After primary acclimatization, the plantlets were shifted into black poly bags filled with sterile pot mixture (farm yard manure; sand; soil 1:1:1 v/v) along with optimum water supply to retain a high humid condition till transplantation in the field.

Assessment of clonal fidelity: The acclimatized micropropagated plantlets were passed though the clonal fidelity assessment using both biochemical and molecular approaches. For biochemical assessment, peroxidase isozyme profile was performed using fresh leaf samples of randomly selected ten clones along with their mother plant. The isozyme banding pattern was observed through 7.5% polyacrylamide gel electrophoresis following the standard procedure of Gantait (2004).

For assessment of clonality at molecular level, ten selected ISSR primers (Gantait *et al*., 2008) were used in polymerase chain reaction amplification. Genomic DNA extraction was carried out from cotyledonary leaves of acclimatized plantlets following the method of Chattopadhyay *et al*. (2008). Twenty five microliter optimized PCR mixture contained 40 ng DNA, 2.5 µL 10X Taq polymerase assay buffer, 3.5 µL 2.5 mM dNTPs, 0.5 U Taq DNA polymerase and 200 ng of primer. The PCR was performed with an initial denaturation at 94°C for 5 min followed by 35 cycles of 45 sec at 94°C, 45 sec at annealing temperature and 90 sec at 72°C and final extension at 72°C for 7 min, 4°C for 5 min was done using Gene Amp PCR system 2400 (Applied Biosystems, USA). The annealing temperature was adjusted as par the Tm of the primer, being used in the reaction. The amplified PCR products, along with 50 bp DNA ladder were resolved by electrophoresis on 1.5% agarose gel in 1X TBE buffer, stained with ethidium bromide (10 µg L^{-1} TBE buffer). The well-resolved and consistently reproducible, amplified DNA fragments as bands, were scored in terms of their appearance or nonappearance and photographed on Gel Logic 200 trans-illuminator system (Kodak).

Data analysis: Complete Randomized Design (CRD) was followed for the *in vitro* culture experiments. Each single explant was considered as an experimental unit. The experiments were carried out in six replications including 20 explants in each and the standard deviation was calculated from the accumulated data. Angular transformations of the percent response values were carried out before the collected data were subjected to Analysis of Variance (ANOVA). Significant difference among the treatments were tested by Duncan's multiple range test (Duncan, 1955) at 5% level using WINDOWSTAT 7.5 (Indostat services, Hyderabad, India) software package from Uttar Banga Krishi Viswavidyalaya, India. For ISSR profiles, the well-resolved and consistently reproducible amplified DNA fragments were scored in terms of their presence or absence. To detect the genetic purity, the resulting banding patterns were compared between DNA samples for each ISSR primer.

RESULTS AND DISCUSSION

***In vitro* propagation:** Observations, documented on response of different explants on shoot bud induction after around a week of culture, clearly signified that amid the three explants inoculated, the shoot tip performed best in all aspect. For shoot tip inoculation in MS basal medium the maximum 85.14% response was recorded (Table 1) and first bud induction was recorded within six days (6.47 days) approximately (Fig. 1A). On the 35th day of culture, around eight multiple shoots developed from a single shoot tip explant with prolific growth. Similar report regarding the

Fig. 1: Micropropagation of stevia. (A) induction of shoot buds, (B) induction and proliferation of multiple shoots, (C) rooting *in vitro*, (D) well rooted *in vitro* generated plantlets, (E) initial stage of acclimatization in sterile sand, (F) advanced stage of acclimatization in sand, soil and farm yard manure (1:1:1 v/v)

efficiency of shoot tip explant on initial culture establishment was also obtained from the study of Kornilova and Kalashnikova (1997) in stevia. Whereas, in contrary to the present account, Bespalhokk *et al.* (1997) and Ahmed *et al.* (2007) reported that the nodal segments from adult *S. rebaudiana* plants cultured for shoot proliferation, produced best result than the shoot tip explants.

For assessment of the PGR efficiency on shoot multiplication, shoot buds were inoculated in MS medium, supplemented with different auxin-cytokinin sources. The monitored performance on shoot multiplication clearly pointed out that a combination of IAA and BAP or either BAP alone does not offer any satisfactory result (Table 2). Both IAA and BAP encompassed an adverse upshot in shoot proliferation as their combinations promoted uninvited callus growth, restraining the usual shoot multiplication. The most noteworthy outcome of the present study was that, auxin displayed an unfavorable consequence on the multiplication frequency as well as on the phenotype of the *in vitro* rising shoots. Whilst BAP alone was used, a reduction in callus growth was observed at the base of the shoots. But after BAP was substituted by KIN as an alternative cytokinin source, the circumstances were radically changed. The preeminent result was obtained in MS medium fortified with the KIN at the concentration of 2.0 mg L^{-1}. Response of shoot tip in multiplication and proliferation was the utmost with 85%. There was no symptom of callus growth and on an average, more than 11 shoots emerged (Fig. 1B) having 11.13 cm length from single inoculants. So, the present study by and large ascertained the efficiency of KIN over BAP in shoot multiplication *in vitro*. Whereas, the earlier experiments of Ahmed *et al.* (2007) and Debnath (2008) established the competence of BAP as an exclusive PGR for multiplication of stevia. Nevertheless, the present report is an innovation on the consequence of cytokinins in shoot multiplication and proliferation

in stevia. During the study of micropropagation in a number of plants, it has been found that KIN is much more effective than BAP which supports the present investigation report on appraisal of better cytokinin source for stevia (Evaldsson *et al*., 1985; Wang, 1986; Gantait *et al*., 2009). The large number of adventitious shoot buds developed in presence of KIN is attributable to the fact that KIN triumphs over apical dominance, releases lateral buds from dormancy and upholds shoot formation (George and Sherrington, 1984).

For root induction and elongation, MS supplemented with IAA or IBA were futile to verify their potentiality in the present study. Roots emerged in the media consisting MS plus auxin, were of dawdling growth rate, thin and hairy (Table 3). Invariably in all these PGR combinations, an increased callus induction was observed with the eminent concentration of auxins. It was interesting to observe that, PGR free MS medium performed the best regarding the induction and growth of healthy roots in an optimal number (Fig. 1C). In the present instance, auxin had proved its adverse effect in rooting by promoting callus growth. The competence of PGR free MS for *in vitro* rooting of stevia in the present report supports the earlier study of Bespalhokk *et al*. (1997).

***Ex vitro* establishment:** After attaining the rapid *in vitro* multiplication rate, successful acclimatization or establishment of tissue culture-raised propagules in soil, is the key parameter of a micropropagation protocol (Ahmed *et al*., 2007). Ultimate triumph of any *in vitro* propagation venture depends on the accomplishment of *ex vitro* adaptation of tissue cultured plantlets. The well rooted plantlets were advanced for an easy two-step acclimatization procedure (Fig. 1D). After the transfer from *in vitro* culture vessels to *ex vitro* plastic cups, filled with sterile sand, the survival rate of 93.09% plantlets was assured (Table 4), after 10 days of growth (Fig. 1E). Sterile sand helped in primary root regeneration and good anchorage to plantlets as an ideal growth medium without any contamination (Gantait *et al*., 2009). Survival percentage was reduced with advancement of time because tissue cultured plantlets were delicate as they have been grown under low intensity artificial light and high humidity. At 20 days after transfer to the polythene bags, containing a blend of sand, soil and farm yard manure (1:1:1 v/v), the survival percentage was reduced to 82.14% (Fig. 1F) which was still satisfactory for the present protocol. In this successful acclimatization of *in vitro* raised plantlets, the retention of high humid condition played a decisive part and the presence of farm yard manure is supposed to be responsible in preservation of moisture.

Clonal fidelity study: Despite the fact that there are several reports available on *in vitro* propagation of stevia (Yukiyoshi *et al*., 1984; Ferreira and Handro, 1988; Sivaram and Mukundan, 2003); the assessment on clonal fidelity has not been conceded out in any of these. So, the present study found to be a significant one in this aspect, as the genetic clonality of *in vitro* raised

Table 4: Response of *in vitro* generated plantlets on *ex vitro* acclimatization

Days after *ex vitro* transfer	Survival percent	Plant height (cm)	No. of leaves	Plant health
0	93.09±2.8a	7.55±0.0c	12.33±1.2c	+
10	90.00±4.4b	7.86±0.1b	18.33±2.4b	++
20	82.14±2.5c	8.40±0.2a	22.33±1.6a	+++
Mean	78.590	7.941	17.666	
CD (5%)	4.278	0.205	2.668	
SE(±)	1.388	0.066	0.849	

+++: Prolific, ++: Good, +: Fair. Data represents Mean±SD of 20 replicants per treatment in seven repeated experiments. Means within columns separated by Duncan's multiple range test p = 0.05; Duncan (1955)

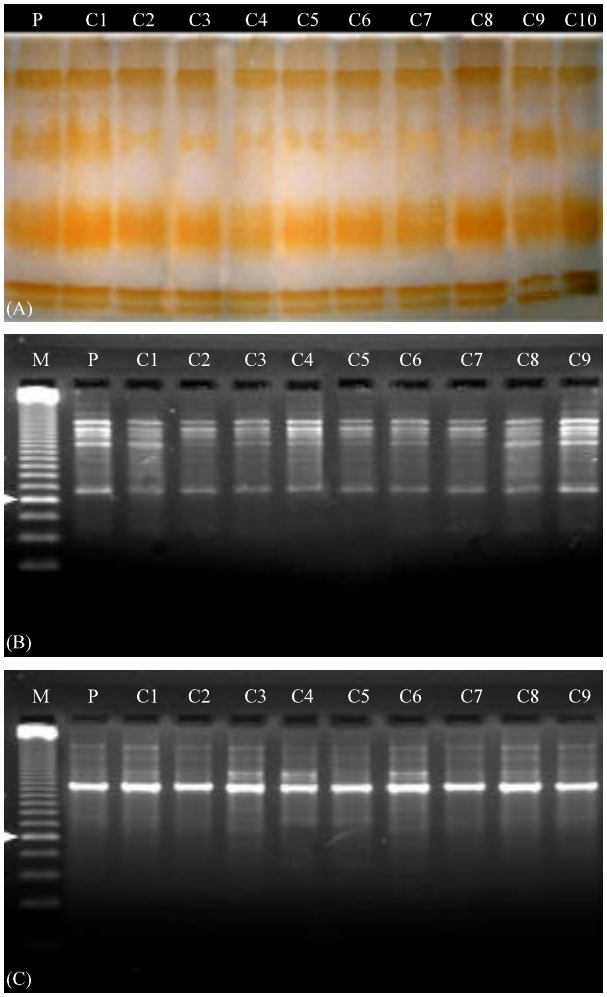

Fig. 2: Clonal fidelity assessment of stevia. (A). Polyacrilamide gel electrophoresis of peroxidase isozyme extracted from *in vitro* regenerated clones (C1-C10) and their mother (P); Agarose gel electrophoresis of ISSR fragments of *in vitro* regenerated clones (C1-C9) with their mother (P) showing monomorphic bands generated by primer IS-7 (5' GTG TGT GTG TGT GTG TA 3') (B) and IS-63 (5' AG AG AG AG AG AG AG AG C 3') (C). Lane M-50 bp ladder (white arrow indicates 250 bp)

propagules was studied in details using both biochemical and molecular markers. Observed banding pattern of peroxidase isozyme revealed that as many as six bands were reproduced and they were monomorphic in nature as compared to their parent and with same Rm values (Fig. 2A). They continued to be in the same group, made by the observations on banding similarity in either number or in intensity. In ISSR profile, among the ten selected primers used, six displayed positive interaction with stevia genomic DNA. But, only four of them appeared to be reproducible. A total number of 240 scorable bands were recorded, ranging in size from 250 bp to 1200 bp. For the sake of brevity profile, two primers IS-7((5' GTG TGT GTG TGT GTG TA 3') and IS-63(5' AG AG AG AG AG AG AG AG C 3') with ten clones and their parent has been presented (Fig. 2B, C). All the reproducible bands for these primers displayed monomorphic patterns for all the clones analyzed.

Although, this study has not detected any genetic change, it is likely that some changes might be occurred have gone undetected, as there is a possibility of point mutations occurring outside the priming sites. The analysis of clones with an increased numbers and types of markers may reveal further clear result. Still, as far as it could be ascertained, the *in vitro* generated clones exhibited their genetic purity with their mother.

CONCLUSION

In conclusion, the aforesaid protocol of *in vitro* mass propagation assures a colossal prospective for exploitation of stevia in a large scale. Furthermore, it will help in saving the labour and cost over raising the plantlets through traditional propagation practices. The explant sources and PGR levels have significant impact on accelerated micropropagation of stevia to regenerate genetically true to the type propagules. So the present study can be considered as a promising approach for commercial level propagation of medicinally important plant stevia.

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