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Genotypic response of *Stevia rebaudiana* accessions to *in vitro* clonal propagation using nodal explants

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ABSTRACT

Genotypic response to *in vitro* clonal propagation was studied in 21 accessions of *Stevia rebaudiana*. Data were recorded with respect to per cent proliferation, root induction and survival during hardening process and Chi-square test for goodness of fit was used to determine the variations regarding response to different nutrient media used for *in vitro* clonal propagation. Nodal explants of *Stevia rebaudiana* can regenerate shoots when cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyl amino purine (BAP; 1 mg/l) and kinetin (Kn; 0.5 mg/l). Rooting of *in vitro* shoots was achieved by adding indole butyric acid (IBA; 0.2mg/l) on half strength MS medium. The accessions C1-4-2, E2-1-6, C1-4-3, E1-2-3 and C1-4-3 of stevia were identified as responsive genotypes to *in vitro* clonal propagation and have potential use in clonal breeding programmes.

Key words: Genotypic response, *In vitro* clonal propagation, Nodal explant, *Stevia rebaudiana*

Stevia rebaudiana (Bertoni) is a perennial shrub belonging to family Asteraceae. The plant originated from the northern regions of South America and grows wild in the highlands of Amambay. It is being commercially cultivated in China, Taiwan, Thailand, Korea, Japan, India and Malaysia. Leaves of stevia contain diterpene glycosides, viz. stevioside and rebaudiosides which are estimated to be 100-300 times sweeter than sucrose (Tanaka 1982). It is a natural source for non-calorific sweetener that passes through the digestive process without chemically breaking down making stevia safe for diabetics. Stevia has various other therapeutic values such as anti-cancerous (Jeppensen *et al*. 2002, 2003) antibacterial and antifungal (Rojas and Miranda 2002). Products of stevia can be added to tea, cooked, baked and processed foods. Its commercial and medicinal value demands for large scale production of stevia plants. Seeds of stevia develop through open pollination show very low germination percentage and propagation by seeds do not allow the production of homogenous population resulting in variability in sweetener level and composition (Nakamura and Tamura 1985, Miyagawa *et al*. 1986). Vegetative propagation by stem cuttings is also limited by the low number of individuals that can be obtained simultaneously from single plant.

To overcome these problems tissue culture is an important technique which has proved to be a potential means to generate true to type planting material in stevia through *in vitro* clonal propagation (Sivaram and Mukandan 2003, Patel and Shah 2009, Ahmed *et al*. 2007). However, the success of *in vitro* clonal propagation may be highly dependent on the genotype of the donor plant, its growth environment and culture conditions and their interactions. Therefore, the present study was undertaken to identify an effective nutrient medium for rapid propagation through nodal explants and investigate the response of genotypes on *in vitro* clonal propagation in different accessions of *Stevia rebaudiana*.

MATERIALS AND METHODS

In the initial experiment a total of seventy five nodal explants of randomly selected plants were cultured on Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with varying concentrations of BAP and Kn (M1 - M15). The medium which gave best response with respect to shoot proliferation was used for proliferation in the subsequent experiment. Similarly, to establish the effective rooting medium a total of thirty regenerated multiple shoots were cut and individual shoots were inoculated in half strength MS medium containing different concentrations of IBA for root induction (RM1 - RM6) and the medium which gave best response with respect to root induction was used for proliferation in the subsequent experiment.

In the second experiment, freshly growing apical shoots were procured from each of the twenty one (six months old plant) accessions for *in vitro* culture. The 21 accessions were

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Table 1 Steviol glycoside profile of stevia accessions selected for *in vitro* studies

Accessions of stevia	Stevioside (%)	Rebaudio-side-A (%)	Total Stevioside + Rebaudio-side-A	Reb-A/ Stev.
C6-4-5	2.7	1.57	4.27	0.58
E3-1-1	4.0	5.3	9.30	1.33
C11-3-2	5.14	3.24	8.38	0.63
C1-4-3	7.6	0.94	8.54	0.12
G7-5-3	5.3		5.3	
C6-4-3	6.0	0.15	6.15	0.03
E3-1-5	3.90	4.74	8.64	1.22
E1-2-3	2.86	6.96	9.82	2.43
C2-1-2	6.1	2.56	8.66	0.42
C2-1-5	11.0	0.16	11.16	0.01
E2-1-6	1.89	4.26	6.15	2.25
C3-4-3	2.72	0.05	2.77	0.02
C1-4-2	5.3	5.3	10.60	1.00
C14-1-9	7.8	0.02	7.82	0.00
C3-2-4	3.2	4.0	7.20	1.25
C3-3-2	5.6	2.99	8.59	0.53
C1-4-4	5.6	3.2	8.80	0.57
C9-4-6	3.44	4.98	8.42	1.45
C10-3-5	3.83	4.64	8.47	1.21
C7-4-3	5.09	0.42	5.51	0.08
C4-1-1	6.0	1.88	7.88	0.31

selected from populations of stevia being maintained under field conditions at CSIR-IHBT, Palampur on the basis of their steviol glycoside profiles estimated through HPLC analysis (Table 1).

Fifty young nodal segments of each genotype were made from the apical shoots and used as explants representing three replications. The explants were washed in tap water and then washed with detergent (Tween-20) for 10-15 min. They were then washed 3-4 times with distilled water. The explants were surface sterilized with 0.1% mercuric chloride for 1 min under laminar air flow and then washed three times with autoclaved distilled water and then cultured on MS medium. The MS medium was prepared with 3% sucrose and 0.8% agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121° C for 20 minutes. Nodal explants were cut from both the ends and dried in filter paper and were cultured on MS media supplemented with different concentrations of growth regulators (BAP and Kn), singly or in combination (Table 2).

Sub-culturing was done after every three weeks interval. Nodal segments from the proliferated shoots were sub-cultured again for further multiple shoot induction. Subsequently, the shoots were cultured on root induction medium for three to four weeks till root formation was observed. Plantlets were transferred to double autoclaved

Table 2 Mean frequency of shoot proliferation and root induction from explants of stevia in different nutrient media

Shoot proliferation media	Concentration of BAP & Kn (mg/l)	No. of explants cultured	Mean shoot proliferation/ explant
M1	1.0+0	50	3
M2	2.0+0	50	2
M3	3.0+0	50	4
M4	4.0+0	50	2
M5	5.0+0	50	2
M6	0+1.0	50	2
M7	0+2.0	50	3
M8	0+3.0	50	2
M9	0+4.0	50	2
M10	0+5.0	50	3
M11	1+0.5	50	7*
M12	2+0.5	50	4
M13	3+0.5	50	4
M14	4+0.5	50	5
M15	5+0.5	50	5
Mean			3.33
Root induction media	*Concentration of IBA (mg/l)*	*No. of explants cultured*	*Mean root induction/ explant*
RM1	0.1	50	2
RM2	0.2	50	9*
RM3	0.5	50	3
RM4	1.0	50	1
RM5	1.5	50	2
RM6	2.0	50	1
Mean			3.00

* Significant at 5% level ($P = 0.05$)

soil mixture containing sand, soil and coco peat (1:1:1). The plantlets were then transferred to green house at 25 ± 2° C temperature and up to 80% relative humidity where successful establishment was achieved after 30 days.

Observations were made with respect to mean shoot proliferation, root induction and survival during the hardening process. The data regarding response of genotypes (21 accessions) was evaluated based on analysis of variance with respect to proliferation, root induction and survival during the hardening process. In order to analyze the data where mean frequencies range from 0-25, square root transformation was done using the formula $(x + 0.5)^{1/2}$, where x is the i^{th} entry in the j^{th} replication.

RESULTS AND DISCUSSION

Different concentrations of cytokinins (BAP and Kn) singly or in combination were used for shoot proliferation from nodal explants. Higher concentration of BAP and Kn does not result in increase in number of shoots. Maximum

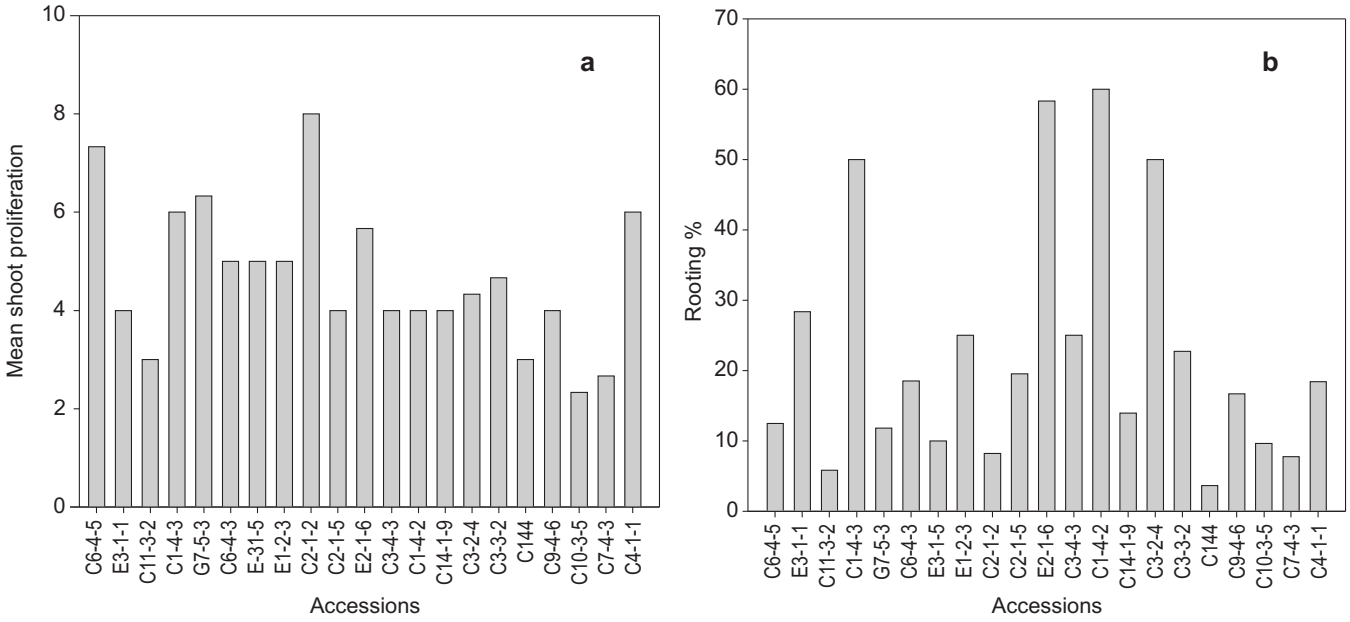

Fig 1 Mean response of stevia accessions to a) *in vitro* shoot proliferation, and b) root induction

shoots were observed on MS medium containing BAP (1 mg/l) and Kn (0.5 mg/l) as shown in Table 1. Best rooting was achieved (Fig 3) when shoots were cultured on half strength MS medium supplemented with IBA (0.2 mg/l). Based on Fisher's t-test, media M11 and RM2 were significantly better from the respective experimental means for shoot proliferation and root induction in stevia.

Data obtained with respect to proliferation, root induction and survival during hardening process were recorded on frequency as well as percentage basis and the data is presented in Fig 1 and 2. The mean shoot proliferation frequency ranged from 8.00 in C2-1-2 to 2.33 in C10-3-5 (Fig 1a). Analysis of variance of the shoot proliferation data indicates significant genotypic variations (Table 3). Heritability estimates for shoot proliferation and root induction were 90.63% and 95.99%, respectively.

Regarding root induction, only eight of the accessions showed considerable root formation while rooting was below 25% in other accessions. The mean root formation frequency ranged from 6.33 in C1-4-4 to 1.33 in E3-1-5 and E3-1-1, respectively. Similar to shoot proliferation, analysis of variance of the root induction data indicates significant genotypic variations and high heritability (95.99%) for the trait (Table 3). In case of survival of plantlets only six

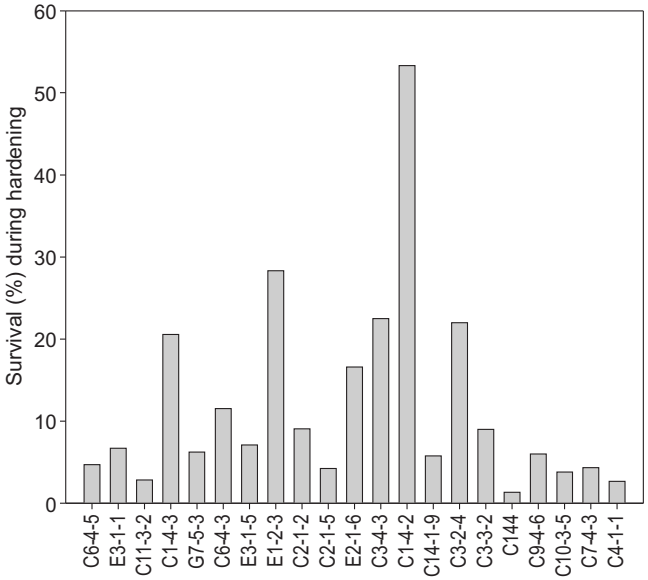

Fig 2 Survival of stevia accessions during hardening process

accessions performed well during the hardening process and the survival frequency ranged from 53.30% in C1-4-2 to 1.34% in C1-4-4. Contrary to shoot proliferation and root induction data, analysis of variance of survival data indicated

Table 3 Analysis of variance for shoot proliferation, root induction and survival after hardening of stevia accessions

Source	df	Variance	F value	CD (P=0.05)	Mean	CV	Heritability (%)
Shoot proliferation	20	6.54	10.68*	1.29	4.68	16.73	90.63
Root induction	20	12.37	24.97*	1.21	3.96	17.76	95.99
Survival during hardening	20	0.177	2.30	NS	1.02	27.04	56.57

* Significant at 5% level (p = 0.05)

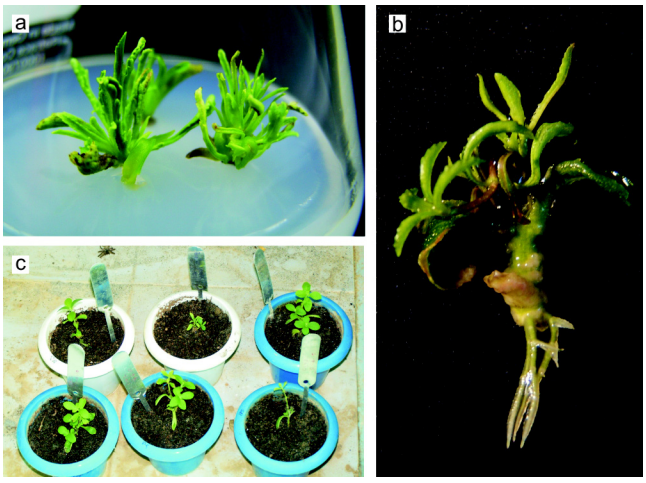

Fig 3 a) Shoot proliferation in nodal explants, b) root induction and c) hardened plantlet of stevia

non-significant genotypic variations and low heritability (56.57%) for survival during hardening (Table 3). The observation signifies high influence of environmental factors affecting survival of plantlets during the hardening process.

Considerable variations were obtained for shoot proliferation and root induction parameters indicating genotypic specificity of stevia accessions to these component traits of *in vitro* propagation. Genotypic response to *in vitro* propagation has also been reported in other plant species such as sugarcane (Cheema and Hussain 2004) and *Phoenix dactylifera* (Al-Khayri and Al-Bahrany 2004). Genotypes with high shoot proliferation such as C2-1-2, G7-5-3 and C6-4-5 did not respond favourably to root induction (Fig 1 and 2), suggesting that the two components may possibly be independent from each other and are under control of different genes leading to independent inheritance of these parameters. Overall, the shoot proliferation and root induction data suggest that genotypic response to *in vitro* clonal propagation constitutes a crucial factor for the success of an improvement program.

CONCLUSION

From the present studies, it is concluded that an efficient protocol is needed for every new variety or clone to get rapid shoot proliferation and root induction. The potential genotypes identified in this study are C1-4-2, E2-1-6, C1-4-3, E1-2-3 and C3-4-3. Though, this particular study comprises a part of investigations on feasibility of *in vitro* propagation for commercial cultivar development, but the breeding material is very useful for developing genotypes responsive to *in vitro* culture.

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