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IN VITRO SHOOT MULTIPLICATION IN *STEVIA REBAUDIANA* BERT., A MEDICINALLY IMPORTANT PLANT

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Summary. *Stevia rebaudiana* Bert., one of the important medicinal plants was subjected to *in vitro* multiplication. MS basal medium supplemented with BAP (3 $mg.l^{-1}$) produced the highest number of multiple shoots (40.54) per explant. However, the maximum shoot length and the highest number of leaves were obtained on MS medium supplemented with BAP (5 $mg.l^{-1}$), IAA (0.5 $mg.l^{-1}$) and GA (1.0 $mg.l^{-1}$). Root initiation and growth was observed on half strength MS medium supplemented with 2 $mg.l^{-1}$ IBA. The present study indicated 40 numbers of shoots after 30 days of culture, thus offering a new opportunity for fulfillment of the increased demand of the plant material in present scenario.

Key words**:** BAP; IBA; rooting; shoot multiplication; sterilization; *Stevia rebaudiana.*

Abbreviations**:** MS – Murashige and Skoog (1962)**;** BAP – 6-benzylaminopurine; IAA – indole-3-acetic acid; IBA – indole-3-butylic acid; GA – gibberellic acid; HSA-MS – half strength agar gelled MS medium; FSA-MS – full strength agar gelled MS medium.

INTRODUCTION

Stevia rebaudiana (family Asteraceae) is a perennial herb cultivated for its high economic value. Traditionally, the plant is used to cure many diseases like hypoglycermia, antihypertenic, diuretic cardiac edema, obesity, high blood pressure, etc. (Melis, 1996; Chan et al., 1998; Ahmed, 2007). Stem extracts of the plant are also known to inhibit the growth of different pathogens (Haebisch, 1992; Ishii, 1995; Liu, 1995; Toskulkao et al., 1995; Kim and Shibata, 1997). The leaf of *Stevia* contains a sweetener compound steviolglyiosides which is extensively used as an alternative of sugar in diabetic food preparation (Yokoyama and Sugiyama, 1990; Chalapathi et al., 1997; Kinghorn and Kim, 1997; Lima

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and Malavolta, 1997; Dwivedi, 1999). It is reported that *Stevia* also contains an antioxidant steviol (Brandle, 1999; Kinghorn, 1999). Therefore, the versatile use of *Stevia* generates high demand to the society. Multiplication of *Stevia* is normally carried out through vegetative propagation by stem cuttings and seeds. Frequently, due to self incompatibility of the flower, the seed production is significantly reduced (Nepovim and Vanek, 1998). The conventional methods of propagation with either seeds or vegetative propagation are extremely slow and take longer time (Ermakov and Kochetov, 1996; Carneiro et al., 1997). Thus, tissue culture techniques provide a viable alternative for the production of disease free plants within a shorter period of time with less cost. Keeping in view the importance of this plant species, there is a need to produce *Stevia* plants through tissue culture. Effect of plant growth regulators on *in vitro* propagation, organogenesis through callus of *Stevia* are available, but the protocol for *in vitro* propagation of the local variety is scanty (Sivaram and Mukundan, 2003; Udin et al., 2006; Ibrahim et al., 2008). Thus, the present investigation was carried out to standardize the protocol for surface sterilization, *in vitro* propagation and establishment of plants to pots of *Stevia rebaudiana.*

MATERIALS AND METHODS

Plants of *Stevia rebaudiana* were obtained from the medicinal garden of Orissa University of Agriculture and Technology (OUAT), Bhubaneswar and maintained in the garden of college premises for periodic utilization.

Surface sterilization.

Nodal segments of the plant were used as explants. The explants were collected from the tender parts of the mature plants. The explants were initially washed with tap water to remove the dirt followed by bavistin (0.2%, w/v) for 30 min. Two chemicals, ethyl alcohol (70%) and $HgCl_2$ (0.1%) were used as surface sterilants. The sterilized explants were washed thoroughly with double sterilized distilled water. These were then cut into appropriate size and cultured on Murashige and Skoog (1962) medium (MS) containing sucrose (3%,w/v) and gelled with agar (0.8%, w/v).

Shoot multiplication.

MS medium supplemented with sucrose and agar was used as a basal medium for soot multiplication. The concentrations of benzyl amino purine (BAP) ranged from 1.0 $mg.l^{-1}$ to 4.0 $mg.l^{-1}$. Other phytohormones such as indole-3-acetic acid (IAA) (0.5 $mg.l^{-1}$ to 1.0 $mg.l^{-1}$) and gibberelic acid (10 $mg.l^{-1}$) were also added separately to the medium for multiplication of shoots. All the chemicals were procured from HI Media Pvt. Ltd. Mumbai, India. The pH of the medium was adjusted to 5.8 using HCl or KOH. The medium was sterilized at 121°C and 1.05 kg/cm^2 pressure. The explants were inoculated aseptically inside the laminar air flow and then incubated at 25 ± 2°C and 16/8 h (day/night) photoperiod under a white fluorescent tube (25 $\mu mol\ m^{-2}s^{-1}$). Ten replicates were maintained for each culture. The shoots differenced from the explants were regarded as the first subculture. The second subculture was obtained from the first subculture upon the same treatment. The total number of

multiple shoots per explant from the 1st and 2nd subcultures, length of shoot and number of leaves emerging per shoot were recorded.

Rooting and Acclimatization.

The shoots having 4 to 5 cm length were inoculated aseptically for rooting. Half strength MS medium with sucrose (3%) and agar (0.8%) was used as a basal medium. Different concentrations of auxins (IAA, 0.25 mg.l^{-1} to 1mg.l^{-1} and/or IBA, 1.0 mg.l^{-1} to 3.0 mg.l^{-1}) were added to the medium as a rooting hormone. Rooted plantlets with 2 to 3 pairs of leaves were removed carefully from the medium and transferred to the plastic pots containing peat moss and garden soil (1:1). The pots were kept inside the glass house at a temperature of 25 ± 3°C, 60 ± 10% relative humidity and natural light (Krupaswamy et al.,2007; Nikam et al., 2008). After a week the potted plantlets were transferred to another pot having only garden soil and forest humus (1:1). Then the plantlets were transferred from the green house to natural conditions. After one month the survival frequency of the plants was calculated. Data were collected from ten independent individuals and each experiment was repeated for three times. The standard error of the means (SEM) and critical difference (CD) were calculated for each experiment (Gomez and Gomez,1984).

RESULTS AND DISCUSSION

The fffect of sterilizing agents on the level of contamination, survival and number of buds per explant in *Stevia rebaudiana* is given in Table 1. The highest percentage of fungal infection was recorded in tap water (control). The best sterilization treatment of the explants was found to include 70% ethyl alcohol for half a minute followed by 0.1% $HgCl_2$

Table 1. Effects of sterilizing agents on the level of contamination, explant survival and number of shoots/explant in *Stevia rebaudiana*. Data represent the means of 10 independent replicates.

Treatments	Fungal infection [%]	Bacterial infection [%]	Death [%]	Aseptic culture [%]	Survival [%]	No. of shoot/ explant
T_1 (tap water)	83.33	16.67	0.12	0.00	_	_
T_2 (70 % alcohol ½ min)	78.24	13.43	8.33	7.33	6.22	1.32
T_3 (70 % alcohol 1 min)	71.23	13.40	6.32	15.34	9.02	1.51
T_4 (0.1 % $HgCl_2$ 2 min)	30.72	9.28	3.41	60.00	56.59	1.89
T_5 (0.1 % $HgCl_2$ 3 min)	18.36	7.61	1.03	74.03	73.00	2.34
T_6 (0.1 % $HgCl_2$ 4 min)	12.81	4.39	12.31	82.81	70.49	2.31
T_7 (T_2 + T_5)	5.81	2.61	1.00	91.58	90.58	2.68
T_8 (T_2 + T_6)	6.19	2.81	7.58	91.00	82.00	2.64
SEM (±)	0.03	0.04	0.06	0.58	0.07	0.03
CD (p= 0.05)	0.09	0.12	0.18	1.76	0.21	0.09

for 3 min. In this treatment the total infection (both bacteria and fungal) was lowest. The highest percentage of survival of explants was also observed upon the above treatment. In addition, the number of days required for shoot emergence from the explant was minimum and the number of shoots was highest as compared to the other treatments. It was earlier reported that 70% ethyl alcohol followed by $HgCl_2$ (0.1%) treatment reduced both bacterial and fungal infections (Viswanath and Jayanthi, 1997). Our results were an agreement with the above report. MS basal medium supplemented with 3 $mg.l^{-1}$ BAP produced the highest member of shoots (Table 2). A positive trend was observed between the concentration of BAP and the total number of shoots per explants up to 3 $mg.l^{-1}$ followed by a decline thereafter. At this concentration the number of multiple shoots per explants was 40.54. The length of the shoot and the number of leaves per shoot were 4.53 cm and 5.03, respectively (Fig. 1a). It was earlier reported that MS medium fortified with 2 mgl^{-1}BAP produced the maximum number of shoots but the reproduced shoots were feeble. In our study, however, it was observed that MS medium fortified with 3 $mg.l^{-1}$ BAP or BAP (5 $mg.l^{-1}$) + IAA (0.5 $mg.l^{-1}$) + GA (1.0 $mg.l^{-1}$) produced the maximum number of shoots (mentioned number) and more than 90 % survival rate. The effect of different concentrations of phytohormones on the development of multiple shoots in *Stevia* is given in Table 3. MS medium supplemented with BAP (5 $mg.l^{-1}$), IAA (0.5 $mg.l^{-1}$) and GA (10 $mg.l^{-1}$) produced significantly the highest number of shoots per explants among all the treatments (Fig. 1b). It was observed that when the concentration of BAP increased from 3 $mg.l^{-1}$ to 5 $mg.l^{-1}$ along with a constant concentration of IAA (0.5 $mg.l^{-1}$) and GA (10 $mg.l^{-1}$), the number of multiple shoots per plant increased form 9.35 to 33.90. A further increase in BAP concentration

Table 2. Effects of different concentrations of BAP on multiple shoot formation in *Stevia rebaudiana*. Data represent the means of 10 independent replicates.

Treatment	Conc. of BAP [$mg.l^{-1}$]	Total No. of shoot/ explant	Total No. of shoot/ single shoot subculture		No. of multiple shoots/ explant	Shoot length [cm]	No. of leaves/ shoot	Survival rate [%]
			1st subculture	2nd subculture				
T_1	1.0	1.45	1.90	1.63	4.50	3.84	4.03	80.10%
T_2	1.5	1.80	2.43	2.12	9.27	4.02	4.31	82.20%
T_3	2.0	2.03	2.67	2.42	13.11	4.15	4.33	85.10%
T_4	2.5	2.48	3.42	2.67	22.65	4.38	4.66	89.00%
T_5	3.0	3.04	4.33	3.08	40.54	4.53	5.03	90.10%
T_6	3.5	2.33	3.08	2.33	16.18	4.49	4.84	89.25%
T_7	4.0	1.67	2.33	1.84	7.16	4.12	4.49	88.0%
SEM(±)	_	0.04	0.12	0.06	1.24	0.02	0.05	0.08
CD (p=0.05)	_	0.12	0.40	0.19	3.76	0.06	0.15	0.18

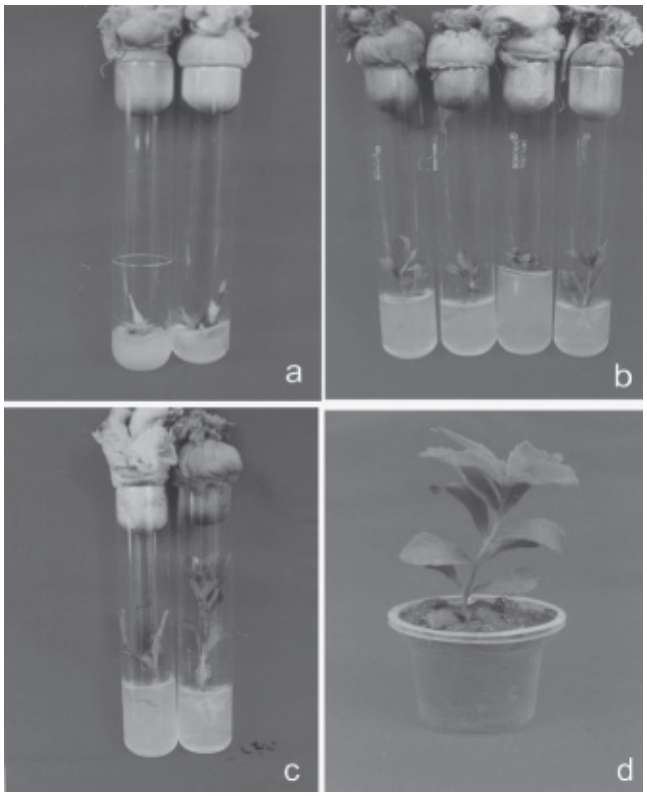

Fig. 1. *In vitro* propagaion of *Stevia reboudiana* – (a) Axillary bud initiation from nodal explant on MS medium + 3% sucrose + 3 mg.l^{-1} BAP; (b) Axillary bud multiplication on MS medium + 3% sucrose + 5 mg.l^{-1} BAP + 0.5 mg.l^{-1} IAA + 10 mg.l^{-1} GA; (c) *In vitro* rooting on half strength MS medium + 3% sucrose + 2 mg.l^{-1} IBA; (d) Three month old plant on plastic pot.

in the medium reduced the emergence of multiple shoots. Upon this treatment, the length of shoots and the number of leaves per shoot were maximum. The role of BAP in shoot differentiation from leaf and cell suspension cultures of *Stevia rebaudiana* has been well studied (Ferreira and Handro, 1988).The regeneration of multiple shoots in the medium with BAP, IAA and GA is comparatively less compared to the medium supplemented with BAP only, but the rate of shoot survival was highest (92.08%) in the latter. Therefore, it was the best treatment for shoot regeneration. The shoots of the 2nd substructure were incubated in a rooting medium. Half strength agar solidified MS medium (HSA-MS) or full strength agar solidified MS medium (FSA-MS) was used as a basal medium. The medium was supplemented with either IBA ranging from 1.0 to 3.0 mg.l^{-1} or IAA ranging from 0.25 to 1.0 mg.l^{-1} (Table 4). Both IBA and IAA were responsible for root initiation. It was observed that HSA – MS supplemented with IBA (2.0 mg.l^{-1}) and HSA–MS supplemented with IAA (0.5 mg.l^{-1}) produced a large number of roots as compared to the other treatments (Fig. 1c). Upon this treatment, early root initiation with longer roots was also observed. Between these two rooting hormones (IBA and IAA), IBA produced the maximum number of roots per shoot.

Table 3. Effects of different phytohormones on the development of multiple shoots in *Stevia rebaudiana.* Data represent the means of 10 independent replicates.

Treatment	Conc. phytohormones			No. of shoots/ explants	No. of shoots		No of multiple shoots/ explant	Shoot length [cm]	No. of leaves/ shoot	Survival rate [%]
	BAP [mg.l^{-1}]	IAA [mg.l^{-1}]	GA [mg.l^{-1}]		1st subculture	2nd subculture				
T_1	3.0	0.5	10	1.84	2.33	2.18	9.35	3.82	4.00	70.20
T_2	4.0	0.5	10	2.58	3.33	2.44	21.00	3.99	4.23	89.10
T_3	5.0	0.5	10	2.73	4.18	2.97	33.90	4.13	4.32	92.08
T_4	6.0	0.5	10	2.18	3.67	2.72	21.76	4.11	4.30	89.80
T_5	7.0	0.5	10	2.04	3.18	2.67	17.32	4.03	4.28	88.40
T_6	4.0	1.0	10	1.67	2.67	1.68	7.45	4.01	4.19	82.70
T_7	5.0	1.0	10	1.52	2.74	2.19	9.12	4.00	4.12	78.80
SEM(±)	-	-	-	0.04	0.03	0.13	0.67	0.03	0.05	0.06
CD (p=0.05)	-	-	-	0.12	0.09	0.39	2.04	0.09	0.15	0.17

Table 4. Rooting response of shoots of *Stevia rebaudiana* after 5 weeks of culture. Data represent the means of 10 independent replicates.

Medium*	Conc. of IBA [mg.l^{-1}]	Conc. of IAA [mgl^{-1}]	Days of root initiation	No. of roots/shoot	Length of the root [cm]
HSA-MS	1.0	-	No root	-	-
HSA-MS	1.5	-	18.33	1.67	0.94
HSA-MS	2.0	-	16.16	2.51	1.36
HSA-MS	3.0	-	16.30	2.33	1.27
FAS-MS	1.0	-	No root	-	-
FAS-MS	1.5	-	2.33	1.31	0.81
FAS-MS	2.0	-	25.67	1.33	0.89
FAS-MS	2.5	-	24.60	1.14	0.83
FAS-MS	3.0	-	23.33	1.09	0.82
SEM(±)	-	-	0.14	0.06	0.03
CD(p=0.05)	-	-	0.41	0.18	0.09
HSA-MS	-	-	No root	-	-
HSA-MS	-	0.25	21.33	1.69	0.92
HSA-MS	-	0.50	16.60	2.18	1.31
HSA-MS	-	0.75	16.67	1.86	1.13
HSA-MS	-	1.00	16.58	1.62	1.09
FSA-MS	-	-	No root	-	-
FSA-MS	-	0.25	29.67	1.09	0.73
FSA-MS	-	0.50	27.33	1.14	0.79
FSA-MS	-	0.75	25.33	1.12	0.74
FSA-MS	-	1.00	24.33	1.04	0.70
SEM(±)	-	-	0.06	0.04	0.03
CD(p=0.05)	-	-	0.18	0.12	0.09

*Medium is supplemented with 3 % of sucrose. HSA-MS: Half strength agar gelled MS medium. FSA-MS: Full strength agar gelled MS medium. "-": No result is observed or absent.

Moreover, the longer roots were found after IBA treatment. Therefore, taking into consideration the above observations, HSA-MS supplemented with IBA 2mg.l^{-1} could be considered as the best medium for rooting. The study revealed that auxin (IBA and IAA) helped to better rooting of *Stevia*. Our results were in agreement with an earlier report that half strength MS medium supplemented with auxin successfully produced roots (Arya et al., 2003). The rooted plantlets from the culture tubes were carefully removed and washed with tap water to remove the traces of agar adhered to them. The plantlets were then planted on plastic cups containing a mixture of finely chopped peat moss and sterilized garden manure (Fig. 1d). Humidity was maintained by covering with transparent polythene. The gradual

exposure of *in vitro* raised plants from laboratory to natural conditions increased the rate of survival. After one month, the rate of survival was calculated as 92.08%. Moreover, the plants transferred subsequently to field conditions grew well and exhibited morphological characters similar to wild plants.

In conclusion, tissue culture of *Stevia* is a viable novel method for rapid multiplication and production of true to the type as well as disease free planting material within a shorter period of time. The present study indicated 40 numbers of shoots, thus offering a new opportunity to meet the increased demand of the plant material in present scenario.

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