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In vitro rapid multiplication of *Stevia rebaudiana*: an important natural sweetener herb

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Abstract. *Deshmukh S, Ade R. 2012. In vitro rapid multiplication of* Stevia rebaudiana*: an important natural sweetener herb. Nusantara Bioscience 4: 105-108. Stevia rebaudiana* Bertoni, belonging to family Asteraceae and natural sweet plant, but due to poor seed viability, fertility, and vigor, *Stevia* cultivation is a challenging task. In the present study, in vitro rapid multiplication method was established for *S. rebaudiana* by inoculating explants on M.S. medium, supplemented with different combination of phytohormone. The maximum number of shoots (18.3±0.8) was obtained on M.S. medium supplemented with BAP + KIN (1.5 + 0.5 mg/L). The highest rooting percentage (95.25) was observed with (IAA 0.1 mg/L). The rooted plants were successfully established firstly in soil with coco peat (1:1) and then directly in ordinary soil.

Keywords: *Stevia rebaudiana*, in vitro culture, multiplication, sweetener, micropropagation.
Abbreviations: IAA: Indole-3-acetic acid, BAP: 6-Benzyl amino purine, KIN: Kinetin, GA: Gibberellic acid. NAA: 1 Naphthalene acetic acid

Abstrak. *Deshmukh S, Ade R. 2012. Perbanyakan cepat secara in vitro* Stevia rebaudiana*: herbal pemanis alami yang penting. Nusantara Bioscience 4: 105-108. Stevia rebaudiana* Bertoni, anggota suku Asteraceae merupakan tanaman pemanis alami, namun karena viabilitas, kesuburan dan kekuatan benih yang buruk budidaya *Stevia* menjadi tugas yang menantang. Dalam penelitian ini metode perbanyakan cepat secara in vitro dilakukan pada *S. rebaudiana* dari eksplan inokulasi pada media MS, dilengkapi dengan kombinasi fitoharmon yang berbeda. Jumlah maksimum tunas (18,3±0,8) diperoleh pada media MS dengan BAP + KIN (1,5 + 0,5 mg/L). Persentase perakaran tertinggi (95,25) diamati dengan (IAA 0,1 mg/L). Tanaman berakar berhasil ditanam pertama kali pada tanah dengan coco peat (1:1) dan kemudian langsung di tanah biasa.

Kata kunci: *Stevia rebaudiana*, kultur in vitro, perbanyakan, pemanis, mikropropagasi.

INTRODUCTION

Stevia rebaudiana Bertoni, the member of the family Asteraceae, is a perennial herb which can be growing up to 1 meter (Kinghorn et al. 1985; Handro et al. 1989; Tadhani et al. 2005). It is natural sweetener plant called as "*sweet weed*", "*sweet leaf*" and "*honey leaf*" (Ahmed et al. 2007). The leaves of *Stevia* are source of glycoside, viz stevioside and rebaudioside which are 100-300 time sweeter than sucrose (0.4% solution) but zero calories (Mousumi 2008), hence it is important medicinal plant and it has been traditionally used for hundreds of year in Paraguay and Brazil in South America continent to sweeten tea and medicine and also used as a sweet treat (Tiwari 2010). It is recommended for diabetes and has been extensively tested on animal and human with no side effects (Megeji et al. 2005). The crude extracts from leaves have been used few decades to sweeten soft drinks and other foods (Komissarenko et al. 1994).

The stevioside, can be used in tea and coffee, cooked or baked goods, processed foods, and beverages, fruit juices, tobacco products, pastries, chewing gum, etc. Stevioside has zero calories and can be used wherever sugar is used, including in bakery. *Stevia* has generated much attention with the rise in demand for low carbohydrate, low sugar food alternative. *Stevia* also has shown promise in medical research for treating obesity and high blood pressure, due to these important medicinal properties the *Stevia* is being cultivated in Japan, Taiwan, Philippines, Hawaii, Malaysia, and overall South America and used in several food and pharmaceutical products (Das et al. 2011).

The main problem in cultivation of *Stevia* is that the plant is heterozygous. Self-incompatible nature of flowers leads to lack of fertilization, poor seed viability, and vigor, due to this plant propagation by seed is not efficient. (Tadhani and Rema 2006; Rathi and Arya 2009). Propagation by seeds does not allow the production of homogeneous population, resulting in great variability in important features like sweetening level and composition (Tamura et al. 1984). Due to such difficulties in cultivation of *Stevia*, tissue culture is the only rapid process for the mass propagation. The present study was carried out to optimize a suitable and efficient protocol for In vitro rapid multiplication of *S. rebaudiana* Bertoni.

MATERIALS AND METHODS

Collection and surface sterilization of explants

Stevia rebaudiana plants were collected from the Government Nursery, Pune University, Pune, Maharashtra, India. The twigs about 5-6 cm were taken and the leaves, auxiliary buds, and apical buds were first washed in running tap water, then treated with 0.1% (w/v) Bavistin solution for 5 min to remove superficial dust particle as well as fungal spores. After that, the explants were treated with 70% alcohol for 2 min followed by 4-5 time wash by double distilled water to remove bacterial contaminants. Sodium hypochlorite (0.3%) also used for the decontamination. It was again treated with 0.1% $HgCl_2$ aseptically for 3-4 min and washed with sterile distilled water 4-5 times.

Inoculation of explants on the multiplication medium

The surface sterilized auxiliary bud were aseptically inoculated vertically on MS medium (pH 5.7) supplemented with specific concentration of growth regulators (BAP, KIN, and NAA) singly as well as in combination, 0.7 % agar was used as a gelling agent and 30 gm/lit sucrose was used as a carbon source.

Culture conditions

All the standard conditions were provided such as photoperiod was 16 hrs light (2000 lux) to 8hrs darkness. The cultures were maintained at 26±1°C and 70% humidity. Subculturing was done for every fortnight and well-grown sub-cultured shoots were further inoculated on multiple shoot formation medium. Regenerated multiple shoots were cut and individual shoots were placed in MS medium containing different concentrations of IBA, NAA and IAA for root induction.

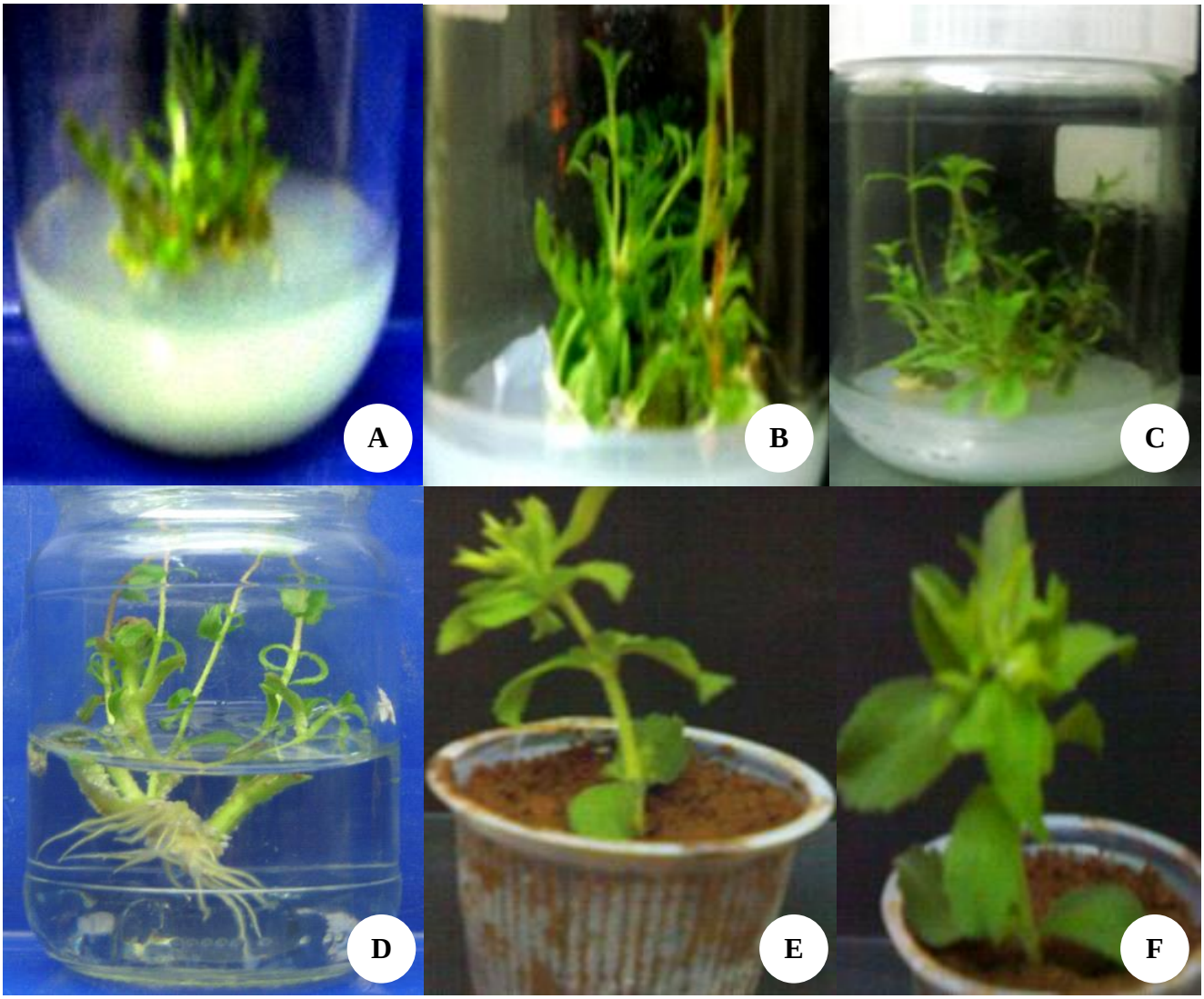

Figure 1. In vitro multiplication of *S. rebaudiana* from nodal explants on MS medium supplemented with BAP + Kin (1.5+1.0). **A.** After 15 days, **B.** After 30 days, **C.** After 45 days of culture. **D.** Adventitious root formation from micro-cuttings on MS supplemented with IAA (0.1 mg/L). **E.** Growth of *Stevia* in plastic pot containing coco peat and soil 1:1. **F.** In ordinary soil.

Hardening of plants

In vitro rooted shoots were kept under normal growth room condition for 7-8 days until the induced roots become partially brown. The shoots were taken out from culture bottle carefully and medium attached to the roots were gently washed out with running tap water. The rooted plants treated with 0.1% bavistin (antifungal) for 1-2 min then transferred on soil: coco peat (1:1) for hardening then directly in ordinary soil (Figure 1).

RESULTS AND DISCUSSION

Shoot-apex were inoculated on MS medium supplemented with different concentration of BAP (0.5, 1.0, 1.5, 2.0 mg/L) and KIN (0.5, 1.0, 1.5, 2.0 mg/L) alone or BAP with KIN or with NAA (0.5, 1.0mg/L) as shown in Table 1. This shows different response during the primary establishment period. After 15 days, multiple shoots emerged directly from auxiliary node of cultured explants. The response of explants to the treatment is presented in Table1. The maximum number of shoots was observed on MS medium containing BAP (1.5 mg/L) among single hormonal set. Similar findings were recorded by (Sivaram and Mukundan 2003). BAP + KIN (1.0 + 0.5 mg/L) and BAP + KIN (1.0 + 1.0 mg/L) also shows significant multiplication. The response was best at BAP + KIN (1.5 + 0.5 mg/L) combination, where highest percentage of explants showing shoot proliferation was found to be 89.25%, whereas highest average number of total shoot was found to be 18.3 ± 0.8, with average length of 4.8 ± 0.8 cm were recorded (Table 1). Similar results have already been reported in *Fragaria indica* (Bhatt et al. 2000). Patil et al. (1996) reported that shoot tips and auxiliary buds produce multiple shoots. When the explants were inoculated on MS medium with IAA + BAP (0.5 + 5.0 mg/L) supplemented with 10 mg/L GA. The numbers of multiple shoots were around 20-25 per plant.

Multiple shoot formation requires the presence of cytokinins in the culture medium (Tadhani and Rema 2006). In the present study, the BAP containing medium is better for the shoot formation at lower concentration as compared to KIN. However, Tamura et al. (1984) reported that high concentration of KIN (10 mg/L) was required for multiple shoot production in *S. rebaudiana*. Progressively higher concentration of BAP resulted in decreasing multiple shoot formation in all the explants of *Stevia*. (Table 1)

Although the process of in vitro rooting is a labor-intensive in the micropropagation studies of *Stevia*, but it seems to be an essential step for plant survival. The addition of auxin at certain level enhances the root formation. Micro cuttings taken from in vitro proliferated shoots were implanted on MS medium containing different concentrations 0.1mg/L, 0.3 mg/L, 0.5 mg/L, 0.8 mg/L, 1.0 mg/L, of IAA, NAA and IBA for rooting. Here each treatment consists of four replications and in each replication 10 explants were used.

Within 6-12 days root initiation starts in the MS medium with IAA 0.1 mg/L and it shows the best response for rooting with highest length of root (4-5cm), number of roots 12-13 and maximum root induction (95.25%) as shown in Table 2. It was observed that the root induction gradually decreased with increased concentration of auxin. There was not any satisfactory root induction in another case except IAA. Similar findings were recorded in *Chrysanthemum morifolium* (Hoque et al. 1995), Pigeon pea (Sivaprakash et al. 1994), *Vitex negundo* (Thiruvengadam et al. 2000) and *Psoralea corylifolia* (Jeyakumar et al. 2002). However, Tadhani et al. (2005) reported 0.1mg/L IBA was the best concentration for rooting.

In vitro rooted shoots were kept under normal growth room condition for 2 weeks until the induced roots become partially brown. The shoots were taken out from growth room and from the culture bottle carefully and gently washed with running tap water. The rooted plant was treated with 0.1% Bavistin for 1 minute and transferred to soil and coco peat (1:1) for primary hardening followed by ordinary soil in natural environment.

Table 1. Effect of auxin and cytokinin concentration on in vitro multiplication.

Growth regulator (mg/L)	Explants proliferation (%)	Average no. of shoots	Average length of shoots (cm)
BAP 0.5	49.50	5.1± 1.7	2.6 ± 0.5
BAP 1.0	55.25	4.2 ± 1.3	2.3 ± 0.4
BAP 1.5	68.00	4.1 ± 1.4	3.3 ± 0.6
BAP 2.0	57.50	4.5 ± 1.5	2.4 ± 0.5
KIN 0.5	42.00	3.6 ± 0.9	2.3 ± 0.4
KIN 1.0	60.25	3.2 ± 0.9	3.4 ± 0.5
KIN 1.5	58.50	3.2 ± 0.9	2.4 ± 1.0
KIN 2.0	52.25	3.6 ± 0.9	1.6 ± 0.6
BAP+KIN (1.0+0.5)	67.25	13.4 ± 1.2	3.5 ± 0.5
BAP+KIN (1.0+1.0)	76.50	14.4 ± 1.7	3.4 ± 0.5
BAP+KIN (1.5+0.5)	**89.25**	**18.3 ± 0.8**	**4.8 ± 0.4**
BAP+KIN (1.5+1.0)	57.50	12.8 ± 0.9	4.4 ± 0.5
BAP+KIN (2.0+1.0)	56.25	11.6 ± 0.8	3.4 ± 0.5
BAP+KIN (2.0+0.5)	59.50	11.3 ± 0.6	1.6 ± 0.6
BAP+NAA (1.0+0.5)	35.25	7.4 ± 0.5	1.6 ± 0.5
BAP+NAA (1.0+1.0)	34.50	4.3 ± 0.4	3.3 ± 0.4
BAP+NAA (1.5+0.5)	37.40	12.8 ± 0.9	2.6 ± 0.5
BAP+NAA (1.5+1.0)	44.50	11.3 ± 0.6	2.7 ± 0.4
BAP+NAA (2.0+0.5)	42.00	11.5 ± 0.5	3.5 ± 0.5
BAP+NAA (2.0+1.0)	52.50	13.9 ± 0.8	1.6 ± 0.5
NAA +KIN (1.0+0.5)	26.00	5.7 ± 0.8	1.7 ± 0.4
NAA +KIN (1.0+1.0)	45.25	5.0 ± 0.8	1.5 ± 0.5
NAA +KIN (1.5+0.5)	56.50	5.7 ± 0.8	1.4 ± 0.5
NAA +KIN (1.5+1.0)	35.25	2.5 ± 0.5	3.6 ± 0.5
NAA+KIN (2.0.+0.5)	42.00	3.7 ± 0.8	2.8 ± 0.6
NAA +KIN (2.0+1.0)	48.25	12.8 ± 0.9	1.4 ± 0.5

Note: ***** Each treatment consists of three replications and in each replication 10 explants were used.

Table 2. Effect of different type of auxin on adventitious root formation.

Auxin	Conc. (mg/L)	Root initiation (days)	Root initiation (%)	Average no. of roots	Average length of roots (cm)
IAA	**0.1**	**6-8**	**95.25**	**12.8 ± 0.6**	**4.7 ± 0.4**
	0.3	10-11	92.0	10.7 ± 0.8	2.4 ± 1.0
	0.5	7-8	85.0	9.5 ± 0.5	1.4 ± 0.5
	0.8	10-12	65.50	7.4 ± 0.5	3.5 ± 0.5
	1.0	9-10	67.75	5.1 ± 0.9	2.4 ± 0.5
NAA	0.1	7-8	70.35	5.2 ± 0.4	2.3 ± 0.4
	0.3	7-8	65.25	4.6 ± 0.5	2.7 ± 0.4
	0.5	7-8	58.50	4.7 ± 0.4	1.4 ± 0.5
	0.8	7-8	62.00	4.5 ± 0.5	2.4 ± 1.0
	1.0	7-8	51.50	3.5 ± 0.5	2.7± 0.6
IBA	0.1	8-10	65.75	7.4 ± 0.5	1.9 ± 0.8
	0.3	10-12	60.25	6.5 ± 0.5	2.4 ± 1.0
	0.5	10-12	45.50	4.5 ± 0.5	1.5 ± 0.5
	0.8	8-9	48.00	4.3 ± 0.4	2.4 ± 0.5
	1.0	8-9	42.75	4.6 ± 0.5	3.6 ± 0.5

Note: *Each treatment consisted of three replications and in each replication 10 explants were used.

CONCLUSION

Stevia is an important sweetener herb. In normal propagation it is very difficult to regenerate due to various reasons like heterozygous nature, self-incompatibility of flowers, lack of efficient fertilization and most importantly poor seed viability, hence there is urgent need to develop efficient protocol for rapid multiplication and thus conservation, because till date no appropriate and efficient method is available for its regeneration. Since tissue culture technology is the only process for the mass propagation, this rapid and efficient regeneration protocol provides a good platform for the multiplication and effective conservation of this important plant.

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