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Full Length Research Paper

Mass production of an economically important medicinal plant *Stevia rebaudiana* using *in vitro* propagation techniques

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Experiments were conducted for standardization of *in vitro* culture technique of *Stevia rebaudiana*, an important non-caloric sweetening herb to explore its potential for micro-propagation and callus culture. Nodal segments of the selected herb as explants were cultured for micro-propagation on MS medium containing 0.1 mg/l N^6-benzyl amino purine for shoot initiation. Maximum plantlets (83.2 ± 0.445033) were found in MS medium treated with 3.5 mg/l N^6-benzyl amino purine at multiplication stage. Young leaves were placed on MS medium containing 2 mg/l 2-4- Dichlorophenoxyacetic acid +1 mg/1 Kinetin was given best result of callusing. Higher regeneration of plantlets (3.8 plantlets/calli) was obtained by placing callus on MS medium with 5 mg/l BA + 1 mg/l NA. Highest rooting average (11.1 ± 0.264052) was recorded on $^{1/2}$MS medium with 100 mg/l activated charcoal. The rooted plantlets were hardened in 1:1:1 ratio of sand: soil: vermicompost and successfully established in soil.

Key words: *Stevia rebaudiana*, *in vitro* propagation, regeneration of callus, non-caloric sweetener, medicinal plant, N^6-benzyl amino purine, 2, 4-Dichlorophenoxyacetic acid.

INTRODUCTION

In ancient Indian traditional Ayurvedic system of medicine, *Stevia rebaudiana* has a long history of use by tribal people. *S. rebaudiana* belonging to the family *Asteraceae*, is a perennial and endemic, medicinal shrub (Sivaram and Mukundan, 2003). *S. rebaudiana* is originnally a South American wild plant (Katayma et al., 1976), 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, and in recent decades it is being cultivated domestically. It is used in its raw leaf form and is now commercially processed into a sweetener. Seed germination of stevia is often poor (Miyazaki and Wantenabe, 1974).

S. rebaudiana is one of 154 members of the genus *Stevia*, which produces sweet steviol glycosides (Robinson, 1930). The first report of its commercial cultivation was in Paraguay in 1964 (Katayama et al., 1976). It is a small hurb perennially growing up to 65 cm tall, with sessile, oppositely arranged lanceolate to oblanceolate leaves, serrated above the middle. The property of the species that called attention to the plant was intense sweet taste of its leaves and aqueous extracts. From the leaves of *Stevia*, steviosides, sweet crystalline diterpene glycosides are extracted. Pure stevioside is non-caloric and 30 times sweeter than sugar (Bhosle, 2004). Other attributes of this natural, high intensity sweetener include its non-fermentable, non-discoloring nature, maintaining heat-stability at 100°C and features a long shelf life. The product can be added to tea and coffee, cooked or baked goods, processed foods and beverages. It is used as a table top sweetener, in soft drinks, baked goods, pickles, fruit juices, tobacco products, confectionery goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets. Stevioside is of special interest to diabetic persons with hyperglycemia and the diet conscious.

The fresh leaves have a nice liquorice taste. It is recommended for diabetics and has been extensively tested on animals and used by humans with no side effects (Megaji et al., 2005). *S. rebaudiana* is a natural plant sweetener known as sweet weed, sweet leaf, sweet herb and honey leaf (Ahmed et al., 2007). Seeds of stevia show a very low germination percentage (Felippe and Lucas, 1971) and vegetative propagation is limited by lower number of individuals (Sakaguchi and Kan,

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Table 1. Effect of different concentrations of BA in MS medium on *in vitro* shoot initiation from nodal segments of Stevia.

S. No.	PGR(mg/l)	No. of shoots/Explants (Mean ±SE)
1	Without PGR	0.3 ± 0.141786
2	0.1 BA	1.55 ± 0.228937
3	0.2 BA	1.35 ± 0.224716
4	0.3 BA	1.25 ± 0.237152
5	0.4 BA	1.15 ± 0.224716
6	0.5 BA	1.15 ± 0.228937
7	1.0 BA	1.05 ± 0.228937

Note: Each treatment consisted of 20 replications. Data (Mean ±SE) were recorded after 15 days of culture.

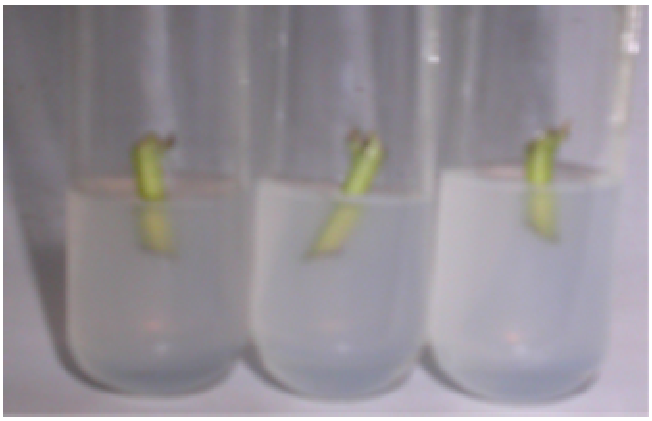

Figure 1. Initiation of *Stevia rebaudiana*

Figure 2. Multiplication of *Stevia rebaudiana.*

1982). Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Nakamura and Tamura, 1985). Tissue culture is the only rapid process for mass propagation of stevia and there have been few reports of *in vitro* growth of stevia (Miyagawa and Fujioka, 1986), *in vitro* propagation from shoot tip and leaf (Akita and Shigeoka, 1994). The present study was carried out to standardize a suitable protocol for *in vitro* propagation of *S. rebaudiana.*

MATERIALS AND METHODS

The pot grown *S. rebaudiana* plants were collected from the State Forest Research Institute Jabalpur (M. P.). The explants (nodal part and young leaves) were cut into small pieces (about 1.5 cm long) and then were treated with 1% savlon for 4 - 5 min with constant shaking and washed thoroughly with running tap water and then washed again thoroughly by adding a drop of liquid soap to remove the superficial dust particles as well as fungal and bacterial spores. They were then surface sterilized twice with sterile double distilled water, 70% alcohol, two times sterile double distilled water, 0.1% mercuric chloride for 2 min followed by 3 - 4 times rinsing with sterile double distilled water inside the Laminar Air flow chamber. Nodal segments were cut vertically and leaf pieces were horizontally placed on MS medium fortified with specific concentrations of different growth regulators adding 3% sucrose (SD fine) and 0.45% Agar Agar. Their pH of the medium was adjusted between 5.6 to 5.8 with KOH before autoclaving at 121°C for 20 min. The culture was incubated at a constant temperature of 25 ± 2°C with 16 h photoperiod (2000 lux) and 8 h darkness, young leaves were placed in dark. Visual observation of cultures was made weekly and the data were recorded.

Nodal segments from the proliferated shoots were subcultured again for further multiple shoot induction after 15 days. Subcultures of multiplied shoots and callus were done at an interval of every 25 – 30 days. Multiple and regenerated shoots were cut and individual shoots were placed in MS medium containing hormones for root induction.

RESULTS AND DISCUSSION

The cultured explants showed more than 80% contamination free cultures when treated with 0.1% $HgCl_2$ for 2 min for surface sterilization. *In vitro* propagation studies showed that the plant hormones play an important role in the initiation, multiple shoot generation, callogenesis, regeneration and rooting. Among the explants used for the culture nodal segments were found to be able to produce shoots (Figure 1) on MS medium prepared with different concentrations of BA (without PGR, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mg/l). The concentration of 0.1 mg/l BA was given the best results (1.55 shoots/explant) (Table 1). Microcuttings taken from the *in vitro* proliferated shoots were transferred on MS medium containing different concentration of BA (2.0, 2.5, 3.0, 3.5, 4.0, and 5.0 mg/l) for multiple shoot regeneration (Figure 2), after 30 days multiple shoots proliferated on 3.5 mg/l BA concentration (Figure 3) and resulted in average 83.2 ± 0.445033 plantlets (Table 2).

Young leaves were placed on MS medium supplemen-

Table 2. Effect of different concentrations of BA in MS medium on *in vitro* shoot proliferation of Stevia. Each treatment consisted of 10 replications. Data (Mean ± SE) were recorded after 30 days of culture.

S. No.	PGR (mg/l)	No. of Plantlets/Explants (Mean±SE)
1.	2.0 BA	26.3 ± 0.56953
2.	2.5 BA	53.1 ± 0.626959
3.	3.0 BA	73.5 ± 0.408325
4.	3.5 BA	83.2 ± 0.445033
5.	4.0 BA	70.5 ± 0.536644
6.	5.0 BA	49.7 ± 0.56953

Note: Each treatment consisted of 10 replications. Data (Mean ±SE) were recorded after 30 days of culture

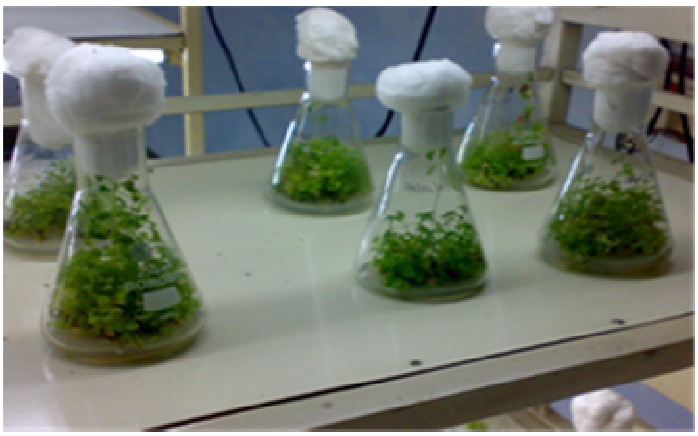

Figure 3. Multiplication of *Stevia rebaudiana*.

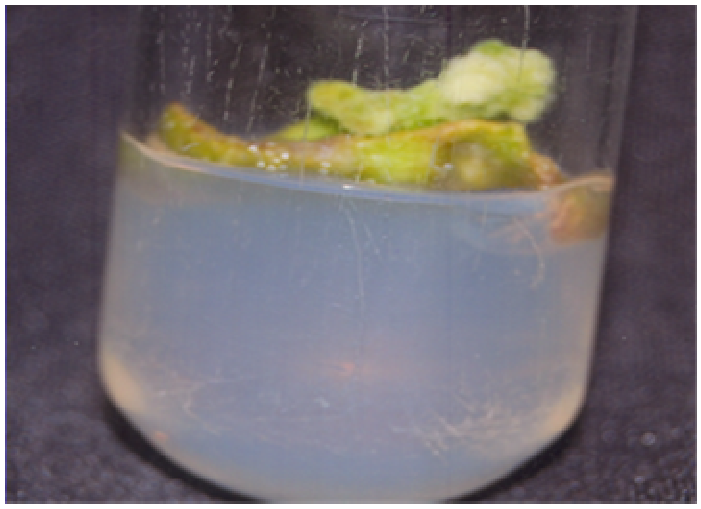

Figure 4. Callus initiation (*Stevia rebaudiana*).

ted with different concentrations of 2,4-D (0.5 1.0, 2.0, 2.5 and 3.0 mg/l) and 2 mg/l of 2,4-D with different concentrations of Kinetin (0.5, 1.0, 1.5 and 2.0 mg/l) and BA

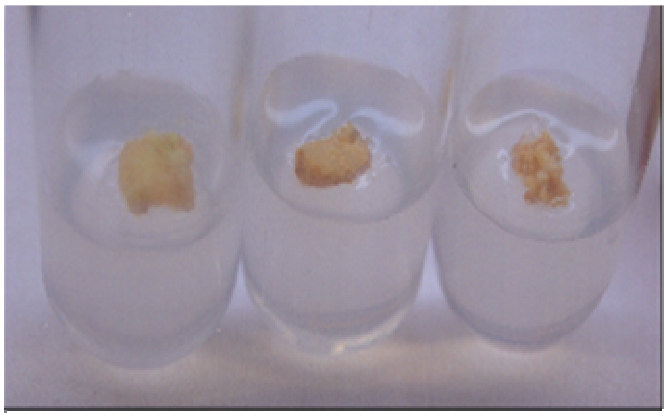

Figure 5. Subculture of Callus (*Stevia rebaudiana*).

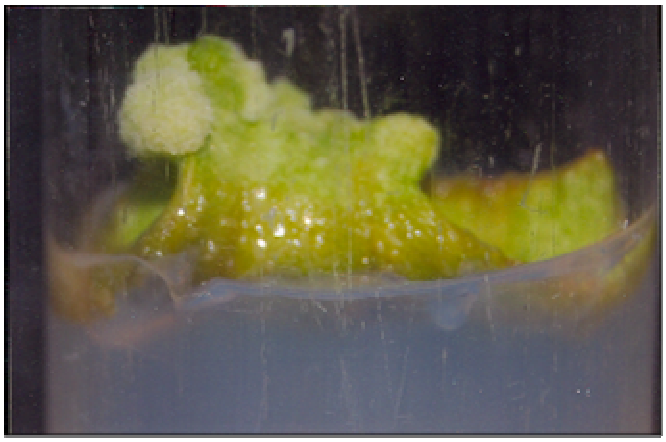

Figure 6. Regeneration of Callus (*Stevia rebaudiana*).

(0.5, 1.0 and 2.0 mg/l) for callus induction (Figure 4). 2.0 mg/l of 2, 4-D with 1.0mg/l Kinetin showed 88% callus induction (Excellent Growth) and after 30 days(Table 3), subculture of callus was done in the same medium (Figure 5). For the regeneration, small pieces of callus called calli were placed on MS medium (Figure 6) containing BA (0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) and 5.0 mg/l BA with different concentrations of NA (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l). 5.0 mg/l BA with 1 mg/l NA gave the best result (Figure 7) with 3.8 plantlets/calli (Table 4).

After 30 days of growth, the elongated shoots were transferred to the rooting medium. Root formation was induced in the *in vitro* proliferated shoots by culturing them on the $^{1/2}$MS medium containing activated charcoal. The best response was observed (Figure 8) in 100 mg/l activated charcoal supplemented with $^{1/2}$MS medium as an average of 11.1 ± 0.264052 of the inoculated explants showed root initiation (Table 5). The plantlets were transplanted to small polythene bags as hardening pots conaining Sand: Soil: Vermicompost (1:1:1). The transplantted plantlets were kept under shade poly house and then were transferred to normal environment conditions.

Table 3. Percent callus induction.

S. No.	2,4-D(mg/l)	Kinetin(mg/l)	BA(mg/l)	% Callus induction	Callus Growth Performance
1	0.5	-	-	0	-
2	1.0	-	-	20	+
3	2.0	-	-	72	++++
4	2.5	-	-	44	+++
5	3.0	-	-	32	++
6	2.0	0.5	-	48	+++
7	2.0	1.0	-	88	++++++
8	2.0	1.5	-	72	++++
9	2.0	2.0		36	++
10	2.0	-	0.5	40	+++
11	2.0	-	1.0	36	++
12	2.0	-	2.0	28	++

Callus not induced = - Very poor = + Poor = ++
Satisfactory = +++ Good = ++++ Excellent = ++++++
Note: Each treatment consisted of 25 replications. Data were recorded after 30 days of culture.

Table 4. Regeneration of Callus.

S. No.	BA(mg/l)	NA(mg/l)	Total calli plated	No of shoot regenerated	Mean % Plantlet regeneration
1	0.1	-	10	0	0 ± 0
2	0.5	-	10	0	0 ± 0
3	1.0	-	10	1	0.1 ± 0.117385
4	2.0	-	10	6	0.6 ± 0.523359
5	3.0	-	10	7	0.7 ± 0.619917
6	4.0	-	10	17	1.7 ± 0.248273
7	5.0	-	10	31	3.1 ± 0.171184
8	5.0	0.1	10	27	2.7 ± 0.203543
9	5.0	0.2	10	28	2.8 ± 0.127152
10	5.0	0.5	10	32	3.2 ± 0.23788
11	5.0	1.0	10	38	3.8 ± 0.190729
12	5.0	2.0	10	21	2.1 ± 0.264052

Note: Each treatment consisted of 10 replications. Data (Mean ±SE) were recorded after 20 days of culture.

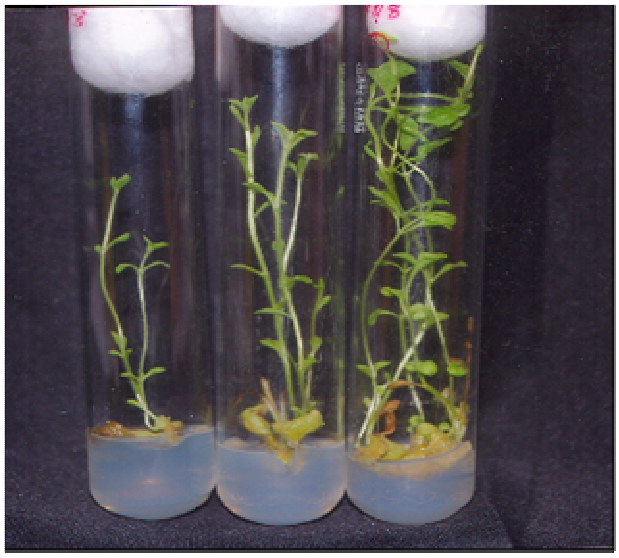

Figure 7. Regeneration of shoot from Callus (*Stevia rebaudiana*).

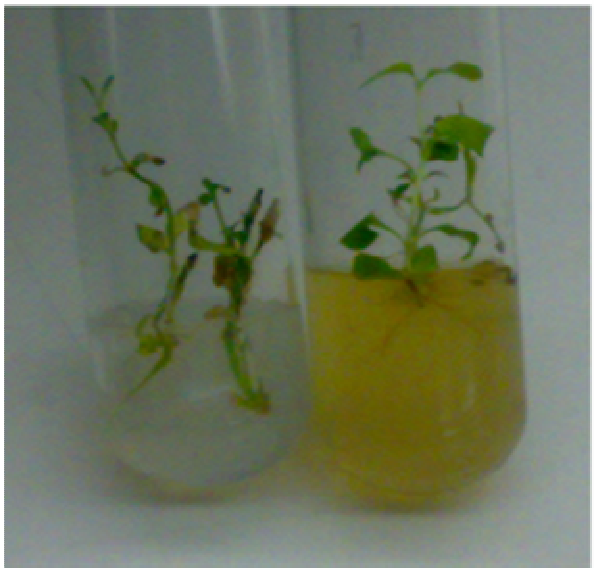

Figure 8. Rooting of shoot (*Stevia rebaudiana*).

Table 5. Rooting response of *in vitro* regeneration excised shoots (10 repeats).

S. No.	Medium	Activated charcoal (mg/l)	No. of roots/ Plantlets (Mean±SE)
1	$^{1/2}$MS	-	5.3 ± 0.248273
2	MS	-	5.9 ± 0.264052
3	$^{1/2}$MS	25	7.1 ± 0.299888
4	$^{1/2}$MS	50	7.9 ± 0.264052
5	$^{1/2}$MS	100	11.1 ± 0.264052
6	$^{1/2}$MS	125	9.1 ± 0.299888
7	$^{1/2}$MS	150	8.3 ± 0.286093

Note: Each treatment consisted of 10 replications. Data (Mean ±SE) were recorded after 20 days of culture.

Through this process of acclimatization, almost 78% survival of the tissue cultured plantlets was achieved.

Conclusion

In this study, experiments were conducted to standardize the source of explants and culture media for multiple proliferation of shoot resulting in mass propagation of homogenous elite plantlets of *S. rebaudiana.* Similar studies on shoot proliferation have been performed by Debnath (2008), Ahmed (2007), Patil (1996), Nepovin and Vanek (1998), Sikach (1998), Akita and Shigeoka (1994) and Sivaram and Mukundan (2003). They also reported that plant hormone is necessary for shooting, elongation and rooting. In most of cases BA was found to be essential for growth and multiple shoot formation. Sivaram and Mukundan (2003) also found out similar response when the elongated shoots were transferred to half strength MS medium.

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