DOI: 10.56511/JIPBS.2016.v3.i03.pp47-56

Journal of Innovations in Pharmaceuticals and Biological Sciences
www.jipbs.com

JIPBS

e-ISSN: 2349-2759 p-ISSN: 2395- 1095

Research article

Micropropagation of *Stevia rebaudiana* Bertoni. through direct and indirect organogenesis

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Abstract

The present research work was undertaken to develop a protocol for micropropagation of *Stevia rebaudiana* Bertoni., an important medicinal plant of Bangladesh. Shoot apex, Leaf and Nodal explants were aseptically cultured on agar solidified Murashige and Skoog (MS) medium supplemented with different growth PGRs (Plant growth regulators). Indirect organogenesis was found in leaf segment and direct organogenesis was observed in shoot apex. But in case of nodal segment both direct and indirect organogenesis were found. Maximum amount of green compact callus produced from nodal explants in MS medium supplemented with 1.0mg/l benzylaminopurine (BAP) + 0.5mg/l indole-3-acetic acid (IAA). The induced calli were cultured on MS medium fortified with different PGRs for shoot proliferation. The maximum number of shoot buds (15.30 ± 0.15) formation was observed in MS medium fortified with 1.5mg/l BAP+0.5mg/l IAA. Multiple shoot buds underwent rapid elongation on elongation media and maximum elongation (6.90 cm) took place on MS with 1.0 mg/l BAP + 1.0 mg/l IAA. Elongated shoot buds produced strong and stout roots (4.40 cm) on half strength MS medium fortified with 1.0 mg/l indole-3-beutyric acid (IBA). The well developed plantlets were successfully transferred to hardening and survival rate was 95%.

Key words: *Stevia rebaudiana,* Micropropagation, Callus, Organogenesis.

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1. Introduction

Stevia rebaudiana Bertoni. Commonly known as names- Stevia, Sweet leaf of Paraguay, Sweet herb, Honey leaf belongs to the family Asteraceae which is estimated to be 100-300 times sweeter than cane sugar [1]. It is a small perennial herb growing up to 65 - 80cm tall, with sessile oppositely arranged leaves, white flower in axillary heads. *S. rebaudiana* is native to the tropical and subtropical South America [2]. *Stevia* was discovered by Antonio Bertoni, a South American Natural Scientist, in 1887. The leaves of *Stevia* are the source of diterpene, glycosides, stevioside and rebaudioside [3]. Stevioside is of special interest to diabetic persons with hyperglycemia and the diet conscious [4]. Now, *Stevia* has

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been introduced as a crop in a number of countries including Brazil, Korea, Japan, Mexico, United States, Indonesia, Tanzania and Canada [5-7]; for food and pharmaceuticals products. Currently *S. rebaudiana* production is centered in China with major market in Japan [8]. The product also can be added to tea and coffee, cooked or baked goods, processed foods, pickles, fruit juices, tobacco products, confectionary goods, jams and jellies, candies, yogurts, pastries, chewing gum and sherbets beverages. *Stevia* also can act as an antimicrobial in pharmacological studies and according to [9], this plant has a negligible effect on blood glucose. *Stevia* leaf extract has the ability to reduce the blood sugar level up to 35.2 % within 6 to 8 hours of ingestion [10]. Therefore, it is attractive as a natural sweetener to diabetes and other carbohydrate controlled diets [11]. As the *Stevia* plant contains zero calorie, it aids in effective weight control and management, also uses for promoting mental activity, increasing energy level and treatment of hypoglycemia, food poisoning and digestion problems.

Seeds of *Stevia* show a very low germination percentage and vegetative propagation is limited by lower number of individuals [12]. Tissue culture is the only rapid process for the mass propagation of *Stevia* and there have been few reports of *in vitro* growth of *Stevia* [13], *in vitro* clonal propagation of *Stevia* was carried by using leaf [14,15] nodal and inter nodal segment [16,17] shoot tip explants [18,19]. The tissue culture is also a powerful tool that can accelerate the genetic breeding [20,21]. This needs to be propagated rapidly to meet up our medicinal demand and also for conservation purpose. The present investigation was undertaken with a view to develop reliable and efficient protocol for rapid and mass scale micropropagation of this plant species study for local environment of Bangladesh.

2. Materials and Methods

Plant Materials and Surface Sterilization

Three months old seedling of *S. rebaudiana* were collected from a nursery of Bangladesh Council for Scientific and Industrial Research (BCSIR),Chittagong and were established in garden pots of Botany Department, Chittagong University. Shoot Apex, Leaf and nodal segments of garden pots grown plants of *S. rebaudiana* were collected and thoroughly washed under running tap water for 10 minutes, treated with liquid detergent for 10 minutes, followed by dipping in 5% (v/v) savlon solution for 10 minutes. The materials were then washed 3-4 times with distilled water for complete removal of detergent and taken under running laminar airflow cabinet and transferred to 500ml sterilized conical flask. After rinsing with 70% ethanol for less than 60 Seconds, they were surface sterilized with 0.1% (w/v) $HgCl_2$ for 5 minutes and washed with sterile distilled water 4-5 times. The surface sterilized explants were cut into small pieces (0.5-1.0cm) with a sterilized surgical blade and then inoculated onto the culture media.

Culture media and conditions for plant regeneration

Murashige and Skoog (MS) basal medium supplemented with different concentration and combination of plant growth regulators (PGRs) were used for induction of organogenesis or embryogenesis. In some cases the multiple shoot buds (MSBs) that developed from either nodal explants or from callus elongated on MS supplemented with different PGRs and for rooting, elongated shoots at a height of 2-4 cm were rescued aseptically from the cultured on rooting medium containing half strength

and one forth strength of MS basal medium fortified with different concentration and combinations Indole-3-butyric (IBA), indole-3-acetic acid (IAA) and α-naphthaleneacetic acid (NAA). In all cases the media were solidified with 0.8% (w/v) agar and pH was adjusted to 5.8 before autoclaving for 30 minutes at 121^0C under 1.1kg/cm^2 pressure. Culture vessels with inoculated explants were maintained under a regular cycle of 14 hours light and 10 hours dark at 25 ± 2^0 C.

Subculture for multiple shoots

Proliferated multiple shoots were rescued very carefully in aseptic conditions and divided into clusters of 2 - 3 shoots using a sterile sharp scalpel. Sub culturing was done on the same or different media for further response at an interval of 15 - 20 days and culture vessels were maintained in the culture room in the same light and temperature conditions.

Rooting of *in vitro* shoot

Experiments of adventitious root formation on the shoots proliferated *in vitro* were conducted only after having sufficient amount of shoot cultures. Different rooting experiments were carried out with half strength MS medium with or without growth regulators to determine the suitable media composition, optimum growth requirements. After 10-20 days, the proliferated multiple shoots were separated and individual shoots were placed in rooting media. The adventitious roots were produced from the cut ends of micro shoots within 2-3 weeks of culture on suitable medium. Elongated shoots at 2-4 cm height were rescued aseptically from the culture vessels and cultured on freshly prepared rooting medium.

Hardening and Acclimatization of plantlets to soil

The well rooted plantlets were transferred to earthen pots containing a mixture of soil and compost(2:1) at relative humidity 90% with light intensity varied 2000-3000 lux and temperature of 28 ± 2^0 C following successive phases of acclimatization. For the purpose, the month of the culture vessels were kept open for one day in the culture room and they were then kept outside the culture room for 6 hours in the next day. Later on those were kept outside the culture room for 12 hours. Finally the seedling were taken out of the culture vessels and rinsed with running tap water for complete removal of medium attached to the roots.

Statistical analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment was replicated thrice. Observations were recorded on the percentage of response, number of shoots per explants and number of roots per shoot. A minimum of 10-15 explants were used for each experiment. Means and standard deviations were calculated for each treatment. The data means ± SD of at least three different experiments were represented.

3. Results and Discussion

Induction of multiple shoot buds directly from shoot apex and nodal segments:

Shoot apex and nodal segments of field grown plants were aseptically cultured with 9 different combinations and concentrations of auxins such as IAA and NAA and cytokinins namely kinetin (Kn) and BAP. Shoot apex and nodal segments underwent direct organogenesis producing multiple shoot buds in some of the media combinations (Table 1). The maximum number of nodal explants (90%) produced multiple shoots on media having 1.5 mg/l BAP + 0.5 mg/l IAA. Here, the average maximum number of shoot per culture was 15.30 ± 0.15 (Figure 1A & B). Such direct

organogenesis was reported to happen in many other medicinal plants including, *Gentiana kurroo* [22], *Vitex negundo* [23], *Wedilia chinensis* [24], *Rauvolfia tetraphylla* [25], *Curculigo orchioides* [26], *Ficus religiosa* [27], *Plumbago zeylanica* [28] and *Punica granatum* [29].



Figure 1. Direct and indirect organogenesis of *Stevia rebaudiana* A&B-Multiple shoot buds initiation and proliferation from nodal explant and shoot apex, C-Callus induction, D-Shoot buds formation form callus, E&F- Elongation of multiple shoot buds, G&H- Elongated shoot buds produced strong and stout roots, I- Acclimatization of *in vitro* grown plantlets in outside environment.

Callus induction from different types of explants:

For the induction of callus tissues, nodal and leaf segments were cultured on MS medium supplemented with different concentration of five PGR namely, IAA, NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), BAP and Kn (Table 2). The nodal and leaf segments gave differential response to different PGR combinations. Morphogenetic differentiation of leaf segments started within 15–25 days after culture and produced light green compact callus tissue. Here, maximum (100%) nodal explant produced callus in media having 1.0 mg/l BAP + 0.5 mg/l IAA. In case of nodal segment initiation of callus formation took place within 15 - 20 days of culture. These callus were green compact (Figure 1C). Nodal segment showed better response in callus formation then leaf segments in different combinations. However, the nature of the callus was almost same in different PGR supplemented media such indirect organogenesis was reported in some medicinal plants including, *Holostema ada-kodien* [30], *Plumbago zeylanica* [28], *Tinospora cordifolia* [31] and *Tinospora cordifolia* [32].

Table 1. Development of multiple shoot buds from shoot apex and nodal segment of *S. rebaudiana* when grown on 0.8% (w/v) agar solidified MS medium supplemented with different PGRs

PGRs combination(mg/l)	Explants	% of explant giving response	Time (d) required for induction of callus	Average* no. of multiple shoot buds sprouted from explants(mean ±SE)
BAP + IAA	NS**	42	15-25	5.20 ± 0.19
0.5 + 1.0	SA**	28	15-20	3.10 ± 0.31
1.0 + 1.0	NA	67	15-18	8.20 ± 0.41
	SA	43	15-18	5.30 ± 0.18
1.5 +0.5	**NA**	**90**	**10-15**	**15.30 ± 0.15**
	SA	55	10-18	7.20 ± 0.10
1.5 + 1.0	NS	85	10-12	10.20 ± 0.33
	SA	68	10-15	6.10 ± 0.19
2.0 + 1.0	NA	48	10-12	7.10 ± 0.51
	SA	43	10-12	4.30 ± 0.12
Kn+NAA	NA	35	15-20	4.50 ± 0.41
0.5 + 1.0	SA	20	15-20	2.80 ± 0.19
1.5 + 0.5	NA	79	10-12	6.70 ± 0.41
	SA	45	10-15	4.50 ± 0.11
1.5 + 0.1	NA	68	10-12	6.20 ± 0.17
	SA	52	10-15	3.80 ± 0.51
2.0 + 0.5	NA	55	10-12	5.20 ± 0.18
	SA	48	10-15	3.10 ± 0.11

***Values are the mean of three replicates each with 15 explants. **NS- Nodal segment, ***SA- Shoot apex.**

Table 2. Data on the induction of callus tissue from the nodal and leaf segments of *S. rebaudiana* when grown on 0.8% (w/v) agar solidified MS medium supplemented with different PGRs

Growth regulators (mg/l)	Explants	No. of explant used	No. of explant gave of response	%of explant giving response	Time (d) required for induction of callus	Colour and texture of callus
BAP+IAA	NS*	20	8	40	15-20	Gc***
0.5 + 1.0	LS**	20	5	25	15-20	Lgc****
1.0 + 0.5	**NS**	**20**	**20**	**100**	**15-20**	**Gc**
	LS	20	12	60	15-20	Lgc
2.0 + 0.5	NS	20	15	75	15-20	Gc
	LS	20	13	65	14-18	Lgc
BAP+NAA	NS	20	15	75	10-15	Gc
1.0 + 0.5	LS	20	8	40	10-15	Lgc
1.0+ 1.0	NS	20	16	80	10-12	Gc
	LS	20	13	65	10-12	Lgc
2.0 + 0.5	NS	20	14	70	10-12	Gc
	LS	20	10	50	10-12	Lgc
BAP+2,4D	NS	15	11	55	15-20	Gc
1.0 + 0.5	LS	15	6	30	15-20	Lgc
1.5 + 1.0	NS	15	15	75	15-20	Gc
	LS	15	11	55	15-20	Lgc

NS- Nodal segment **LS- Leaf Segment, Gc- Green compact,****Lgc- Light green compact**

Development of multiple shoot buds from different types of calli:

In order to promote differentiation the callus tissues were further grown in a broad spectrum of auxin and cytokinin supplemented MS media (Table 3). It is evident that white callus did not undergo any kind of differentiation and finally died although in some of the media it initially multiplied. On the other hand light green, green and compact callus multiplied and differentiated producing shoot buds (Figure 1D) on MS supplemented with 0.5 - 2.0 mg/l BAP in combination with 0.5 - 1.0 mg/l IAA or NAA. The maximum shoot formation was observed in medium containing 1.0 mg/l BAP + 0.5 mg/l IAA. Development of multiple shoot buds from the callus also noted in other medicinal plants including, *Thevetia neriifolia* [33], *Abrus precatorius* [34], *Jatropha curcas* [35].

Elongation of multiple shoot buds:

In order to induce rapid elongation, the multiple shoot buds that produced directly and indirectly from the different types of explants underwent elongation when cultured on the elongation media (Table 4). The highest rate of elongation (6.90 cm) was recorded when grown on the medium supplemented with 1.0 mg/l BAP and 1.0 mg/l IAA. Figure 1E & F shown the rapid elongation of shoot buds on elongation medium. Similar result was also founded in other medicinal plants *Vitis vinifera*[36], *Jatropha curcas*[37], *Helicteres isora*[38].

***In vitro* rooting and acclamatization**

In order to get complete plantlets, 2 to 3 cm long *in vitro* grown shoots were separated and transferred to rooting media. Half and one fourth strength MS basal medium fortified with different concentrations of auxins (NAA, IAA and IBA) was used for rooting experiment. Data were recorded 5 weeks after inoculation. Response of shoots to rooting was very much dependent on the concentrations and combination of auxins provided (Table 5). The highest mean number (12.00 ± 0.25) and mean length (4.40 cm) of root per culture (Figure 1G & H) was noted in half strength MS medium supplemented with 1.0 mg/l IBA. Similar result was also reported in other medicinal plants *Rhinacanthus nasutus* [39], *Emblica officinalis* [40] *Clerodendrum indicum* [41] and *Cinnamomum camphora* [42].

Table 3. Results of further culture of induced callus of *S. rebaudiana* on 0.8% (w/v) agar solidified MS medium supplemented with wide spectrum of PGR combination.

PGRs combination(mg/l)	% of explants showing proliferation	Time(d) required	Average* no of multiple shoot buds sprouted from explants(mean±SE)
BAP+ IAA 0.5 +0.5	32	15-25	4.20 ± 0.51
0.5 +1.0	38	15-25	4.50 ± 0.32
1.0+0.5	**95**	**15-20**	**6.10 ± 0.17**
1.0 + 1.0	78	15-20	6.00 ± 0.19
1.5 + 0.5	65	10-15	5.78 ± 0.21
1.5 +1.0	53	10-18	4.80 ± 0.23
2.0+ 0.5	48	10-20	3.50 ± 0.14
BAP+NAA 0.5 +0.5	29	15-20	3.80 ± 0.07
1.0 +0.5	71	15-20	5.30 ± 0.15
1.5 +.05	63	10-20	4.80 ± 0.19
1.5 +1.0	57	10-18	4.01 ± 0.05

*** Values are the mean of three replicates each with 15 explants.**

Table 4. Data on the elongation of direct and indirectly produced shoot buds of *S. rebaudiana* when grown on 0.8% (w/v) agar solidified MS medium supplemented with wide spectrum of PGR combination.

PGRs combination(mg/l)	Average* initial length (cm) of individual shoot bud (mean ± SE)	Average* length(cm) of multiple shoot bud after 30 days of culture(mean±SE)
BAP+IAA		
0.5+0.5	1.10 ± 0.21	2.80 ± 0.31
0.5 + 1.0	1.30 ± 0.23	3.00 ± 0.19
1.0 + 0.5	1.80 ± 0.31	4.20 ± 0.21
1.0 + 1.0	**3.10 ± 0.14**	**6.90 ± 0.24**
2.0 + 0.5	1.40 ± 0.09	3.20 ± 0.28
2.0 + 1.0	1.00 ± 0.07	2.00 ± 0.18
BAP + NAA		
0.5 + 0.5	1.00 ± 0.51	2.20 ± 0.42
0.5 + 1.0	1.50 ± 0.34	2.90 ± 0.51
1.0 + 0.5	1.20 ± 0.19	3.30 ± 0.25
1.0 + 1.0	1.60 ± 0.17	4.20 ± 0.11
2.0 + 0.5	1.00 ± 0.21	3.10 ± 0.28
2.0 + 1.0	1.10 ± 0.24	2.30 ± 0.17
Kn + IAA		
0.5 + 0.5	1.10 ± 0.13	2.30 ± 0.31
0.5 + 1.0	1.50 ± 0.33	2.80 ± 0.33
1.0 + 0.5	1.30 ± 0.41	3.11 ± 0.21
1.0 + 1.0	1.90 ± 0.07	4.00 ± 0.52
2.0 + 0.5	1.20 ± 0.10	3.80 ± 0.19
2.0 + 1.0	1.00 ± 0.23	2.40 ± 0.17

*** Values are the mean of three replicates each with 15 explants.**

Table 5. Data on the development of roots in elongated multiple shoot buds of *S rebaudiana* when grown on 0.8% agar solidified rooting media.

PGRs	mg/l	Half strength		
		% of Rooting	Number* of roots per shoot	Average length of root(cm)(mean±SE)
IAA	0.5	69	4.64 ± 0.35	2.20 ± 0.18
	1.0	80	5.10 ± 0.11	2.40 ± 0.21
IBA	0.5	90	9.20 ± 0.21	3.42 ± 0.16
	1.0	**100**	**12.00 ± 0.25**	**4.40 ± 0.17**
NAA	0.5	70	6.20 ± 0.25	2.75 ± 0.31
	1.0	62	3.70 ± 0.07	1.80 ± 0.18
IAA	One fourth strength			
	0.5	48	2.10 ± 0.37	1.90 ± 0.41
	1.0	55	2.30 ± 0.19	2.10 ± 0.38
IBA	0.5	62	2.15 ± 0.08	1.80 ± 0.31
	1.0	70	1.10 ± 0.51	2.00 ± 0.17

*** Values are the mean of three replicates each with 15 explants**

The well rooted plantlets were then transferred to outside natural environment through sequential phases of acclimatization. The regenerated plants were finally transferred to earthen pots (Figure-1I) containing a mixture of soil and compost (2:1). Over 95% of the plants survived after transplantation to the garden.

Conclusion

In conclusion, the present investigation reports an efficient and reproducible regeneration protocol via. direct and indirect organogenesis of *Stevia rebaudiana* Bertoni. The method is flexible, allowing incorporation of different types of explants (nodal segment, shoot apex and leaf segment) with BAP, Kn, NAA, 2,4-D and IAA effective in both callus and multiple shoot buds proliferation. MS medium containing 1.5 mg/l BAP+0.5 mg/l IAA was the best for shoot proliferation. Between the two explants nodal segment gave better response than leaf segments and MS fortified with1.0 mg/l BAP+0.5 mg/l IAA was the best for callus induction. Half strength MS medium fortified with 1.0 mg/l IBA was found to be the best treatment for root formation in *S. rebaudiana.* The protocol developed for seedlings of *S. rebaudiana* can be used reliably for propagation in a commercial scale and *ex situ* conservation of this valuable medicinal plant species.

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