In Vitro Clonal Propagation of Stevia Rebaudiana Bertoni Through Node and Shoot Tip Culture

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Abstract

A reliable rapid large scale micropropagation method has been established from the node and shoots tip explant of *Stevia rebaudiana* Bertoni. Experiments were conducted to standardize the culture media with plant hormone for multiple shoot proliferation and rooting for obtaining plantlets with uniform characteristics like mother plant in terms of growth and habits. Different concentrations and combinations of auxins (IAA) and cytokinins (BAP, Kin) were used in MS for the above purpose. Maximum shoot regeneration was found in MS treated with 2.0 mg/l BAP both in node and shoot tip explants. In the above combination, nodal explants produced 14-16 initial shoots. Shoot tip explants produced 10-12 shoots. For *in vitro* rooting, different concentrations of IBA and NAA were used. Higher rooting percentage was recorded on MS fortified with 0.5 mg/l IBA. The rooted plantlets were hardened and successfully established in the soil. About 85% of the regenerated plantlets survived in the natural condition.

Keywords: *In Vitro* culture, Stevia rebaudiana Bertoni, Callus, tissue culture

1. Introduction

Stevia rebaudiana Bertoni is a rare medicinal herb belongs to the family Asteraceae. The plant is perennial, about 60-75 cm tall, leaf is sessile and oppositely arranged, flower is white and seed is very small. It is originally a South American wild plant [1]. This plant was cultivated hilly area in Paraguay. At first M.S. Bertoni reported this plant in 1887. *S. rebaudiana* Bertoni is a sweet herb indigenous to the elated terrain of north eastern Paraguay near its borders with Brazil [2].The leaf extract of stevia is very sweet. The leaves of stevia are the source of the diterpene glycosides, viz. steviosides and rebaudiosides [3]. Pure steviosides are non-caloric and 300 times sweeter than sugar [4]. Now it is being cultivated in Japan, Taiwan, Philippines', Hawaii, Malaysia and over all South America for food and pharmaceutical products [5]. The product can be added to tea, coffee, coke or beverages. Currently, about 750-1000 of *S. rebaudiana* are use by Japan, Brazil and other nations in variety of foods including soft drinks, sea foods, pickled vegetables etc [6]. Use of stevioside as a natural sweetener has been reported to be safe [7]. It is a special interest to Diabetics patient and diet conscious. Seed of stevia show a very low germination percentage [8-10] and vegetative propagation is limited by lower number of individuals. Propagation by seed does not produce homogenous populations; resulting in great variability is important feature like sweetening level and composition [11]. Moreover, for commercial purpose when large scale propagation is necessary, the conventional way of production is not adequate to fulfill the required demand. Hence, there is a need to standardize a quicker method of propagation by *in vitro* culture techniques. So, tissue culture is only the alternative method for mass propagation of *S. rebaudiana*. Earlier *in vitro* propagation of stevia has been reported through different explants [12-14]. The climatic condition, soil type of Bangladesh is very favorable for commercial propagation of this important plant. Considering its importance and to overcome the problem of its propagation through conventional methods, the present study was undertaken to develop a simple, rapid and economical protocol for the production of plantlets on a large scale from the node and shoot tip explants of *S. rebaudiana*.

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2. Materials and Methods

Healthy one to two months old pot grown *Stevia reboudiana* Bertoni plants were collected from the BRAC agricultural farm, Gazipur and conserved the plants in the experimental garden of the Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka for obtaining explants. Shoot tip and nodal explants were collected from these plants. The explants were initially washed thoroughly under running tap water and afterwards rinsed several times with distilled water. Then explants were cleaned by Trix (Commercial detergent, Reckitt Benckiser Bangladesh Limited), mixed with Tween 80 and finally by distilled water for several times. These were surface sterilized for 2 minutes in 70% ethanol, 10 minutes in 1.5% sodium hypochlorite in which 0.01% Tween-20 was added also as surface sterilent. Explants were then treated with 0.1% $HgCl_2$ solution for 8-10 minutes followed by five washes with sterilized distilled water. Surface sterilized explants were cut into convenient size and placed on MS medium containing different concentrations of BAP alone or in combination with Kn and NAA. For induction of roots, regenerated shoots (3-4 cm) were excised and transferred to MS supplemented with various concentrations IBA and NAA.

For all experiments, pH of the medium was adjusted to 5.8 and solidified by 0.4% phytagel and dispensed into culture vessels. The culture vessels containing medium were then autoclaved at 121ºC and at 1.05 $kgcm^{-2}$ pressure for 20 minutes. Following inoculation, all the cultures were kept in a growth room with 16 h photoperiod having light intensity

of 2000 lux (approx.) from white florescent tube light and at temperature of 26 ±1ºC. When required, subcultures were carried out at an interval of four weeks. Data was recorded on multiple shoot regeneration from node and shoot tip explants, percentage of explants regenerated, number of shoots per culture, days to rooting, percentage of cultures rooted, number of roots per shoot and survival percentage of rooted plants at natural condition etc. The regenerated plantlets after developing sufficient root system in the *in vitro* condition were deemed ready to transfer to soil. The plantlets were carefully removed from the culture vessels. The roots of the plantlets were gently washed under running tap water to remove agar attached to the root zone. Immediately after washing, they were transferred to small polythene bags containing a mixture of soil, sand and compost in 1:1:1 ratio. The plantlets in the poly bags were covered with thin polythene bags to check sudden desiccation. The inner sides of these bags were sprayed with water at every 24 hours to maintain high humidity around the plantlets. The polythene bags were gradually perforated to expose the plantlets to the outer normal environment and subsequently removed after seven days. By this time the plantlets were become established in the soil. They were finally transferred to the experimental field.

3. Results and Discussion

Two type of explants viz., nodal and shoot tip explant of *Stevia rebaudiana* Bertoni were cultured on MS supplemented with different concentrations of auxin (NAA) and cytokinins (BAP, Kn) singly or in various combination for optimizing multiple shoot regeneration. Within three weeks multiple shoot emerged directly from the cultured explants. The explant showed different responses to various combinations are shown in Table 1.

Table 1. Effect of different concentrations and combinations of auxin (NAA) and cytokinins (Kn and BAP) on multiple shoots regeneration from nodal and shoots tip explants of *Stevia rebaudiana*. Data were recorded after five weeks of culture.

Supplements (mg/l)	Type of explants Inoculated	No. of explants inoculated	No. of explants regenerated	% of explants induced shoot	Average no. of initial shoots/explant	Days to shoot initiation
BAP						
0.5	Node	10	7	70	7	12
	Shoot tip	10	7	70	6	12
1.0	Node	10	7	70	7-8	10
	Shoot tip	10	7	70	7-8	10
2.0	Node	10	9	90	14-16	11
	Shoot tip	10	9	90	10-12	10
2.5	Node	10	8	80	8-9	10
	Shoot tip	10	8	80	9	10
BAP+NAA						
1.0 + 0.2	Node	10	6	60	5-6	12
	Shoot tip	10	6	60	5	12
1.0 + 0.5	Node	10	6	60	4-5	14
	Shoot tip	10	5	50	4	12
1.0 + 1.0	Node	10	5	50	Callus	-
	Shoot tip	10	4	40	Callus	-
1.0 + 1.5	Node	10	4	40	Callus	-
	Shoot tip	10	4	40	Callus	-
BAP+ Kn						
0.5	Node	10	6	60	5-6	12
	Shoot tip	10	6	60	5-6	10
1.0	Node	10	8	80	8-10	11
	Shoot tip	10	8	80	8-10	10
2.0	Node	10	7	70	6-7	12
	Shoot tip	10	7	70	7	12
2.5	Node	10	6	60	6	12
	Shoot tip	10	5	50	6	11

Results of this study indicate that among the combinations only BAP and BAP with Kn produced shoot regeneration from most of the explants and the media containing BAP with NAA responded less in shoot regeneration. Among the various hormonal supplements used, in both nodal and shoot tip explants, the best shoot induction was observed in MS fortified with 2.0 mg/l BAP (Table 1). In this combination highest percentage of explant (90%) showed

shoot proliferation both for shoot tip and nodal segment (Fig. 1). After development of *in vitro* shoots, they were sub-cultured and maintained in the same media composition for obtaining multiple shoots. Averages of 14-16 numbers of shoots were regenerated from nodal explant (Fig. 2). Whereas 10-12 shoots regenerated from shoot tip explants (Fig. 3) after sub-cultured in the same medium. Several investigators reported multiple shoot induction using different concentrations of BAP [15]. They got best response in case of multiple shoot regeneration from *in vitro* micro cutting of *S. rebaudiana* using 0.5 mg/l BAP. However, the result of the present investigation slightly different with this investigation. In the present study, comparatively higher concentration 2.0 mg/l BAP were found suitable for large scale shoot initiation and further multiplication. This difference may be due to the difference physiological condition of the explants. The effects of BAP and Kn were studied on multiple shoot regeneration in stevia. Seventy five percent of explant (Both Node and Shoot tip) showed shoot proliferation in MS contaning 1.0 mg/l BAP and 0.5 mg/l Kn. In this combination about 8-10 shoots were regenerated from both the explants. So, only BAP was found superior to BAP added with Kn combination. The regenerated shoots in BAP and Kn combination, however vitrified in nature. Hoque *el. al*. 1998 obtained similar response in their work on *Chrysanthemum* using Kn in combination with BAP. The response was poor and considerable amount of callus was formed with BAP and NAA combination. Similar results have already been reported by Ahmed *et. al*. 2007 in *S. rebaudiana*. So, in the present study 2.0 mg/l was found to be ideal concentration for high frequency multiple shoot induction. The regenerated shoots increased double to triple when they were subcultured in the same medium. In the present study,

Figs. 1-6. *In vitro* plant regeneration in *Stevia rebaudiana* Bertoni, (1) Induction of shoots from shoot tip and nodal segments when they were inoculated on Murashig and skoog (MS) fortified with 2.0 mg/l BAP, (2) Development of multiple shoots when shoot tip were sub-cultured in the same medium mentioned in 1, (3) Multiple shoots regeneration from nodal segments at the same media compositions, (4) Rooted shoots on half-strength MS supplemented with 0.5 mg/l IBA, (5) Rooted plant after shifted in polybag and (6) Well hardened plant in earthen tub.

experiments were conducted to standardize the culture media with plant hormone for multiple shoot proliferation for obtaining plantlets with uniform characteristics like mother plant in terms of growth and habits. Similar studies on shoot proliferation have been reported by several scientists [16-18].

Individual shoots from a multiple shoot mass were separated and transferred on to rooting medium Different concentrations of IBA and NAA on MS were used as rooting medium. In case of IBA in the all concentrations, roots appeared after one or two weeks. About four weeks, roots were developed but the root induction gradually decreased with increasing the concentration of IBA. At 2-2.5 mg/l IBA callus induction was observed from the cut end of the shoots. On the other hand, NAA did not significantly induced higher quantities of root. Callus induction was observed when NAA was used. The best response was achieved when excised *in vitro* grown shoots were sub-cultured on MS added with 0.5mg/l IBA (Table 2). Developing healthy roots at the cut end of the regenerated shoot is presented in Fig. 4 [19] Earlier few authors also reported maximum root induction in Stevia using 0.5 mg/l IBA [20]. It has been reported that IBA is a suitable auxin for adventitious root induction and it was also found superior to IAA or NAA for its more stable in nature [21- 22].

Table 2. Effect of different concentrations IBA and NAA on root induction from micro cutting in *Stevia rebaudiana*. Data were recorded after four weeks of culture. (+, ++ indicates slight, considerable callusing respectively at the base of micro cuttings).

Supplements (mg/l)	Number of shoots inoculated	Average No. of roots from each shoot	Days to initiation of roots	Callus formation at the cutting base
IBA				
0.2	8	10	10	-
0.5	8	12	9	+
1.0	8	8	10	++
1.5	8	4	12	++
2.0	8	3	11	-
NAA				
0.2	8	6	12	-
0.5	8	5	10	+
1.0	8	-	-	++
1.5	8	-	-	++
2.0	8	-	-	+++

All the rooted plantlets were taken out from the culture tubes and thoroughly washed under tap water to remove the agar. Then the plantlets were transferred to poly bags (Fig. 5) containing soil sand and cow dung in the ratio of 2:1:1. During the initial period of acclimatization for maintaining high humidity, the plantlets were kept in growth room covered by transparent poly bags. Gradually the plantlets shifted to out door condition (Fig. 6). About 85% plantlets survived in natural condition. The results of the present investigation demonstrated the establishment of an *in vitro* regeneration protocol for *S. rebaudiana* and this technique appears to be commercially suitable for large scale clonal propagation.

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