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Utilization of liquid medium for rapid micropropagation of *Stevia rebaudiana* Bertoni

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Abstract

Liquid shoot culture of *Stevia rebaudiana* Bertoni in MS medium containing BAP (1.5 mg/l) and IAA (0.5 mg/l) was developed and evaluated in relation to shoot multiplication, on average 37 new shoots per explants were obtained within 3 week. Shoot lets were regenerated from nodal explants of stevia through axillary shoot proliferation. For rooting different concentrations of IAA, IBA and NAA were used and highest rooting percentage (63%) was recorded on MS medium with IAA (1.0 mg/l). The rooted plantlets were hardened and successfully established.

1. Introduction

Stevia rebaudiana Bertoni is a perennial herb belongs to the Asteraceae family. It is a natural, non-caloric sweet-tasting plant used around the world for its intense sweet taste. The sweet herb of Paraguay, stevia produces sweeteners in it's leaves are natural plant products [1-3]. Leaves of this plant produce zero-calorie diterpene glycosides (stevioside and rebaudioside) [4-6], a non-nutritive, high-potency sweetener and substitute to sucrose, being sweetener than sucrose. It is recommended for diabetes and has been extensively tested on animals and has been used by humans with no side effects. Stevia is likely to become a major source of high potency sweetener for the growing natural food market in the future [2]. Now it is being cultivated in Japan, Taiwan, Philippines, Hawaii, Malaysia and overall South America for food and pharmaceutical products. Products can be added to tea and coffee, cooked or baked goods, processed foods and beverages, fruit juices, tobacco products, pastries, chewing gum and sherbets. In Japan alone, 50 tones of stevioside are used annually with sales valued in order of 220 million Canadian dollars [7]. Seeds germination of stevia is often poor [8-12]. Therefore, there are basically two options for multiplication; tissue culture and stem cutting.

It is now evident that plant tissue culture is an essential component of Plant Biotechnology which offers novel approaches to the production, propagation, conservation and manipulation of plants [13]. The success of *in vitro* culture depends mainly on the growth conditions of the source material [14, 15], medium composition and culture conditions [16] and on the genotypes of donor plants.

In-vitro propagation of *S. rebaudiana* in solid culture has been also reported by other authors [17-21]. However, very few reports are found about liquid culture system for stevia shoot multiplication. The establishment of culture in liquid media has several advantages, such as faster growth and multiplication rate [22], lack of impurities from agar, dilution of exudates from the explants [23], uniform dispersal and better availability of nutrients and growth regulators [24]. The liquid system could be useful for increasing the scale of production (towards scaling up for bioreactor study) and cost reduction (agar is one of the most expensive ingredients in the medium). For the above mentioned reasons we decided to develop the liquid culture of *S. rebaudiana* shoots for micropropagation [25-30]. The present study was carried out to develop a suitable protocol for rapid micropropagation of *S. rebaudiana* by using liquid medium.

2. Materials and Methods

2.1. Plant materials and surface sterilization

The twigs (about 5-6 cm) of shoots of pot grown *S. rebaudiana* plants were collected from the Greenhouse, Department of Botany, Annamalai University, Chidambaram, Tamailnadu, India. The

twigs with node explants were washed in running tap water and then washed again thoroughly by adding a few drops of Tween-20 to remove the superficial dust particles as well as fungal and bacterial spores. They were then surface sterilized with 0.1 % mercuric chloride for 3 min followed by rinsing them five times with double distilled water inside the laminar air flow chamber. Nodal segments (with a single axillary bud) about 0.5-0.8 cm were prepared aseptically and were implanted vertically on MS medium [31] fortified with specific concentrations of growth regulators (BAP, Kn and IAA) singly or in combination adding 30 g/l and 0.8 % agar (Himedia, Mumbai). The pH of the medium was adjusted to 5.8 with 0.1 NaOH before autoclaving at 15 psi and 121°C for 20 min. The cultures were incubated at a constant temperature of 25±2°C with 16 h photoperiod (2000 lux). Subcultures were done every 28 days interval. Nodal segments from the proliferated shoots were subcultured again for further multiple shoot induction.

2.2. Establishment of liquid shoot culture

Shoot tips of multiple shoot culture of *S. rebaudiana* on MS medium [31] supplemented with BAP (1.5mg/l) and IAA (0.5mg/l) were inoculated into liquid medium of the same composition, but excluding agar. Static liquid shoot culture was initiated by placing shoot tips into 250 ml conical flasks containing 25 ml of MS liquid multiplication medium. The liquid medium was adjusted to pH 5.7 before being sterilized by autoclaving at 15 psi and 121°C for 15 min. All cultures were kept at 25±2°C under a 16 h photoperiod provided by cool white fluorescent lamps. The shoots from static liquid culture were further subcultured every three weeks and used in the experiments described in the work.

2.3. Rooting and transplantation

Shoots that developed in liquid multiplication medium were transferred individually into half strength MS agar (0.7%) medium supplemented with IAA (1.0 mg/l).The shoots were maintained for 4 weeks under the same culture condition as for development of roots. After this time, the percentage of rooted shoots was recorded. Rooted plantlets were transplanted to pots filled with sterilized mixture of sand, vermicompost and soil (1:1:1) and grown for 5 weeks in greenhouse conditions to determine the percentage of plants that survived.

3. Results and Discussion

Shoot tips of *S. rebaudiana* were incubated in liquid MS medium supplemented with BAP (1.5mg/l) and IAA (0.5mg/l). The liquid cultures under static conditions were used to determine the effect of liquid media on *S. rebaudiana* shoot proliferation. Our experiment showed that static liquid culture was effective in growth and multiplication of stevia shoots tips with callus. The shoots were partially submerged in liquid medium and no hyperhydricity symptoms were observed, when the time spent by shoots in multiplication medium took no longer than 3 weeks. At this time, on average, 37 shoots per explant were formed and the multiplication rate in the following ten subcultures under the described conditions remained at the same level. Also on the agar-gelled medium with the same growth regulators, stevia responded with average number of 3-11 shoots per explant, but the time required for the shoot induction was longer (5 weeks) [32-35].

Table 1. Effect of BAP, Kn and IAA of growth regulators for shoot proliferation, Table 2: Effect of BAP and IAA in MS liquid medium for shoot multiplication, Table 3: Effect of IAA, IBA and NAA in half-strength MS medium for root formation

Growth regulators (mg/l)			Shoot formation (%)	No of total shoot per culture Mean±S.E.	Average length of shoot per culture (cm) Mean±S.E.
BAP	Kn	IAA			
0.5	-	-	25	3.7±0.3	3.8±0.4
1.0	-	-	52	8.2±0.4	4.7±0.2
1.5	0.5	-	89	11.5±0.3	6.5±0.6
2.0	0.1	-	70	7.6±0.2	5.7±0.4
2.5	-	-	56	5.3±0.2	4.8±0.2
3.0	-	-	45	4.8±0.5	4.2±0.4
5.0	-	-	32	4.2±0.4	3.7±0.3
-	1.0	0.2	48	4.0±0.3	5.6±0.1
-	2.0	0.2	57	6.7±0.2	7.2±0.6
-	5.0	0.2	35	2.8±0.3	6.3±0.3
1.5	-	0.5	67	7.3±0.2	7.8±0.1
2.0	-	0.2	48	5.6±0.4	6.5±0.2

Growth regulators (mg/l)		Shoot formation (%)	No of total shoot per culture Mean±S.E.	Average length of shoot per culture (cm) Mean±S.E.
BAP	IAA			
0.5	0.2	67	14±0.2	4.6±0.3
1.0	0.2	84	25±0.2	4.5±0.2
1.5	0.5	93	37±0.5	5.2±0.4
2.0	0.5	80	25±0.3	5.0±0.5
2.5	0.2	72	19±0.4	4.3±0.2
3.0	0.2	65	12±0.2	4.0±0.3

Growth regulators (mg/l)	Concentration (mg/l)	Root formation (%)	No. of total roots/culture Mean±S.E.	Average length of roots/ culture (cm) Mean±S.E.
IAA	0.2	38	2.7± 0.5	3.2 ± 0.6
	0.5	47	3.4± 0.3	4.5 ± 0.2
	1.0	63	7.5± 0.4	5.4 ± 0.3
	2.0	40	4.6± 0.2	4.0 ± 0.2
IBA	0.2	27	2.1± 0.6	2.8 ± 0.3
	0.5	44	2.8 ± 0.7	3.2 ± 0.4
	1.0	50	4.7 ± 0.4	4.9 ± 0.3
	2.0	23	3.0 ± 0.2	3.2 ± 0.2
NAA	0.2	32	2.4± 0.2	2.7 ± 0.5
	0.5	48	3.2 ± 0.3	3.3 ± 0.3
	1.0	58	5.0 ± 0.3	4.7 ± 0.2
	2.0	27	3.4 ± 0.4	3.1 ± 0.3

With respect to morphology, stevia shoots grown in liquid medium were characterized by higher number of leaves, larger leaf areas and more number of multiple shoots were formed than compared to those cultured on the agar-solidified medium and a decrease in the average shoot length in the liquid

medium was observed. The average length of these shoots was 5.2 cm (table 2), as against 7.8 (table 1) cm in agar culture. Moreover, shoots from liquid culture were difficult to root. Only 63 % (table 3) of the shoots formed roots within 4 weeks on ½ MS agar medium supplemented with IAA (1.0 mg/l), whereas the percentage of shoot multiplied on agar solidified medium was 89%. As already mentioned, in our study, application of liquid medium was associated with decreased shoot length, and the effect can probably be responsible for the reduced rooting of the shoots. On inhibition of rooting of shoots, which were multiplied in liquid culture has been also reported for *Centaurium erythraea*, *Salvia officinalis* and *Curcuma longa* [33-36].

When stevia plantlets were transferred into the soil and grown in the greenhouse no differences were observed in growth and morphology between the plants coming from the liquid media during multiplication stage and those multiplied on the solid medium. Survival rate of the plantlets was above 80%.

Figure: Micropropagation of *S. rebaudiana* (a) Direct regeneration of shoots (b) Initiation of multiple shoot formation (c) Induction of multiple shoot formation in liquid medium (d) Root regeneration (e) Hardening of regenerated plantlets

In vitro rooted shoots were kept under normal growth room conditions for 4 weeks until the induced roots became partially brown. The shoots were then taken out from the growth room, kept under room temperature for 15 days and then taken out from the culture tubes carefully and the medium attached to the roots was gently washed out with running tap water. The plantlets were transplanted to pots filled with sterilized mixture of sand, vermicompost and soil (1:1:1) treated with 0.1% Agrason (fungicide). The transplanted plantlets were kept under shade for 15 days and then were transferred to normal environmental conditions. Through this process of acclimatization almost 77% survival was achieved. By using the method described above, hundreds of clonal plants can be produced from one nodal explant by continuous subculturing of shoot propagules. The multiplication rate was achieved significantly large by using liquid medium.

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