Bionature, 30 (2), 2010 : 89-95

AN EFFICIENT *IN VITRO* MULTIPLE SHOOTING METHOD OF *STEVIA REBAUDIANA* BERTONI.

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ABSTRACT

The small miracle plant *Stevia rebaudiana* Bert. is commercially important due to presence of a natural sweetner, stevioside (diterpene glycoside), a potent alternative to sucrose. Poor seed germinability limits its reproduction to only vegetative propagation by stem cuttings. This makes micropopagation most reliable method. Axenically grown cotyledonary leaves produce profuse calli (10mg/lit NAA-0.5mg/lit Kinetin), which subsequently develop microshoots. Vigorous microshooting (max 72.65, microshoots/explant) was noticed at moderate concentrations of Kinetin and BA (Kinetin 3mg/lit- 5mg/lit, BA 0.5mg/lit-3mg/lit) with maximum shoot length in minimum shoot initiation time. Most of such microshoots rooted efficiently (20 roots /shoot; 4cm root length) within 5-6 days of culture at high IBA concentrations (5mg/lit- 10mg/lit). Such efficient microshoots are being tested for their acclimatization and field establishment potential through routine hardening procedures. The objective of the study is to develop an elite line (microshoots) with high regeneration, good field transfer potential with sustained stevioside production.

Key words: Stevia, Micropropagation, Multiple shooting

Introduction

Stevia (*Stevia rebaudiana* Bertoni.), a semi-bushy perennial herb belongs to the family Asteraceae. The plant is also known as "Sweet leaf (in USA)", "Sweet honey leaf (in Australia)", "Sweet herb of Paraguay", which is estimated to be 300 times sweeter than sucrose (Kinghorn, 1987). *Stevia* is native to eastern Paraguay & have been used as a sweetener for many years. Guarani tribes of Paraguay and Brazil used *Stevia rebaudiana* as a sweetener and source of medicine. *Stevia* has been introduced as a crop in a number of countries including Japan, China, Korea, Mexico, United States, UK, Indonesia, Canada and all over South America (Lee *et al.* 1979; Donalisio *et al.* 1982; Brandle *et al.* 1992.).

Stevioside, a diterpene glycoside forms the largest part of sweetener molecules present in the leaves. Other compounds present but in lower concentration are steviolbioside 2, rebaudioside A4, B5, C6, D7, E8, F9 and dulcoside A 10 (Kennelly, 2002; Starrat *et al.* 2002). Stevioside is highly recommended for diabetes, hypoglycemia,

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high blood pressure & obesity because it remains unmetabolized. It has been extensively tested on animals and humans with no side effects (Megaji *et al..,* 2005).

Progress towards large scale commercialization has been slow, largely due to difficulties in cultivating the crop. Poor germinability of seeds does not allow it as a means of propagation and vegetative propagation by stem cuttings, is limited due to low availability of plantlets. Therefore, microprogation is the most reliable and suitable method for propagation of *Stevia* (Darekar, 2004). The present attempt has been made to identify the suitable method for production of large number of microshoots within minimum time span. The focus of the paper is to develop an easy micropropagation protocol with high reproducible efficiency, for this medicinally important crop.

Material and Methods

Seeds of *Stevia rebaudiana were* collected from the nursery of Govt. Cinchona Factory located at Mungpoo (altitude 5000ft), Darjeeling, West Bengal. Axenically grown cotyledon leaves were used as explants in the experiment. For this, seeds were surface sterilized with 0.1%(w/v) mercuric chloride and then soaked in distilled water for 2-3 hrs. Soaked seeds were then kept in filter paper inside the petri-plates for germination. Cotyledonary leaves were then thoroughly washed with sterile distilled water followed by surface sterilization with 0.1% mercuric chloride for 2-3 minutes. After surface sterilization, the explants were rinsed three times in sterile double distilled water.

Full MS (Murashige & Skoog, 1962) medium supplemented with plant growth regulators (PGR) like 1- Napthеleneacetic acid (NAA) in combination with 6-Benzyladenine (BA) or Kinetin (Kn) and MS with 6-Benzyladenine (BA) or Kinetin (Kn) singly or in combination with each other at various concentrations were prepared for shoot initiation. Subsequent rooting of the multiple shoots was carried out in MS supplemented with Indole-3-butyric acid (IBA).

The culture medium contained 3% sucrose (w/v) and it was gelled with 1.0% (w/v) agar-agar. The pH of the media was adjusted to 5.6-5.9 by 0.1 (N) NaOH. The gelled medium was dispensed into culture tubes, plugged with non-absorbent cotton and then autoclaved at 121°C for 15-20 minutes under 15 lb/ square inch pressure. The culture tubes were kept in the culture room illuminated with white fluorescent light (2000 lux) under 16/8 hr light/dark condition. Subculturing was made routinely after every four weeks of inoculation. After transfer of microshoots into rooting medium, the basal segments were further subcultured in a fresh medium (with same PGR combination) for next cycle of shoot proliferation to maximize number of microshoots.

Results and Discussion

Callusing and shoot induction-

Cotyledonary leaves placed in MS media with NAA-Kinetin combination showed profuse callusing. Higher concentrations of NAA (above 5.0 mg/lit) & lower concentrations of Kinetin (0.5 - 3.0 mg/lit) produced better callusing. Among other concentrations tested, only 10.00 mg/lit NAA-0.5 mg/lit Kinetin generated

profuse white, friable & regenerating calli, which subsequently sprouted microshoots (Fig C & D). Moreover, a high concentration of NAA, beyond 10mg/lit, stimulated rhizogenesis exclusively, from the calli. (Table 1). Generally, NAA-BA combination supported only callusing. Both lower to moderate concentrations of NAA-BA (0.5-5.0mg/lit) showed best callogenesis, out of which the combinations of 5.0 mg/lit BA -1.0 mg/lit NAA & 5.0 mg/lit BA- 3.0 mg/lit NAA triggered remarkable callusing. Contrarily, a high concentration range of BA-NAA (10 mg/lit BA with 5-10 mg/lit NAA) responded poorly. It was also noticed that MS with only BA showed direct organogenesis (shooting) from explants, as evidenced by emergence of microshoots at 0.5 - 3.0 mg/lit BA. Particularly BA at 1.0 mg/lit enhanced no. of shoots/explant (52.30), reduced the time to emergence of shoots (3-3.5 Weeks) and increased shoots length (2.16 cm) considerably (Table 2). Kinetin (0.5 mg/lit - 10.00 mg/lit) too, showed direct shooting from explants and specifically 5.0 mg/lit Kinetin responded best [number of shoots (62.30), time to emergence of shoots (2.5-3 Weeks) and length of shoots (2.73cm)] (Table 2). Kinetin-BA combination proved to be better in comparison to when they were used singly. The different combinations of Kinetin-BA (5.0 mg/lit Kinetin with three different BA levels 0.5, 1.0, 3.0 mg/lit) responded best among other

TABLE 1. INDUCTION OF CALLOGENESIS & ORGANOGENESIS OF *STEVIA REBAUDIANA* IN MS MEDIUM WITH DIFFERENT PGR COMBINATIONS.

Growth Regulators (mg/lit)	Callusing Response	Nature of Organogenesis
Kinetin 0.5 + NAA 0.5	+ +	-
Kinetin 0.5 + NAA 1.0	+ +	-
Kinetin 0.5 + NAA 3.0	+ +	-
Kinetin 0.5 + NAA 5.0	+ + +	-
Kinetin 0.5 + NAA 10.00	+ + + +	Only shooting
Kinetin 0.5 + NAA 12.00	+ + + +	Only rooting
Kinetin 0.5 + NAA 15.00	+ + + +	Only rooting
Kinetin 0.5 + NAA 20.00	+ + + +	Only rooting
Kinetin 1.0 + NAA 0.5	+ +	-
Kinetin 1.0 + NAA 1.0	+ +	-
Kinetin 1.0 + NAA 3.0	+ +	-
Kinetin 3.0 + NAA 1.0	+ +	-
BA 0.5 + NAA 0.5	+ +	-
BA 0.5 + NAA 1.0	+ +	-
BA 0.5 + NAA 3.0	+ +	-
BA 1.0 + NAA 0.5	+ +	-
BA 1.0 + NAA 1.0	+ +	-
BA 3.0 + NAA 0.5	+ +	-
BA 3.0 + NAA 1.0	+ +	-
BA 3.0 + NAA 3.0	+	-
BA 5.0 + NAA 0.5	+ +	-
BA 5.0 + NAA 1.0	+ + +	-
BA 5.0 + NAA 3.0	+ + +	-
BA 5.0 + NAA 5.0	+ +	-

+ Compact slow growing callus, + + green Fast growing callus, + + + white friable callus, + + + + friable callus & regeneration, - No organogenesis. Data had taken after 4 weeks of inoculation.

TABLE — 2 REGENERATION OF MULTIPLE SHOOTS OF *STEVIA REBAUDIANA* IN MS SUPPLEMENTED WITH BA & KINETIN (ALONE OR IN COMBINATION).

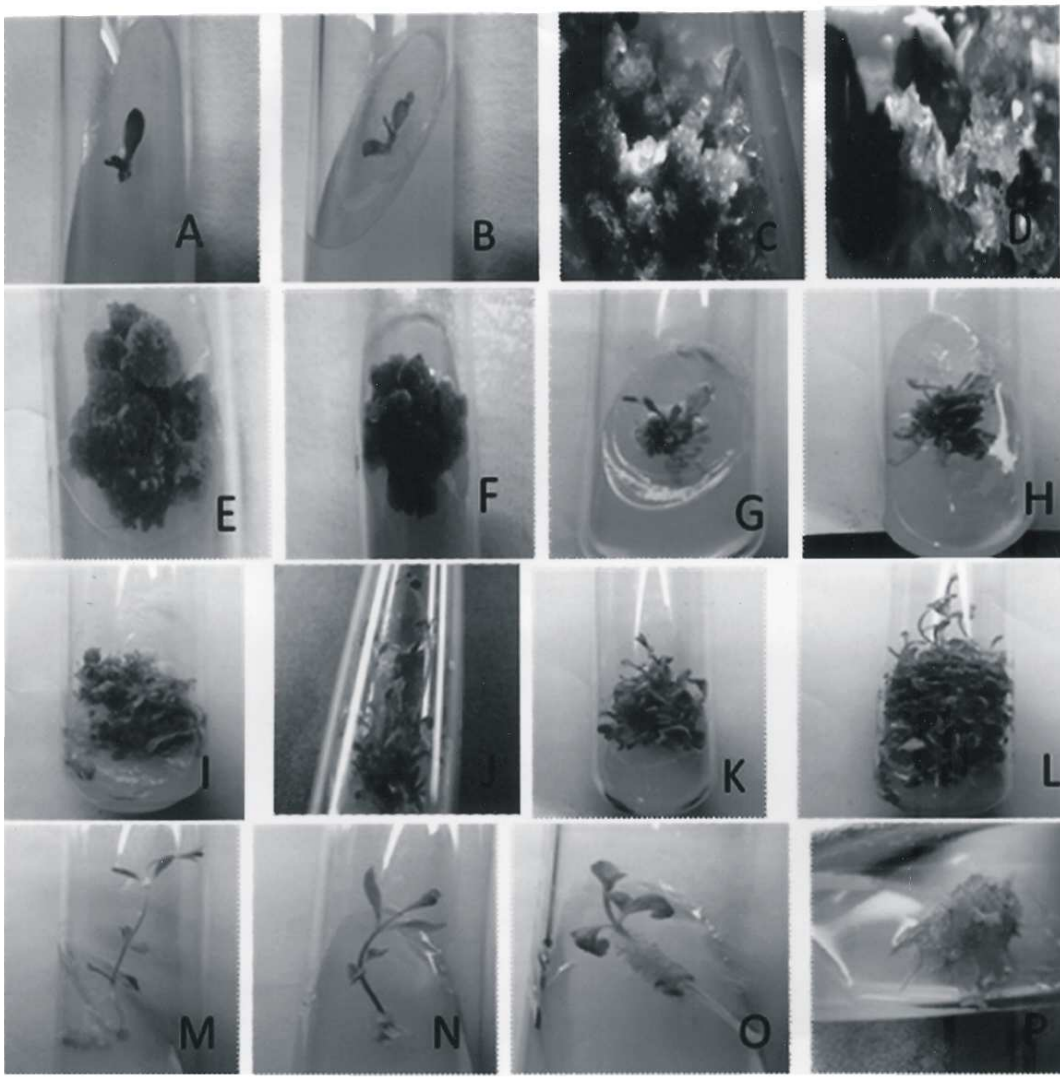
Growth regulators (mg/lit)	No. of shoots / explants*	Time to emergence of shoots (week)	Length of shoots*(cm)
BA 0.5	39.33 ± 1.67	3-3.5 weeks	1.93cm ± 0.09
BA 1.0	52.30 ± 0.9	3-3.5 weeks	2.16 cm ± 0.15
BA 3.0	43.66 ± 1.55	3-3.5 weeks	2.03 cm ± 0.04
BA 5.0	-	-	-
BA 10.00	-	-	-
Kinetin 0.5	46.62 ± 0.87	3-3.5 weeks	2.26 cm ± 0.18
Kinetin 1.0	47.66 ± 1.88	3-3.5 weeks	2.33 cm ± 0.17
Kinetin 3.0	57.60 ± 1.13	3-3.5 weeks	2.30 cm ± 0.2
Kinetin 5.0	62.30 ± 0.9	2.5-3 weeks	2.73 cm ± 0.24
Kinetin 10.00	58.36 ± 1.12	2.5-3 weeks	2.63 cm ± 0.24
Kinetin 0.5+BA 1.0	45.33 ± 1.11	2.5-3 weeks	2.23 cm ± 0.17
Kinetin 1.0+BA 1.0	53.63 ± 0.87	2.5-3 weeks	2.33 cm ± 0.17
Kinetin 3.0+BA 3.0	59.60 ± 1.13	2.5-3 weeks	2.66 cm ± 0.2
Kinetin 5.0+BA 0.5	**72.65 ± 1.11**	**2-2.5 weeks**	**3.2 cm ± 0.13#**
Kinetin 5.0+BA 1.0	68.32 ± 1.56	2-2.5 weeks	3.03cm ± 0.15
Kinetin 5.0+BA 3.0	62.30 ± 1.9	2-2.5 weeks	2.93 cm ± 0.17

* The values of shoot number and shoot length represent mean ± mean deviations.
\# Best result

TABLE — 3 ROOTING RESPONSE OF THE MULTIPLE SHOOTS OF *STEVIA REBAUDIANA* IN MS MEDIUM CONTAINING IBA. INITIAL SHOOT LENGTH 2.5 - 3.0 CM, ROOT TYPE - TAP ROOT, SHOOTS ARE THREE WEEKS OLD.

IBA (mg/lt)	Plan t height* (cm) explants*	No. of roots/shoot*	Days to emergence of root (weeks)	Length of root(cm)*	Callusing under shoot base
0.25	16.34 ± 0.88	7.3 ± 0.56	8-9	3.43 ± 0.37	+
0.5	18.23 ± 0.51	7.3 ± 0.56	6-8	3.23 ± 0.17	+
1.0	21.3 ± 0.43	15.66 ± 0.88	5-7	3.86 ± 0.11	+
3.0	14.41± 0.38	17.60 ± 1.13	5-7	3.96 ± 0.15	+ +
5.0	10.26 ± 0.51	19.66 ± 0.88	5-6	4.25 ± 0.16	+ +
10.00	7.6 ± 0.26	21 ± 0.66	5-6	4.66 ± 0.18	+ + +

\+ Scanty callus, + + moderate callus, + + + profuse callus.
*The values of plant height, root number & root length represent mean with ± mean deviations



Callogenesis, multiple shooting & rooting of *Stevia rebaudiana* Bertoni

Fig. A & B	Regenerating explants in MS medium
Fig. C & D	Profuse friable and regenerationg calli in MS medium containing Kinetin (0.5mg/lit) + NAA (10.00 mg/lit)
Fig. E & F	Callusing in MS medium containing BA (5.0 mg/lit) + NAA (3.0 mg/lit)
Fig. G & H	Induction of multiple shoots in MS medium with BA (1.0 mg/lit).
Fig. I & J	Shoot multiplication in MS medium with Kinetin (5.0 mg/lit).
Fig. K & L	Regeneration of multiple shoots in MS medium with Linetin (5.0 mg/lit) /+ BA (0.5 mg/lit).
Fig. M & N	Rooting of microshoot in MS medium with IBA (3.0 mg/lit).
Fig. O	Callusing at microshoot base during rooting in MS medium with IBA (5.0 mg/lit).
Fig. P	Profuse call using during rooting in MS medium with IBA (10.0 mg/lit).

concentrations tested so far. Amongst them, a tremendous regeneration of microshoots [number of microshoots (72.65); reduced time to emergence of shoots (2-2.5 weeks) and increased length of shoots (3.2cm)] was noticed directly from cotyledonary explants at Kinetin 5.0-BAP 0.5 mg/lit combination. This remarkable microshoot production indicates very high meritematic activity coupled with sustained cyto-differention.

Root induction - As a routine, Microshoots were further tested for their rooting potential in IBA containing MS. Profuse rooting was visible from microshoots regenerated previously in MS with Kinetin-BA. A range of 0.25 mg/lit - 10.00 mg/lit IBA used for rooting experiment. It has been noticed that number of roots per microshoots increased with higher concentration of IBA (in 0.25 mg/lit IBA no. of roots /shoot is 7.3 and at 5.0mg/lit IBA the ratio increases to 19.66). It has also been noticed that amount of the IBA-induced characteristic callusing from microshoot base has gradually increased with higher concentration of IBA (above 5mg/lit). It has been found that, at 10.00 mg/lit IBA concentration, the amount of callusing from microshoot base becomes too high and stimulated only roots from regenerating calli. On an average, a moderate concentration of, IBA-treated (0.5-5.0 mg/lit) microshoots showed good response in number of roots, days to emergence of roots, length of roots and importantly amount of callusing from shoot base. This is of particular impotance to us because such juvenile plantlets become the best candidate for hardening and field transfer (Table 3). The present paper demonstrates a simple protocol for micropropagation of *Stevia rebaubidiana*, which could repeatedly generate very high number of microshoots with good regeneration potential.

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