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Somatic embryogenesis in *Stevia rebaudiana* Bertoni using different concentration of growth hormones

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SUMMARY

In vitro propagation of *Stevia rebaudiana* through somatic embryogenesis was successfully achieved from axillary buds using nodal and leaf as explants in basal medium (MS) with vitamins, sucrose (30g/l), agar (0.9% w/v) and supplemented with 2, 4- D (2.0 mg/l) + BAP (0.2mg/l) + TDZ (0.2mg/l). These conditions yielded friable callus cultures. Callus sub cultured on medium with reduced concentration of 2, 4- D (1.0 mg/l) became embryogenic. Organogenesis of embryonic callus was then achieved by eliminating the agar and modulating the mediums with hormones BAP (1.0 mg/l) + IBA (0.5mg/l) and increasing concentration of sucrose concentration up to 40g/l for 1 week followed by transfer of mature embryonic callus to ½ strength MS medium containing IBA (1.5 mg/l) were 83% of embryos developed into micro shoots. Through sequential hardening process, well rooted plantlets with survival rate (95%) were established in the field.

Key words : *In vitro,* Somatic embryogenesis, *Stevia rebaudiana*, Growth hormones, Organogenesis

Stevia, one of the 950 genera of the *Asteraceae* family is a genus of more than 200 species family, it grows up to 1 meter with an extensive root system and brittle stem producing small, elliptic leaves. Members of *Stevia* comprise mostly of herbs but also shrubs and trees. Originally it is said to be native to subtropical South America (Paraguay and Brazil) (Soejarto *et al.*, 1982). The Guarani Indians of Paraguay were the first to exploit this sweetener for mate tea. It has been cultivated domestically in continental China, Taiwan, Thailand, Korea, Brazil and Malaysia (Brandle and Rosa, 1992, Fors, 1995). Pure extract stevioside is non–caloric and 300 times sweeter than sugar with a delicious and refreshing taste (Bhosle, 2004). The other attributes of this natural, high intensity sweetener include non-fermentable, non-discoloring, maintain heat stability at 100° C and features a lengthy shelf life. The leaves are the source of the diterpene glucoside vis. stevioside, rebaudioside A and C, and along with it also contain rebaudioside A and C, dulcoside. Various studies have found the leafs to contain protein, fibers, carbohydrates, iron, phosphorous, calcium, potassium, sodium, magnesium, zinc, rutin (flavonoid), true vitamin A, vitamin C and an oil which contain 53 other contitutents. In addition to its sweetening property it has therapeutic values such as antihyperglycemic, anticanerous (Jeppensen *et al.*, 2002, 2003), antihypersensitive agent (Chan *et al.*, 1998) contraceptive (Melis, 1999) and prevention of dental caries (Fujita H *et al.*, 1979). *Stevia* can also inhibit bacterial and fungal growth (Cerda-Gercia-Rojas and Pereda Miranda, 2002).

The main problem in cultivation of these plants is that they are heterozygous and self- incompatibility leads to low germination percentage and with that vegetative propagation too is limited by the lower number of individuals that can be obtained simultaneously from a single plant (Sakaguchi and Kan, 1982). To overcome all these, multiplication and improvement of this medicinal plant though tissue culture may be an alternative for rapid mass propagation of *Stevia*. Somatic embryogenesis and organogenesis have been the common pathways for clonal propagation of superior medicinal plant species. Somatic embryogenesis enables large numbers of plantlets to be produced within a short span of time.

MATERIALS AND METHODS

The methods of plant tissue culture were the standard method as described in plant cell, tissue and organ culture fundamental methods (Gamborg and Phillips, 2004).

Plant material and sterilization:

The leaves were excised from both mature field grown of *Stevia rebaudiana* plant collected from "Swariya Musli" farm, and controlled cultured *in vitro* plants. The leaf disc (15mm diameter) were cut into small pieces and were washed thoroughly under running tap water, then soaked in labolene (a commercial neutral detergent Quligens, India) (5% v/v) for 5 minutes. After

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washing in double distilled water, the segments were surface sterilized using 0.1% (w/v) mercuric chloride solution for 2-3 minutes.

Culture media and condition:

The sterilized segments were thoroughly washed 3 times (5 times each) with sterile double distilled water. Sterilized segments cut into appropriate size (0.5-1 cm) were cultured on sterile MS (Murashige and Skoog, 1962) medium fortified with 2, 4 –D, BAP and TDZ alone or in combination. Calli developed on MS medium supplemented with reduced concentration of 2, 4 –D. Efficacy of growth hormone for initiation of organogenesis in embryonic callus was then tested using BAP. For maturation, half- strength MS agar were fortified IBA was tested effective. All media were supplemented with sucrose 30g/l, and 0.8% agar. The pH of the medium was adjusted between 5.6-5.8 before the addition of agar. The media were sterilized by autoclaving at a pressure of 1.06 kg cm^{-2} (121^0C) for 20 minutes. Solid medium cultures were incubated either in 16/8 h photo period (25µmol m^{-2} s^{-1}) or in darkness. All cultures were incubated at 25±2^0C. Fresh weight of callus was recorded by first removing the agar particle and blot drying the callus (using filter paper) and then weighing using an electronic balance. Twenty cultures were raised for each treatment, and the best treatments were repeated twice.

Transplantation and statistical analysis:

Healthy and well developed plantlets with 14-15 cm long were removed from the culture vessels and washed thoroughly in distilled water, they were then transferred to plastic small bags containing a mixture of sterile soil, sand and vermiculite in ratio (1:1:1v/v) and were kept in green house for 15 days before transferring to field. The plantlets were watered at every 2 days intervals and were kept at net house. Data with regarded to frequency of multiple shoot formation, number and height of shoots (mean ± SD) was recorded after 30 days of culture.

The analysis consists of mean values and standard error that are given in table's mean were compared using students t- test at α = 0.001 and 0.05.

RESULTS AND DISCUSSION

In this study we focused on production of embryogenic cultures, which were proliferated, friable, rapidly growing and regenerable. Among leaf and nodal sections tested, leaf bases were responsive to embryogenesis and or/ organogenesis. Other sections turned brown an eventually died (data not shown).

Callus induction:

Leaf explants induced callus on MS medium fortified with growth regulators 2, 4-D, NAA, IBA, TDZ alone or in combinations. On growth free MS medium, the sub cultured calluses derived from any source turned white and eventually necrotic. The amount of somatic embrogenic callus initiation was highest 96% in combination with MS+2, 4-D (2.0mg/l) +BAP (0.2mg/l) +TDZ (0.2mg/l) with number of somatic embryos 40.

Table 1 : Effect of growth hormone on induction of somatic embryogenic callus after 4 weeks in *Stevia rebaudiana* Bertoni

Sr. No.	MS+Growth hormone(mg/l)	Somatic embryogenic callus initiation(%)	No. of somatic embryos
1.	Control	0	-
2.	2,4-D(0.5mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	76%	28
3.	2,4-D(1.0mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	85%	30
4.	2,4-D(1.5mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	90%	36
5.	2,4-D(2.0mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	96%	40
6.	IBA(0.5mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	55%	12
7.	IBA(1.0mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	69%	18
8.	IBA(1.5mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	77%	20
9.	IBA(2.0mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	60%	8-9
10.	NAA(0.5mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	30%	2
11.	NAA(1.0mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	47%	5
12.	NAA(1.5mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	55%	3
13.	NAA(2.0mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)	33%	-

* Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks.

Note-

-As BAP and TDZ showed effective growth among all concentrations (data not shown).

BAP (0.2mg/l) + TDZ(0.2mg/l) in this experiment kept constant.

-Callus growth and maintainace in 2, 4-D alone for 10-15 days.

Table 2 : Efficacy of Growth hormone and increase sucrose concentration on maturation and initiation of organogenesis of somatic embryos from 3-4 weeks old callus derived from leave explants

Sr. No.	MS + Growth hormone(mg/l)	Number of shoots/callus	Shoot length(cm)	(%)Calli forming shoot
1.	Control	0.1±0.01	-	-
2.	BAP(0.5mg/l)+IBA(0.2mg/l)	16.4±0.4	4.0±.0.33	77%
3.	BAP(1.0mg/l)+IBA(0.5mg/l)	20.8±0.2	5.1±0.39	90%
4.	BAP(1.5mg/l)+IBA(1.0mg/l)	18.7±0.7	4.5±0.30	88%
5.	BAP(2.0mg/l)+IBA(1.5mg/l)	13.0±0.3	3.6±0.28	65%
6.	Kn(0.5mg/l)+IBA(0.2mg/l)	2.2±0.4	0.7±0.13	13%
7.	Kn(1.0mg/l)+IBA(0.5mg/l)	3.1±0.6	1.3±0.17	22%
8.	Kn (1.5mg/l)+IBA(1.0mg/l)	3.9±0.9	2.2±0.41	29%
9.	Kn (2.0mg/l)+IBA(1.5mg/l)	3.4±0.3	1.7±0.13	23%

* Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks.

(Table 1). This combination showed friable proliferated callus with greenish yellow colored, smooth and compact which produced somatic embryos. The cells were generally, small and with higher amount of cytoplasm and starch grains. Only the embryogenic compact region was selectively removed and transferred /sub-cultured and maintained in fresh culture. Increased BAP resulted in green and hard callus. Auxin plays vital role in callus induction specially 2, 4-D. In this present experiment 2, 4-D was mainly stressed. The potential of 2, 4-D as the most efficient growth regulator has well documented in several plant species (Choi *et al.,* 2002, Kumar *et al*., 2002, Vikrant and Rashid 2002, Martin 2003) experiments showed the role of 2, 4-D in callusing. Callus induced on MS fortified with 2.0 mg/l 2, 4-D was found to be optimal for induction, maturation and conservation of embryos similar promoting effect in *Hemidesmus indicus* (Sarassan *et al.,* 1994), *Eleutherococus sessiliflorus* (Choi *et al.,* 2002) and Cuminum cyminum (Tawfik and Noga 2002). TDZ a substituted urea with both cytokinin activity and auxin activity (Mok *et al.,* 1982, Visser *et al.,* 1992, Huetteman and Preece, 1993, Murthy *et al.,* 1998).

Callus growth and maintainace:

Sub-culturing of profused calli was grown and maintained on medium with reduced concentration of 2, 4-D (1.0 mg/l) for 10-15 days. In baffalograss, stimulation of somatic embryogenesis has been achieved by stepwise decrease in 2, 4-D and addition of BAP during subculture or transfer.The addition of BAP was introduced in the next step.

Maturation and organogenesis:

The medium containing auxin alone and no cytokinin or cytokinin alone and no auxin yielded root or shoot cultures respectively. For differentiation of calli basal medium (MS) supplemented with MS+ BAP (1.0mg/l) + IBA (0.5mg/l) followed by increasing concentration of sucrose content up to 40 g/l. The mean number of shoots per callus 20.8±0.2 with shoots length 5.1±0.39 (Table 2). Embryo induction occurred at early stages while shoot regeneration occurred at later stages. Auxin induces callus formation and proliferation and somatic embryogenesis while cytokinin induces mostly shoot and root differentiation and elongation (De Klerke *et al.,* 1997, Guohua, 1998). The calli weight was noted 1425± 0.80.

As reported earlier, cytokinin when combined with auxin showed enhanced shooting. Initiation of shoot primodia was observed within 2-3 weeks of transfer, giving rise to shooting 90%. The addition of increased amount of sucrose also played vital role, with increased in weight, size and growth of callus.

Rooting and plant regeneration:

Supplementations of shoot medium with auxin were tested (IBA, NAA and IAA). IBA induced shoot cultures

Table 3 : Maturation of shoot primodia derived from embryonic callus in ½ strength MS medium

Sr. No.	½MS+Growth hormone(mg/l)	Shoot rooted (%)	No. of roots per shoots	Field survival rate (%)
1.	Control	-	-	-
2.	IBA (0.5 mg/l)	60%	2	70%
3.	IBA (1.0 mg/l)	78%	4-5	90%
4.	IBA (1.5 mg/l)	83%	8-9	95%
5.	IBA (2.0 mg/l)	70%	1-2	85%
6.	NAA(0.5 mg/l)	40%	-	-
7.	NAA(1.0 mg/l)	54%	2	44%
8.	NAA(1.5 mg/l)	73%	4	60%
9.	NAA(2.0 mg/l)	33%	-	-

* Values are mean ± standard error of three replicates with ten cultures per replicate; data scored after three weeks.

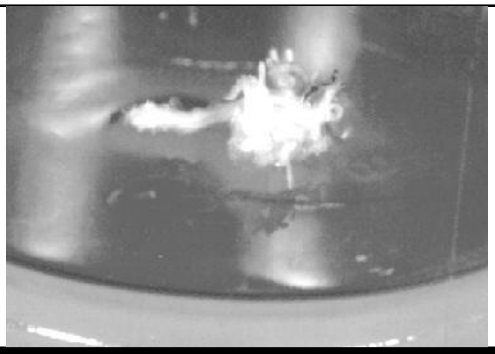

Fig. A : Induction of somatic embryogenic callus after 4 weeks 2,4-D (2.0mg/l)+BAP(0.2mg/l)+TDZ(0.2mg/l)

Fig. C : Maturation of shoot primodia IBA (1.5 mg/l)

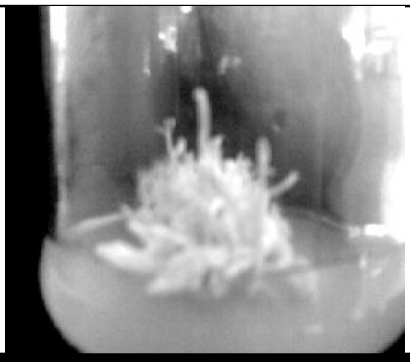

Fig. B : Maturation and initiation of organogenesis of somatic embryos from 3-4 weeks old callus BAP (1.0mg/l)+IBA(0.5mg/l)

Fig. D : Rooting

Fig. E : Acclimatiztion of *Stevia rebaudina Bertoni*

Fig. 1 : Somatic Embryogenesis of *Sievia rebaudiana Bertioni* whole cycle (A to E)

to grow roots; it possesses both shooting and rooting property. IBA was more suitable for root induction than IAA and NAA, when cultured on half –strength MS solid medium fortified with IBA (1.5 mg/l), each shoot primodia 83% per shoots developed an average of 8-9 roots within 3-4 weeks (Table 3). The effectiveness of IBA in rooting has been reported for medium plants like *Hemidesmus indicus* (Sreekumar *et al.,* 2000) and *Aloe polyphylla* (Abrie and von Staden 2001). Rooted shoots were transferred directly to plastic small bags containing a mixture of sterile soil, sand and vermiculite in ratio (1:1:1) and were kept in green house for 15 days before transferring to field. The plantlets were watered at every 2 days intervals and were kept at net house.

The present protocol describes here the micro propagation of *Stevia rebaudiana* Bertoni through callusing facilitates the rapid propagation of this valuable, medicinal plant.

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