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ORIGINAL RESEARCH ARTICLE

Studies on Micropropagation of *Stevia rebaudiana* Bert.

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ABSTRACT

Stevia rebaudiana Bert. can regenerate shoots when cultured on Murashige and Skoog (MS) medium supplemented with 6 - benzyladenine BA (8.87 µM and indole-3-acetic acid (5.71 µM). Rooting of the *in vitro* derived shoots could be achieved following subculture on to auxin-containing medium. A survival rate of 70% was recorded at the hardening phase on the substrate cocopeat. The presence of the sweet diterpeneglycosides, viz., stevioside and rebaudioside, was confirmed in the *in vitro* derived tissue of *stevia* using MS techniques, callus cultured on agar-solidified (MS)medium supplemented with (BA) (8.87 µM) and indole-3-butyric acid (9.80 µM) showed the highest sweetener content. In the initiation process the lower concentration of mercuric chloride treatments with lesser timings was found to be the best. High ratios of leaf, stem are desirable in cultivated *stevia* because of the low stevioside concentrations in stem culture. To get more multiplication with good shoot proliferation of *stevia*,the M_3 and M_4 combinations was found to be the best**.** *Stevia* is an obligate short day plant therefore enhanced vegetative growth under long day conditions is not surprising. To get erect, shoots with broader leaves the S1 combination was the best. The *stevia* production would be best situated in a long-day environment where vegetative period is longer and Steve oil glycoside yields will be higher. In order to germinate, embryos must have functional shoot and root apices capable of meristematic growth. Experiments are being performed in order to develop suitable media for maturation and germination of somatic embryos from *stevia* leaves. Plants can initiate flowering after a minimum of four true leaves have been produced.

Key wards: Microprapagation . *Stevia* . Tissue culture. *Invitro* propagation, Callus formation.

1. INTRODUCTION

Stevia (Stevia rebaudiana Bert.), the popular family member of Asteraceae, is a sweet, medicinal herb of Paraguay, consisting a non-caloric natural sugar, alternative to artificially produced sugar substitutes. The *stevia* leaf is 300 times sweeter than sugar (sucrose) obtained from sugar beet, sugarcane etc., with a zero-calorie value [17].The *stevia* leaves are the significant resource of diterpene glycoside, like rubsoside, steviolbioside dulcoside,rebaudiosides and stevioside [19]. These compounds, stevioside ranks top in dramatically accelerated use in health concerns related to dental cares, diabetes and obesity. The sweetness is due to stevioside, the most abundant glycoside [13]. The sweet compounds pass through the digestive process without chemically breaking down, making stevia safe for diabetic and obese people. Recently, *stevia* attained a better awareness owing to its superior sweetness and the curative values for restraining the diterpene glycosides of *stevia* are being used in human [5]. Additionally, the diterpene glycosides of *stevia* are being used in array of foods products and beverages. The extract from *stevia* leaves formally have been accepted as food supplement in a number of countries like Brazil, Korea and Japan [15]. The *stevia* leaves are treated as prospective source in the area of pharmacology and pharmacognosy [2].In Asia, Japan was the first to commercialize the stevioside as a sweetener in medicine industries. Later, the cultivation of *stevia* has been extended to quite a lot of countries in Asia, along with USA, Canada and several countries of Europe [4]. Though in recent times, *stevia* is being popularized in a number of countries, but large-scale propagation techniques are yet to be standardized to restore the quality and quantity of plants [9]. Propagation through seeds is very difficult due to self-incompatibility which results in sterile seeds. Hence in vitro propagation appears as an alternative technique for rapid multiplication of

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stevia within a short span of time [7]. Further *in vitro* propagation through direct organogenesis provides a rapid reliable system for a production of large number of genetically uniform disease free plantlets, irrespective of the season [11]. Although, earlier attempts have been made for the propagation of *stevia* through tissue culture [3,14,1,8]. But a considerable effort is still required to make it more practicable.

The present study has been undertaken to develop a novel protocol for *in vitro* accelerated mass multiplication with an efficient acclimatization procedure and to assess the clonal fidelity of *in vitro* generated propagules through biochemical and molecular markers [12,20].

2. MATERIALS AND METHODS

The present study was collected from Faculty of agriculture, Annamalai University, annamalainagar, Tamilnadu. The species (*Stevia rebardiana Bert.*) was identified with help of herbarium in botanical survey of India, Coimbatore. Micropropagation was done in Sun flow Biotech, Coimbatore, Tamilnadu.

2.1. Culture media

Major and minor nutrients were used according to the description of (16) supplemented with only sucrose (20 g/ L).$CaCL_2$(166.2 mg/ L) and 0.8 agar. The pH of the medium was adjusted between 5.6 – 5.8 before gelling with agar (0.6% W/V) and autoclaved at 121^0 C under 15 Ib pressure for 15 – 20 min.

2.2. Preparation of stock solution

1. The macronutrients are dissolved in 500ml double distilled water.
2. The micronutrients are dissolved in 250 ml of double distilled water.
3. I was dissolved in 250 ml of double distilled water.
4. The vitamins were dissolved in 100 ml of double distilled water.

Agar and sucrose were weighted as when added. But stock solutions were prepared and stored in refrigerator, stock solution of vitamins were prepared and stored in a deep freezer chamber.

2.3. Precautions in the preparation of media

1. All the stock solution was stored in glass bottle[s] under refrigeration.
2. Iron stock was stored only in an amber coloured bottle.
3. The bottle must thoroughly washed by double distilled water.
4. Details about the date and concentration of all stock solution were labeled with glass marking pen.

2.4. Growth regulators

The following growth hormones were supplemented to the media individual as well as combination at different concentrations as maintained below:

1. NAA and BAP at the concentration of 0.5 to 3.0 mg/L.
2. NAA and BAP combination at the concentration of 0.5 to 0.3 mg/L.
3. NAA + GA_3 and BAP + GA_3 combination at the concentration of each 0.5 mg/L.

2.5 *Sterilization of explants*

The shoot tip explants were washed thoroughly in running tap water for 30 minutes and placed in detergent solutions. After that the explants were washed thoroughly with double distilled water. Then the explants were transferred in front of laminar airflow and disinfected with 0.1 percent v/v aqueous mercuric chloride ($HgCl_2$) for a period of 8 minutes. Finally, they were rinsed with several changes of sterilized distilled water. The disinfected plantlets were taken for inoculation.

2.6. Inoculation

Solidified MS basal medium containing B5 vitamins, 0.8 % agar, 3 % sucrose and different combination of growth regulators as previously specified (0.5 to 3.0 mg/L) were used .The shoot tip were inoculated vertically on the MS medium containing different concentration of BAP (0.5 to 3.0 mg/L). Before, starting inoculation all the laboratory instrument such as media containing were transferred to laminar airflow chamber and the platform surface of the chamber was swapped with 70% alcohol after swapping the UV light switched on for 30 minutes. After that the UV light became switched “off” and “on” the ordinary light. Before inoculation hands were rinsed with 70 percent alcohol. Then the explants were inoculated on the medium. The instruments used aseptic manipulations were sterilized by dipping in 70 percent alcohol followed by flaming and cooling.The inoculations were carried out in vicinity of flame. The explants has been put in it and immediately covered with cotton plug. Each treatment represented three cultures tube. The cultures were kept under 16 hrs light/ day (2400 Lux) photoperiod at 25± 2^0C. The shoot regeneration was assessed after 4 weeks in culture length of 2.0 and above. The subsequent sub culture was made on the medium, which shared maximum shoot regeneration.

3. RESULTS AND DISCUSSION

This review summarizes the existing literature and provides a baseline for new research. During sterilization methods, out of eight treatments

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where sterilants were used, the $NaOCL_2$ was found to be fair compared to $HgCL_2$.The concentration of mercuric chloride (T_1 and T_2) the response percentage was 40% and 50% respectively and the dried percentage was also very high. In other wise lower concentration of mercuric chloride treatment with lesser timings found to be the best, in which T_4 out of 20 but sterilized 80% of the explants responded.

In $NaOCl_2$ treatments, lower concentration could not control the contamination and response percentage was also less. In higher concentration of 10% of $NaOCl_2$ for 15 minutes T_7 and 12 minutesT_8. The 20 and 10 percentage was respectively response, but mortality percentage was very high (**plate 1**). Effect of growth hormones and number of plants formed in the *stevia rebaudiana* Bert. was fourteen and concentration of media were tried to study the multiplication of plants. Different concentrations of cytokinins were tried with an auxin (NAA). The *stevia,* the lower concentration of benzyl aminopurine shows good growth, while in higher concentration the plants develop retardant (i.e. the growth was suppressed) by the more concentration of cytokinin [18]. But the crop plant likewise potato, banana, lily, onion and other normal plants, the concentration of cytokinin, especially benzyl aminopurine increases, the multiplication ratio (6:10). There was substantial increase from 0.5 mg to 1 mg BAP, but the ratio was same even with increase of 0.5 mg BAP. The ratio was come down or reduced further by increase of BAP. It was noticed that the multiplication ratio was good in M_1 and M_2 and it was increased further M_3 and M_4 where 0.5 mg BAP was increased. In M_7 and M_8 if ratio was worst and drastically come down than M_1 and M_2 and also the cultures started drying and the callus also was drying after 2 weeks. In 0.1 mg Kinetin the ratio 1:1.5, which was very low, but when it was increased gradually and the ratio was 1:2.5 in 2.5 mg kinetin. It was also observed that the increase of cytokinin concentration did not produce any callusing and stunted growth of plants. Different media viz., MS, B5,MS – B5 containing NAA and BAP (each 1.5 mg/L were tested for the influence of shoot regeneration. Among the two different conditions NAA and BAP showed maximum percentage of response, whereas NAA and kinetin regenerated maximum number of adventitious shoot along with MS – B5 medium. Some of the medium also supported the growth of healthy shoots with maximum mean shoot length while the other media produced lesser number of regenerated shoots in the order of MS and B5. The medium containing NAA and BAP was observed to induce shoot and root simultaneously whereas in the medium containing NAA and kinetin, shoot formation occurred first and it was followed by root formation. The roots are lesser in number and thicker than the plants of medium containing NAA + BAP medium. The shoot tip explants at lower level of BAP (0.5 mg/L) produced single shoot. Multiple shoot observed from 1.0 -2.0 mg/L BAP. Maximum response as well as healthy shoot was noticed at 1 mg/L BAP. Hence multiple shoot from 1 mg/L BAP containing medium were used for the present experiment. Medium containing NAA was equally good for embryogenesis. But the rate and percentage of differentiation was comparatively very lees. Moreover, the differentiated embryo had a tendency to from callus. Among different concentrations of NAA (0.5 – 3 mg/L). The different concentrations of IBA or IAA (0.5 mg/L) inhibited differentiation and suppressed the somatic embryogenesis. Somatic embryogenesis was induced if *in vitro* leaves of *stevia robaudiana* Bert. were cultured on high sucrose medium containing 2,4-D and BA somatic embryos were fist observed 15 days after initiation. The majority of the somatic embryos appeared to from directly without intermediate callus formation. Medium with 1 mM was superior to 5 mM of BA according to the number of somatic embryos induced (**Table 1**). The levels of 2, 4-D tested in this study induced similar numbers and percentage of somatic embryos (**Table 1 & plate 5**).In these work, high levels of sucrose have been used with success here and in various protocols for induction of somatic embryogenesis in other members of Asteraceae family. When the embryos were transferred to media without growth regulators on a lower concentration of sucrose (30 mg/L). They developed a root but failed to from the shoot apex. In order to germinate, embryos must have functional shoot and root apices capable of meristematic growth. More experiments are being performed in order to develop suitable media for maturation and germination of somatic embryos from *stevia* shoots.

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Table 1: Effect of $HgCl_2$ and $NaOCl_2$ on the number and percentage of buds formed during initiation of *Stevia rebaudiana* Bert.

Treatment code	Treatment	No. of buds inoculated	No. of buds responded	No. of buds contaminated and dried	Responding %	Remarks
T_1	0.1% $HgCl_2$:12 min	20	10	10	50	Fair
T_2	0.1% $HgCl_2$: 15 min	20	12	8	40	Fair
T_3	0.05% $HgCl_2$: 15min	20	6	14	70	Good
T_4	0.05% $HgCl_2$:12 min	20	4	16	80	Best
T_5	7.5% $HgCl_2$:15 min	20	10	10	50	Fair
T_6	7.5% $HgCl_2$:12 min	20	12	8	40	Fair
T_7	10% $HgCl_2$:15 min	20	16	4	20	Poor
T_8	12% $HgCl_2$:12 min	20	18	2	10	Poor

Table 2: Effect of growth hormones on the number and ratio of plants formed during multiplication of *Stevia rebaudiana* Bert.

Media Code No.	No. of plants inoculated	No. of plants multiplied	Multiplication ratio
M_1	20	46	1:2.3
M_2	20	42	1:2.1
M_3	20	68	1:3.4
M_4	20	38	1:1.4
M_5	20	58	1:2.9
M_6	20	60	1:3
M_7	20	42	1:2.1
M_8	20	56	1:2.8
M_9	20	66	1:3.3
M_{10}	20	50	1:2.5
M_{11}	20	30	1:1.5
M_{12}	20	38	1:1.9
M_{13}	20	48	1:2.4
M_{14}	20	64	1:3.2

Table 3: Effect of growth hormones on the numbers of roots and length of shoots formed during shooting and rooting stage in *Stevia rebaudiana* Bert.

Trial code	Media combination	No. of. Shoot inoculated	Length of shoots(Cm)	No. of Roots formed	Remarks
S1	½ MSB+0.02BAP+0.2mg IBA	20	6	5	Best
S2	½ MSB+0.01BAP+0.2mg IBA	20	5	3	Fair
S3	½ MSB+0.05BAP+0.1mg IBA	20	4	3	Fair
S4	½ MSB+0.05BAP+0.2mg IBA	20	4	4	Best
S5	½ MSB+0.05BAP+0.4mg IBA	20	4	6	Best
S6	½ MSB+0.02BAP+0.6mg IBA	20	4	Clusters	Poor
S7	½ MSB+0.02BAP+0.8mg IBA	20	4	Clusters	Poor
S8	½ MSB+0.02BAP+1.0mg IBA	20	4	Clusters	Poor

Plate-1:*Stevia* inoculation after one week initial period of explant

Plate-2: *Stevia* with slight growth after two week initial period of explants

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Plate-3:*Stevia* with shoot formation after six weeks interval

plate-4:*Stevia* multiplication after eight weeks interval

Plate-5:*Stevia* mother plant

4. CONCLUSION

In present investigation protocols for *In vitro* propagation of *Stevia rebaudiana* Bert.. Have successfully been standardized and changes in total soluble protein contents and peroxidase activity during different stages of callus growth have been estimated. As the sweet herb, is an important medicinal plant, it is becoming an endangered species due toits infertile and small sized seed. The methods of vegetative propagation are not efficientto save this rare plant. Therefore protocols developed in present investigation are not only useful for its mass scale propagation but also conservation of germplasm.

REFERENCES

1. Ahmed, M.B., M. Salahin, R. Karim, M.A. Razvy and M.M.Hannan *et al.,* .An efficient method for in vitro clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bert.) in bangladesh. Am. Eurasian *J. Sci.* Res., 2:(2007) 121-125.
2. Anbajhagan, m., M. Kalpana, R.Rajendran, V. Natarajan and D .Dhanavel, . *In vitro* production of *Stevia rebaudiana* Bert. Emir. *J. Food Agric.,* 22:(2010)216-222.
3. Bespalhokk, J.C., L.G.E. Vicira and J.M. Hashimoto, . Embryogenic callus formation and histological studies from *Stevia rebaudiana* Bert. Floret explants.rev. Bras. *Fisiol.,* 5:(1997)51-53.
4. Brandle,J.E., A.N.Starratt and M.Gijzen, . *Stevia rebaudiana Bert.*: Its agricultural,biological and chemical properties. Canad.*J.Plant Sci*., 78:(1998)527-536.
5. Chalapathi, M.V. and S.Thimmegowda, S.Sridhara, V.R.Ramakrishna Parama and T.G.Prasad, 1997. Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bert.) a future crop of India. *Crop Res*., 14:(1997)347-350.
6. Chattopadhyay, K.,S.Bhattacharyya, N.Mandal and H.K. Sarkar, . PCR-based characterization of mungbean (*Vigna radiata L.*) genotypes from Indian subcontinent at Intra and inter-specific level.*J.Plant Biochem. Biotec nol*., 17:(2008)141-148.
7. Das, K.,R. Dang, S. Khanam and P.E.Rajasekharan, .*In vicro* methods for production of steviosides from *stevia* .Ind.*J.Nat. Prod*.,21 :(2005)14-15.
8. Debnath, M., Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana.J.Med.Pl.Res*., 2:(2008) 45-51.
9. Dhananjay, S. and J. Deshpande, Commercial Cultivation of Medicinal and Aromatic Plants. *Himalaya Publishing House, New Delhi*, pp:(2005) 296-298.
10. Gantait,S., N.Mandal, S.Bhattacharyya and P.K.Das, *Invitro* Mass Multiplication with pure genetic identity in *Anthurium*

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andreanum Lind. *Plant Tissue Cult. Biotech*., 18:(2008)113-122

11. Gantait,S., N.Mandal, S.Bhattacharyya and P.K.Das, *Invitro* Mass Multiplication with genetic clonality in elephant garlic(*Allium ampeloprasum)*. *J. Crop Weed,* 5:(2009)100 -104.
12. George,S. and S.L. Sherrington, Tissue culture in forest trees: Clocal propagation of *Tectona grandis* L.(teak) by tissue culture. *Plant Sci.Lett.*, 17:(1984)259-268.
13. Kinghorn, A.D., Food Ingredient Safety Review : *Stevia rebautiana* Leaves. *Herbal Research Foundation, Boulder, CO USA*.(1992).
14. Kornilova, O.V. and E.A. Kalashnikova, Clonal micro propagation of Stevia (*Stevia rebaudiana* Bert.)*Cercetari Agrton Moldova*, 30:(1997)80-85.
15. Mizutani,K. and O.Tanaka, Use of *Stevia rebaudiana* Bert. Sweeteners in Japan. In: *Stevia*: The Genus *Stevia* (Medicinal and Aromatic- plants Industrial Profile), Kinghorn, A.D., (Eds.). *Taylor and Francis, London*, pp:(2002) 178-195.
16. Murashige, T. and F.Skoog, A revised medium for rapid growth and bioassay with *tobacco* tissue culture. *Physiol. Plant.,* 15:(1962) 473-497.
17. Richman, A.S.,M. Gijzen, A.N.Starralt, Z.Yang and J.E. Brandle, Diterpene synthesis in *Stevia rebardiana* Bert. recruitment and up-regulation of key enzymes from the gibberellin biosynthetic pathway. *Plant J.,* 19:(1999)411-421.
18. Sivaram, L. and U. Mukundan, *In vitro* culture on *Stevia rebaudiana* Bert.In Vitro Cell.Dev. *Biol. Plant*, 39:(2003) 520-523.
19. Starratt, A.N., C.W. Kirbyb, R.Pocsa and J.E. Brandle, Rebaudioside F, A diter pene glycoside from *Stevia rebaudiana* Bert. *Phytochemistry,* 59 :(2002)367-370.
20. Wang, W.C., Clonal propagation of *Stevia rebaudiana* Bert. Through axillary shoot proliferation *in vitro*. *Plant cell Tissue org. Cult.,* 6: (1986)159-166.

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