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An Efficient Organogenesis Based Micropropagation of Medicinal and Natural Sweet Leave Plant *Stevia rebaudiana* Bert. in Kingdom of Bahrain

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Abstract: *Stevia rebaudiana Bert. is a small shrub, bearing small leaves which are 30 times more sweeter than sugar. A rapid and efficient plant micro-propagation technique of Stevia rebaudiana based on leaf derived callus induction and shoot differentiation, via individual shoot culture and multiple microshoot induction has developed. Several plant tissue culture media based on modified Murashige and Skoog (MS) medium supplemented with different plant growth regulators (PGRs) were used for callus induction, callus differentiation, microshoot induction and root development. 87% leaf explants showed callus induction in presence of 0.9 µM 2,4-dihydroxyacetic acid (2,4-D), 2.2 µM N6-benzylaminopurine (BAP) and 1.1 µM Indole-3-acetic acid (IAA) and the highest shoot differentiation from callus tissue (8.42 per explants derived calli) was observed in presence of 2.9 µM IAA and 8.8 µM BAP. The maximum number of microshoot formation (15.63 ± 1.03 per shoot) and the highest plant regeneration capacity (132 plant / explant) were noticed in presence of 1.1 µM IAA and 8.8 µM BAP.*

Keywords: *Growth regulators, Micropropagation, Regeneration, Stevia*

I. Introduction

Stevia is a perennial, medicinal, endemic, small shrub that originated in Paraguay and indigenous to the northern region of South America, gradually it has been cultivated in several countries for use as an effective alternate to sugar for a long time. Botanically, it is *Stevia rebaudiana* (Bertoni), a member of Asteraceae family. The leaves of *Stevia* is used as an alternative of artificial sweetening agents. It has a sweet taste which is 30 times stronger than natural sugar but surprisingly without any calories. Hence it is named as "hypocaloric bio-sweetener". The sweetener extracted from Stevia is known as Stevioside, which is 300 times sweeter than cane sugar. The leaf of Stevia contains 15 - 20 % of Stevioside and is considered as natural non calorie sweetener (Brandle *et al*., 1998; Chalapathi and Thimmegowda, 1997). Stevia is the safest means of natural sweetener, can be used as an artificial sweetener in diet soda, tea, fruit juice, chewing gum, and other food beverages. Stevia has been used with success to treat many ailments including diabetes, high blood pressure, digestion ailments, addictions, topically for acne and other skin ailments including weight loss aid. The first report of commercial cultivation of Stevia started in Paraguay in 1964, which is its place of origin. The introduction, establishment and commercial utilization for sweetening and flavoring food of Stevia was started in Japan during 1968. Later on the cultivation spread out other Asian countries, African countries, UK, Mediterranean region, North America and all over South America (Soejarto *et al*., 1982; Saxena and Ming, 1988; Lewis 1992; Nepovim and Vanek, 1998). The use of Stevia as herbal product started in the market since 1970s and 1980s and its extracts have been allowed for using as a dietary component as sweetening agent was approved by Food and Drug Administration (FDA, 1995, 2007).

The cultivation of Stevia on a large scale needs germination of large number of seeds, but generally the seeds show a low germination rate, moreover plants show great variation in plant growth level and sweetening level (Miyazaki and Wantabe, 1974; Sakaguchi and Kan, 1982; Tadhani *et al.,* 2006). Vegetative propagation by stem cutting is not promising effort, more over it is destructive and is limited due to its slowness to obtain large number of plants concurrently from a single plant. To mitigate this difficulties, plant tissue culture technique is the best alternative and has been utilized to conserve, multiply, medicinally important plants in a large scale. Large number of endangered, rare, medicinally important plants have been multiplied using *in vitro* techniques. The plant tissue culture techniques play an important role in conservation of biodiversity and plant production (Sadeq *et al*., 2014 a; 2014 b; 2014 c; Pathak and Abido, 2014). There are different types of culture methods using different organs and technique which varied with desire objectives of plant production and regeneration either, in the process or organogenesis or somatic embryogenesis (Abo El-Nil, 1997; Sharma and Batra, 2006; Debnath *et al*., 2008). Different explants of *Stevia rebaudiana* were used for micropropagation such as stem tips, node, intermodal segment, leaf etc. (Tamura *et al.,* 1984; Bespalhok *et al*., 1998; Sivaram and Mukundan, 2003; Uddin *et al.,* 2006; Deshmukh and Ade, 2012). Micropropagation techniques and culture process of

different explants show differential response under *in vitro* culture media, hormonal balance etc., due to difference in clones and varieties growing under different environmental conditions in various countries for their adaptation issue (Ferreira and Hondro, 1988; Raji and Osman, 2012). Regardless of being several available protocol, different clones of the same species show differential responses under *in vitro* culture. I have developed an efficient plant production protocol using leaf explants of *Stevia* in the Kingdom of Bahrain.

II. Materials and Methods

2.1. Explant resource

One months old two young Stevia *rebaudiana* Bert. plants (15-20cm height) grown in soil pots were collected form Ramkrishna Mission Ashram, Narendrapur, Kolkata, India. The collected plants were kept in the plant growth room for three weeks for acclimatization and growth. The young leaves of three week old plants were used as explants for *in vitro* culture initiation.

2.2. Surface sterilization of explants

Young leaves were surface sterilized following modified method (Pathak and Hamzah, 2008), were washed in running tap water for 2-3 minutes to remove dust particle from their surface. Then leaves were kept successively for 2 minutes in 1% of Lux solution and 0.5% Mercuric chloride solution and the respected solutions were discarded in each step and were washed thoroughly (5-6 times) using autoclaved distilled water. Finally the leaves were washed with 70% ethanol for 30 seconds and were transferred to autoclaved distilled water until final excise and transfer to culture media.

2.3 Culture media and culture conditions

The media used throughout the culture of Stevia *rebaudiana* were modified Murashige and Skoog (MS) media (1962) supplemented with 3% sucrose, 0.3% casein hydrolysate, 1 mg/L Nicotinic acid, 1 mg/L Pyridoxine HCL and 10 mg/L Thiamine HCL. The pH of the media were adjusted to 5.8 before adding 0.8% agar and autoclaved at 121°C, 20 minutes at 15 psi. Modified MS culture media were fortified with different combinations and concentrations of plant growth regulators (PGRs) according to the nature of plant tissue in the culture media in different stages. The cultures were maintained at 16/8 h (light/day) cycle with cool, white, fluorescent light intensity of 2000-2500 LUX, temperature of 21±2 °C and 50-60% humidity.

2.4. Treatment for callus induction from leaf culture

Surface sterilized leaf segments of *Stevia* sp. were used for culture initiation in modified (#1) MS media without any growth regulator and modified MS media supplemented with (#2) 0.9 µM 1,4-dihydroxyacetic acid (2,4-D) + 2.9 µM 3-indoleaceticacid (IAA) + 0.9 µM 6-benzylaminopurine (BAP), (#3) 0.9 µM 2,4-D + 2.9 µM IAA + 2.2 µM BAP, (#4) 0.9 µM 2,4-D + 5.8 µM IAA + 2.2 µM BAP, (#5) 0.9 µM 2,4-D + 5.8 µM IAA + 4.4 µM BAP for 3 weeks.

2.5. Shoot differentiation from callus

Callus growing in media #4 were cultured three weeks for shoot differentiation in media (#1), (#6) 2.9 µM IAA + 4.4 µM BAP, (#7) 2.9 µM IAA + 8.8 µM BAP, (# 8) 2.9 µM IAA +13.3 µM BAP, (# 9) 5.8 µM IAA + 4.4 µM BAP, (#10) 5.8 µM IAA + 8.8 µM BAP, (#11) 5.8 µM IAA +13.3 µM BAP.

2.6. Multiple microshoot induction and growth

The regenerated microshoots were cultured for three weeks in media (#1), (#12) 1.1 µM IAA + 2.2 µM BAP, (#13) 1.1 µM IAA + 4.4 µM BAP, (#14) 1.1 µM IAA + 8.8 µM BAP, (#15) 2.2 µM IAA + 8.8 µM BAP, (#16) 2.2 µM IAA + 13.3 µM BAP for multiple shoot induction from elongated callus derived shoot. Newly proliferated multiple microshoots developed from nodal segments were cultured in media (#14) for three weeks for multiplication of microshoots.

2.7. Root induction and transfer of regenerated plants

Microshoots (1-2 cm) were cultured in rooting media weeks (#1), (#17) 2.9 µM IAA, (#18) 2.6 1-naptheleneacetic acid (NAA), (#19) 1.7 µM IAA + 1.6 µM NAA, (#20) 2.9 µM IAA + 2.6 NAA for two weeks. Rooted plantlets were washed in autoclaved distilled water to remove adhered culture media from their rooting surface and transplanted in plastic pots containing autoclaved potting soil and were covered with plastic bags containing few holes for gradual acclimatization in plant culture room (25 ± 3°C, 16 hours photo period and 8 hour dark period, 40-50% relative humidity).

2.8. Date analysis

The experiments were carried out using completely randomized design (CRD) with three replications of each experiment and 8-10 explants per replication (depending on stage of experiments). Data was collected in three to four weeks interval which has been mentioned in different steps of culture. Based on morphological response, such as leaf derived callus induction, microshoot regeneration from differentiated callus, multiple shoot induction, elongation of shoot, root induction and plantlet regeneration capacity were analyzed based on data collected in each step. Plant regeneration capacity of explants was calculated based on number of shoot differentiation from explants derived callus, then number of microshoot induction of those differentiated shoots and their multiplication. Statistical analysis of data were carried out using STATISTICA Version 13 (Stat Soft

Inc.). Means of three replicates per treatment were compared with control as well as multiple treatment groups. Mean comparisons and one way analysis of variance (ANOVA) with Tukey HSD Test was performed using Fisher's least significant difference (LSD) test at $P \leq 0.05$.

III. Results and Discussions

3.1. Culture initiation and shoot induction

The percentage of callus induction from leaf segments in modified MS media supplemented with BAP, 2,4-D and IAA has presented in Fig.1. The highest response of 87% callus initiation from leaf segments was obtained in media containing 2.2 µM BAP, 0.9 µM 2,4-D and 1.1 µM IAA. The callus induction capacity of leaf, node and internode of Stevia were compared in presence of different concentration of 2.4-D and the maximum callus induction was happened in presence of 3mg/L (Uddin *et al.*, 2006). The callus development capacity from *Stevia* seeds were compared in presence of different PGRs while the maximum callus was induced in presence of 2.5 mg/L IAA and 1.5 mg/L BAP together with 1.5mg/L 1-Napthaleneacetic acid (Keighobadi *et al.*, 2014).

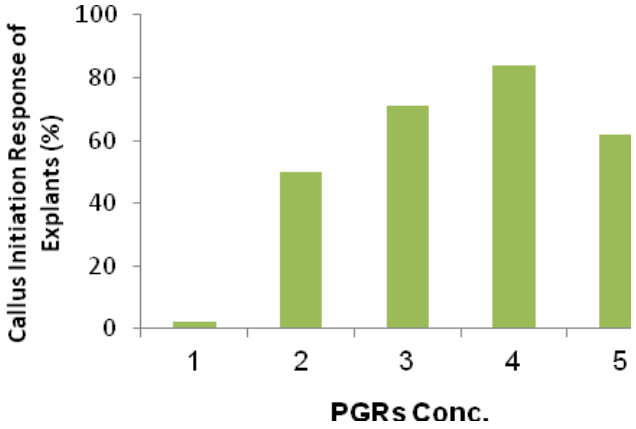

Figure 1. Effect of different plant growth regulators (PGRs) supplemented to modified MS media for callus initiation response from leaf explants of *Stevia rebaudiana* after three weeks of culture. Results are percentage of explants response in callus initiation.

In the flow chart of plant micropropagtion of *Stevia rebaudiana* (Fig.2), leaf derived calli (a,b) were transferred to modified MS media containing various concentrations of BAP and IAA and their shoot initiation frequency has shown in Fig.3. The highest shoot differentiation response was observed from the callus tissue in presence of 2.9 µM IAA and 8.8 µM BAP shown in Fig.2 (c,d).

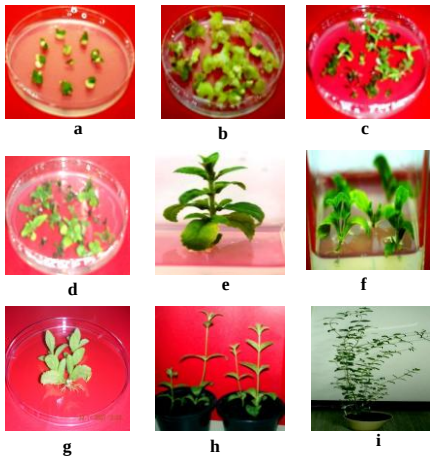

Figure 2. Stage of micropropagation of *Stevia rebaudiana* Bert. (a) Leaf Explants showing callus initiation response; (b) Explant derived callus showing growth and shoot differentiation; (c,d) Differentiated shoots are growing for shoot elongation; (e) Elongated shoots showing multiple microshoot development; (f) Culture of microshoot; (g) Microshoot showing root development; (h) Rooted plantlets growing in soil pots; (i) Mature plant growing in growth room.

The interactive effects of culture media on mean number of shoot induction per callus unit were statistically significant ($P \leq 0.05$) has shown in Fig.3. The effect of PGRs on shoot height variation were compared and the maximum height were observed in 2.9 µM IAA with 8.8 µM BAP followed by 2.9 µM IAA with 13.3 µM BAP (Fig.4) and their interactive effect were statistically significant ($P \leq 0.05$). The differential response of callus tissue in the initiation of shoot was observed in presence of different concentration of BAP and IAA and those were statistically analyzed. The combination of IAA and BAP giving better response than other PGRs combinations for shoot induction and multiplication from different explants sources has reported in several studies of different plant species (Bhatt and Dhar, 2000.; Sen *et al*., 2009; Sadeq *et al.*, 2014c).

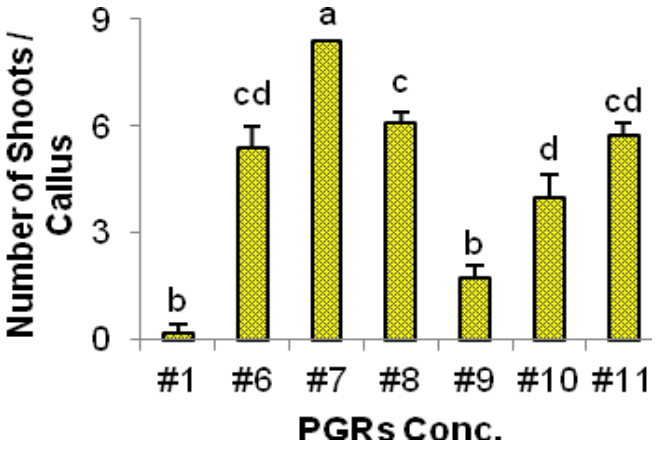

Figure 3. Effect of various plant growth regulators (PGRs) to modified MS media on *in vitro* shoot initiation response from callus differentiation of *Stevia rebaudiana* after three weeks of culture. Results are means with standard error of shoots differentiated per callus of three sets of individual experiments. Means followed by the same letter are not significantly different using Fisher's LSD test at $P \leq 0.05$.

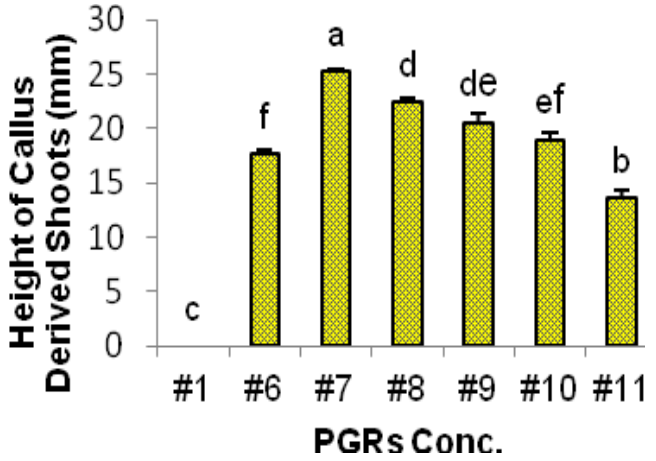

Figure 4. Effect of various plant growth regulators (PGRs) to modified MS media on growth of callus derived shoots of *Stevia rebaudiana* after three weeks of culture. Results are means with standard error of shoot height of three sets of individual experiments. Means followed by the same letter are not significantly different using Fisher's LSD test at $P \leq 0.05$

3.2. Microshoot differentiation and plantlet regeneration

The efficient plant regeneration capacity of leaf derived callus was achieved via microshoot induction of callus derived shoot and their further growth and culture has shown in Fig.2 (e,f). The various concentration of BAP and IAA were used for microshoot induction of callus derived shoot (Fig.5). The interactive effects of culture media on mean number of microshoot induction with standard errors (per shoot) were statistically significant ($P \leq 0.05$) has shown in Fig.5.

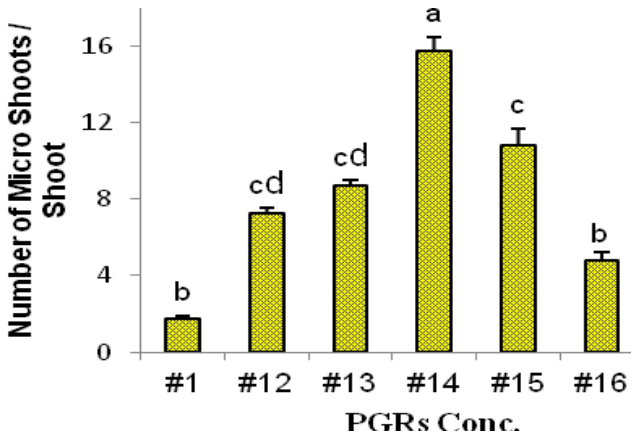

Figure 5. Effect of various plant growth regulators (PGRs) to modified MS media on microshoot differentiation and development of *Stevia rebaudiana* from cultured shoots after three weeks of culture in respected media. Results are means with standard error of shoot numbers of three sets of individual experiment. Means followed by the same letter are not significantly different using Fisher's LSD test at $P \leq 0.05$.

Analysis of variance revealed different PGRs concentrations significantly affect microshoot induction frequency and the highest number of microshoots (15±2) developed in presence of 1.1 µM IAA with 8.8 µM BAP. Similarly, BAP with IAA is very effective combination on multiple shoot induction from nodal segment culture of *Melissa officinalis* (Mohebalipour *et al.,* 2012), *Aloe barbadensis* (Baksha *et al.,* 2005), *Heliotropim kotschyi* (Sadeq *et al.,* 2014a) etc. while Kinetin is less effective in inducing multiple shoot in compared to BAP in *Vriesea* sp (Silva *et al.,* 2009), *Bacapa* sp (Gurnani *et al.,* 2012). The maximum number of strong and long roots (3±1) was observed (Fig.2g) in the MS media supplemented with 1.7 µM IAA and 1.6 µM NAA in compare to other media (Fig.6). The effect of IAA was more prominent than only NAA in several plants were observed (Ujjwala, 2007; Saini *et al.,* 2011), although, there are several reports where indolebutyric acid (IBA) played important role (Hwang, 2006; Trauttman and Visser, 1990). The plantlet regeneration capacity was compared based on callus derived shoot regerneration frequency and multiplication of microshoots in different culture media (Fig.7). The highest plantlet regeneration capacity (132 plantlets/explant) was observed in the presence of 1.1 µM IAA with 8.8 µM BAP. Similarly, other studies in different plants also showed that higher ratio of BAP in compare to IAA in the basal media is very helpful combination in regenerating plantlet either from callus or from nodal explant (Baksha *et al*., 2005; Makunga and Staden, 2008; Sadeq *et al.,* 2014b). Fig. 2 (h,i) showed growth of regenerated Stevia plants in soil.

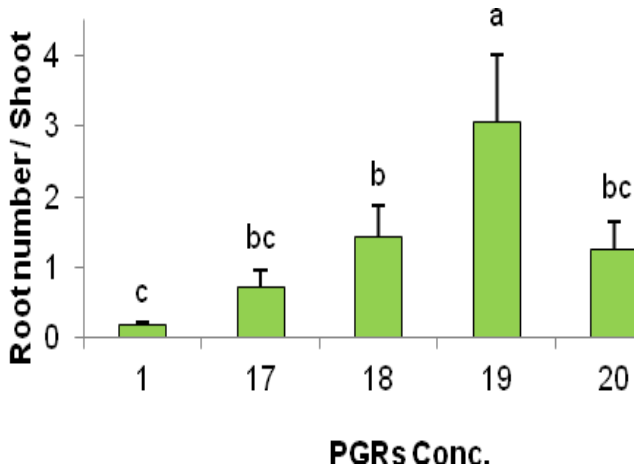

Figure 6. Effect of various plant growth regulators (PGRs) to modified MS media on *in vitro* root initiation response of transferred shoot of *Stevia rebaudiana* after three weeks of culture. Results are means with standard error of roots developed per shoot of three sets of individual experiments. Means followed by the same letter are not significantly different using Fisher's LSD test at $P \leq 0.05$.

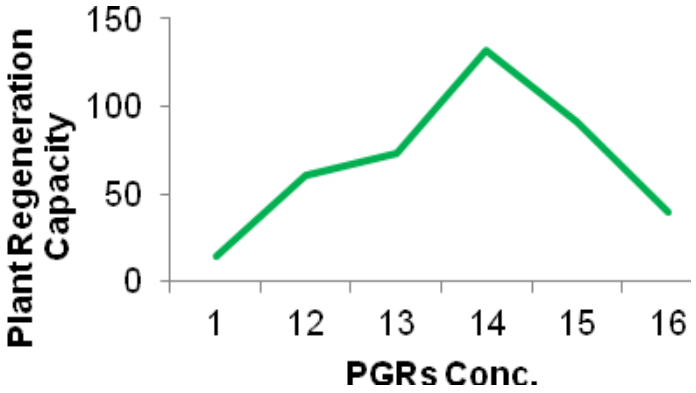

Figure 7. Effect of PGRs combinations on plantlet regeneration capacity of *Stevia rebaudiana* in different culture media.

IV. Conclusion

Considering the great importance of leaf of *Stevia rebaudiana*, rapid multiplication and plant production using this two step organogensis based response in the process of micropropagation is very competitive, innovative idea and efficient protocol. Moreover, introduction of this type of micropropagation technique is unique and can be useful for other plants and being the first time report in the Kingdom of Bahrain.

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