Indian Journal of Plant Sciences ISSN: 2319–3824(Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jps.htm
2016 Vol.5 (4) October-December, pp. 1-6/Abraham and Smrithi
Research Article

IN VITRO PROPAGATION OF SWEET HERB; *STEVIA REBAUDIANA* BERT

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ABSTRACT
An efficient plant regeneration protocol has been developed from leaf explants of *Stevia rebaudiana*, an economically and medicinally important herbaceous plant species belonging to the family Asteraceae. Plant regeneration and multiple shoot proliferation was initiated from leaf explants cultured on MS medium supplemented with various concentrations of 6-benzyladenine (BA) and kinetin (KN) in combination with Auxins (IAA and 24,D). The maximum percentage (81.3%) response with average number of shoots (9.32±0.14), was achieved with medium containing 2 mg/l KN+0.5mg/l IAA. Shoot culture was established by repeated subculturing of newly formed shoots. Rooting of shoots was achieved on half-strength MS medium. Well-developed plantlets transferred to plastic pots containing soil and cocopeat (1:1) showed 80% survival. This protocol can be used for commercial propagation and for future genetic improvement studies.

Keywords: *Stevia Rebaudiana, In Vitro Propagation*

INTRODUCTION
The sweet herb of Paraguay, *Stevia rebaudiana* is becoming a major source of sweetener with no calorific value. The *S. rebaudiana* is commonly known as sweet leaf, sugar leaf, or simply Stevia. The sweet herb *Stevia* is becoming a major source of natural sweetener as an alternate of sugar. Stevia belong to *asteraceae* family. It is rapidly replacing the chemical sweetener like Saccharine and Aspartame. The plant is native to tropical and subtropical regions of North America and South America. There are near about 240 species of *Stevia* genus. It is grown widely in countries like Brazil, Colombia, Paraguay and Venezuela. Countries like Japan and Korea; where it is widely used as a sweetener.

Stevia crop has good potential in India. There is high potential of return as compared to traditional crop. Recently FSSAI, Govt. of India approved extracts of Stevia as an alternative for traditional sweeteners (The Gazette of India, 2015). Diabetes currently affects 246 million people worldwide and India has the largest number of people with diabetes i.e. 40.9 million (Agarwal *et al.*, 2010). Some people switch to artificial sweeteners, but these man-made chemicals cause more health problems than they cure. These chemicals attack vital organs that could lead to serious complications after prolonged use. Considering the rapid increase in diabetic patients in India, especially in Kerala due to changes in food habits and lifestyles, Stevia will be sole alternative for sucrose as a natural sweetening agent in future. Stevia helps to treat many ailments like high blood pressure, hyperlipidemia, obesity, skin diseases and digestive disorders (Gregersen, 2004 and Savita *et al.*, 2004). It has no side effects and is safe for consumption (Ferri *et al.*, 2006). As an exclusive source of natural sweetening agent and has wide range of medicinal values, there will be huge demand in the market for planting material. Today *Stevia* is ready to become 'King' in the market of sweetener. As demand of low carbohydrate sweetener is rising day by day. The extract of Stevia Rebaudioside-A is around 300-400 times sweeter than normal sugar. The sweetness of Stevia also felt for long time. There are two compounds in Stevia leaves 1.) Stevioside (10%-20%) and 2.) Rebaudioside-A (1-3%). The Stevioside is stable at 100 °C this is main advantage of Stevioside over other sweetener.

The methods adopted for large scale Stevia plant production includes seed germination, vegetative propagation, and tissue culture techniques. A few reports suggesting poor germination rate of black viable seeds are also present (Goettermoeller and Ching, 1999). 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 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. Now the focus is on the plant tissue culture techniques that offer a simple, rapid, economical, and efficient way of plant production, propagation, manipulation, and conservation.

Various explants like shoot apex, nodal and leaf explants were used to find their potential for regeneration. A study in 2003 has reported a higher rate of shoot regeneration from these explants by making use of varying concentrations of 6-Benzyl adenine (BA) and Indole-3-acetic acid (IAA) (Sivaram and Mukundan, (2003). Various reports have recorded good regeneration from nodal explants. However, the success rate of regeneration from leaf and internodal explants was less (Mitra and Pal, 2007, Jain *et al.*, 2009 and Sadeak *et al.*, 2009). Moreover, the reported protocols may not work with all varieties or ecotypes available. At present we are not able to propagate materials collected from Kerala and Karnataka with known media composition.

In the present study, report is being given on *in vitro* regeneration of *S. rebaudiana* from leaf explants and field establishment. These could be used for mass propagation and also for germplasm conservation leading to effective plant propagation.

MATERIALS AND METHODS

Stevia was collected from farmers of Wayanad district of Kerala, Gundalpett and Managalore of Karnataka. The plant populations were maintained at three localities 1. Sir Syed College Garden, 2. Thalassery and 3. Thamarassery to avoid the loss of plants due to natural causes.

The attempts were directed to establish the *in vitro* cultures following standard tissue culture protocols. We have tried varying concentrations and combinations of cytokinins and auxins in basal Murashige and Skooge (MS) media. Initially above 30% contamination rate was reported due to high precipitation and as the work progresses it has been reduced to below 10%. At least 30 replicas were made for each experiment. The explants used were internodal as well as leaf. Among explants leaves have given quick response. So, for further experiments we have restricted our trial with leaf explants only.

Plant Material and Sterilization

The leaves were excised from both mature field grown of *S. rebaudiana* plant 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 Qualigens, India) (5% v/v) for 5 minutes. After washing in double distilled water, the segments were surface sterilized using 0.1% (w/v) mercuric chloride solution for 3-5 minutes.

Shoot Initiation and Multiplication

The sterilized segments were thoroughly washed 3-5 times with sterile double distilled water. Sterilized segments cut into appropriate size (0.5-1 cm) were cultured on sterile MS medium (with 3% sucrose solidified with 0.8% agar; The pH of the media was adjusted to 5.8) supplemented with BA and Kinetin at different concentrations and in different combinations with auxins (IAA and 2,4 D). The successful combinations are listed in the table-1. For maturation, half- strength MS agar were fortified IAA was tested effective. The media were sterilized by autoclaving at a pressure of 1.06 kg cm^2 for 20 minutes. Solid medium cultures were incubated either in 16/8 h photo period. All cultures were incubated at 25 ± 2^0C. Thirty cultures were raised for each treatment, and the best treatments were repeated twice. Every two weeks observations were made and data on survival percentage were recorded. Average shoot number and shoot length were also recorded.

Rooting

Auxin IAA and NAA (0.5 and 1 mg/l) were used for root initiation. Later it is observed that hormone free MS media is enough for rooting.

In Vitro Acclimatization

The healthy and well developed plant lets were removed gently from the vials and washed with sterile distilled water to remove traces of culture media and transplanted on sterile potting mixture

(cocopeat:sand 1:1). Plantlets were covered with plastic bags and maintained in shaded condition at around 25 ^{0}C. The humidity is regulated by making holes on plastic bag after 2-3 days. The plant lets were irrigated with ½ strength MS media and were kept in green house for 15 days before transferring to field. The hardened plantlets were planted in the field after 2-3 weeks under shade nets.

Statistical Analysis

Data with regarded to frequency of multiple shoot formation and number of shoots (mean ± SD) was recorded after 30 days of culture. The analysis consists of mean values and standard error.

RESULTS AND DISCUSSION

S. rebaudina plants were efficiently regenerated from leaf explants. When leaf explants from field-grown mature plants of *S. rebaudina* were cultured on MS medium supplemented with various concentrations of BA, KN, IAA and 2.4 D the emergence of the shoot buds was observed at 18–22 days after inoculation (Figure 2). Data on different growth parameters from different treatments were recorded 4 weeks after culture initiation following first subculture (Table 1). The cytokinin type and concentration greatly influenced axillary shoot regeneration from leaf explants. Of the two cytokinins tested, KN-treated explants achieved higher regeneration than those treated with BA.

Table 1: Effect of Different Concentrations of Growth Regulators in MS Medium on Multiple Shoot Induction from Leaf Explants of *Stevia rebaudiana*

Sl. No.	Growth Hormones (mg/l)				% of Shooting	Average Number of Shoots	Mean Shoot Length
	BA	IAA	Kn	24 D			
1	3	1			23	3.12±0.23	1.05±0.11
2	2.5	1			19.8	2.01±0.35	1.22±0.07
3	2	1			19	2.02±0.11	0.87±0.08
4	1.5	1			8.3	2±0.13	1.33±0.16
5	1	1			0	0	0
6	0.5	1			0	0	0
7		0.5	3		43	6.45±0.29	2.08±0.13
8		0.5	2.5		41.5	4.8±0.24	1.53±0.11
9		0.5	2		81.3	9.32±0.14	1.89±0.12
10		0.5	1.5		18.2	14.13±0.11	1.73±0.15
11			1	2	3	2.04±0.17	0.63±0.03
12			0.5	2	0	0	0
13			0.25	2	0	0	0
14				3	0	0	0
15				2.5	0	0	0
16	3			1	12	1.80±0.31	0.73±0.04
17	2.5			1	9.3	2.1±0.13	0.58±0.06
18	2			1	5.2	1.2±0.10	0.53±0.02
19	1.5			1	0	0	0
20	1			1	0	0	0
21	0.5			1	0	0	0

The 2.0 mg/l KN in combination with 0.5mg/l IAA treatment yielded maximum regeneration (81.30%) and maximum number of multiple shoots (9.32±0.14). At KN concentrations higher than 2.0 mg/l, the number of shoots as well as the percentage of response were lower (Table 1). Reduction of the number of shoots generated from explants at lower than optimal KN concentrations have also been noticed. The synergistic effect of auxin and cytokinin has been demonstrated in several medicinal plants, viz. *Santolina*

canescens, *Rauvolfia tetraphylla*, *Bupleurum fruticosum* and *Rotula aquatic* (Cascado *et al.*, 2002; Faisal and Anis, 2002; Fraternale *et al.*, 2002; Martin 2003). In accordance with these reports, the present investigation also exemplifies the positive modification of shoot induction efficiency by an auxin (IAA) in combination with cytokinin (KN). However, when IAA was replaced with 2,4D, a significant decrease in induction and multiplication of shoots was noticed. The combination of 2,4D produced friable yellow callus (Figure 1). The mass production of callus can be utilized for the exploitation of secondary metabolites *in vitro* (Satish *et al.*, 2012).

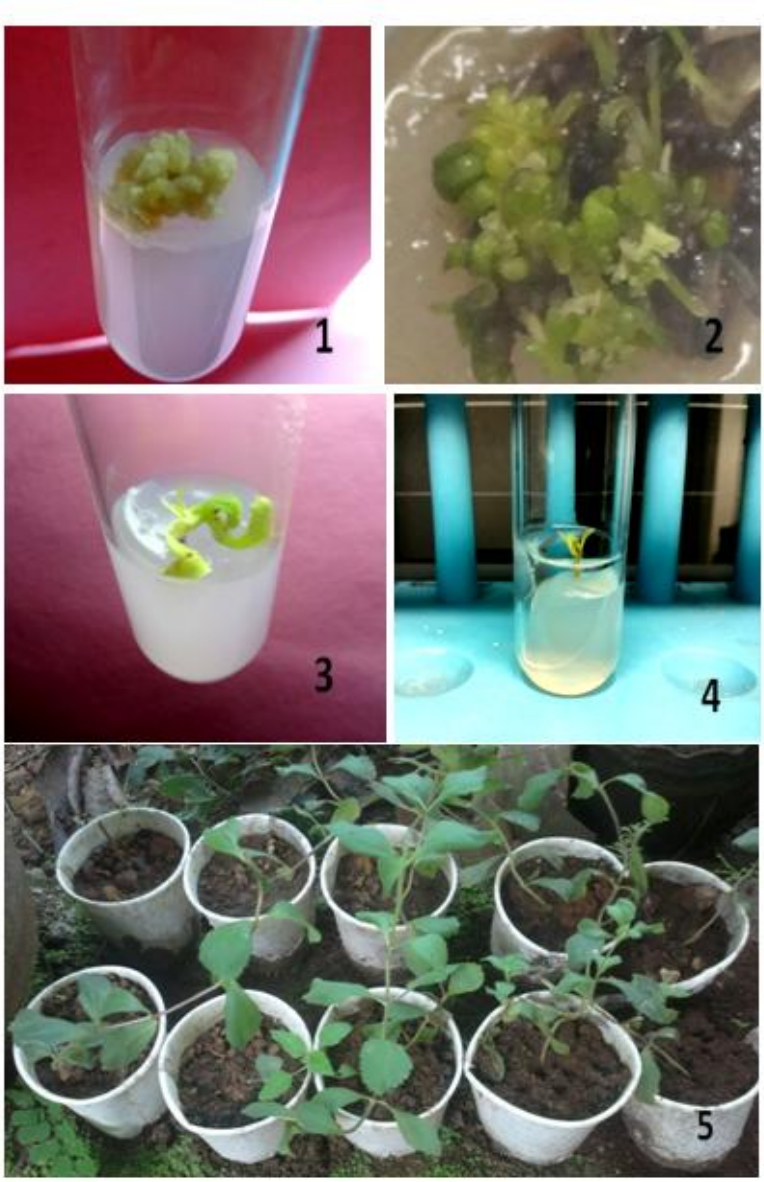

Figure 1-5: *In Vitro* Clonal Propagation of *Stevia rebaudiana*

1. Callus Induction on Leaf Explants on MS Media with 0.5 mg/L KN + 2 mg/L 24,D
2. Shoot Proliferation from Leaf Explants on MS Media Supplemented with 2 mg/L KN + 0.5 mg/L IAA
3. Isolated Plantlet for Rooting on Hormone Free MS Medium
4. Rooted Plantlets on ½ MS Medium
5. Plantlets Developed *In Vitro*, Transferred to Pot for Hardening

In several medicinal plants (Kukreja *et al.,* 1990, Sen and Sharma, 1991 and Vincent *et al.,* 1992) the BA is found most effective in inducing multiple shoot formation. In the present study BA in combination with IAA showed shoot regeneration but with less number of shoots per explants. BA 3 mg/l + 1mg/l IAA showed 23% regeneration with (3.12±0.23) shoots. As the BA concentration decreases the number shoots and also reduced percentage of response (Table 1). Previous reports show that BA is efficient in axillary shoot multiplication in *S. rebaudiana* (Filho *et al.,* 1993 and Ahmed *et al.,* 2007). The combination of BA with 2,4D has given hard callus in very less quantity. The combination of BA with IAA and 2, 4 D also given shoot in less frequency. Shoot regeneration from callus is noticed in MS+BAP (3mg/l)+IAA (1mg/l). Among the explants used for the culture leaf segments were found to be able to produce profuse callus.

The results of callus production and multiplication are presented in Table 1. Inter-nodal segments initiated callus earlier than leaf and nodal explants. The highest amount of callus was found in the MS medium with 2 mg/L 2,4-D. Uddin *et al.,* (2006) studied callus culture from leaf, nodal and inter-nodal segments on MS medium containing 2,4-D.

Most of the cases rooting were observed along with shooting. Other cases regenerated shoots were rooted on hormone free MS media (Figure 3&4). About 80% hardening efficiency was obtained with *in vitro* generated shoots (Figure 5).

The result of the experiment and other earlier research report clearly support the possibility of propagating *S. rebaudiana* by adopting *in vitro* techniques (Jagatheeswari and Ranganathan, 2012; Taware *et al.,* 2010; Banerjee and Sarkar, 2009). The climatic requirements of this tropical elite medicinal plant indicate that it can be introduced in the Kerala. The unique selling points of *Stevia* sweetener are very strong in Kerala due to the presence of diabetic and other metabolic disease including obesity. *In vitro* propagation can become an important alternative to conventional propagation and breeding procedures for wide range of plant species. By using the method described above, hundreds of clonal plants can be produced from one leaf explant by continuous subculturing of shoot propagules. Our results provide a basis for further research in micropropagation of other genotypes of *S. rebaudiana*. Currently our attempts are directed to enhance the sevioside content by in vitro mutagenesis and exploring the chances of production of stevioside from callus.

ACKNOWLEDGEMENTS

The authors are thankful to the Kerala State Council for Science, Technology and Environment, Government of Kerala for financial support.

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