Vol. 18(25), pp. 540-547, 19 June, 2019
DOI: 10.5897/AJB2019.16849
Article Number: 4B7342161315
ISSN: 1684-5315
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African Journal of Biotechnology

Full Length Research Paper

Efficient low cost seesap (CSUP) technique for micropropagation of newly introduced sweetener plant, *Stevia rebaudiana* Bertoni

Pubudini Lakshani Thilakarathne[1], Sriyani Edussuriya Peiris[2] and Pathirana Chathurangi Lankika[2]

[1]Faculty of Science and Technology, Horizon Campus, 482/B, Suhada Mawatha, Millenium Drive, Chandrika Kumaranathunga Mawatha, Malabe, Sri Lanka.
[2]Sri Lanka Institute of Information Technology, New Kandy Road, Malabe, Sri Lanka.

Received 28 April, 2019; Accepted 12 June, 2019.

Stevia rebaudiana **is a non-caloric sweetening herb, which has a high potential for commercialization. During this study low cost media sterilization, seesap (CSUP) technique which applies sodium hypochlorite was used as an alternative to autoclaving. *In vitro* cultures were established using nodal segments collected from stevia mother plants. These *in vitro* shoots were cultured on Murashige & Skoog (MS) medium supplemented with 16 combinations of 6- benzyl amino purine (BAP) and kinetin (KN) with 3% sugar at 5.8 pH, to investigate the *in vitro* shoot multiplication. The developed shoots were transferred to half MS medium supplemented with 0, 1 and 2 mg/L Indole butyric acid (IBA) with 3, 4 and 5% sugar in nine combinations at 5.8 pH, to investigate the rooting. The well rooted stevia plantlets were transferred to Jiffy™ bags for hardening. The results showed that the combination of 1.0 mg/L BAP and 1.5 mg/L KN resulted in the highest number of shoots (11.8 shoots/shoot). The combination of 2.0 mg/L IBA with 5% sugar in half MS medium resulted in the highest root induction (7.2 roots / plant) and root elongation (1.02 cm). The plants grown in culture medium containing 5% sucrose gave 100% survival rate at acclimatization.**

Key words: Stevia, micropropagation, shoot induction, root induction, acclimatization, low cost seesap (CSUP).

INTRODUCTION

Stevia (*Stevia rebaudiana* Bert.), the popular family member of Asteraceae, is a sweet, medicinal herb of Paraguay, containing 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 (Richman et al., 1999). The stevia leaves are the significant resource of diterpene glycoside like

*Corresponding author. E-mail: pubudinithilakarathne@gmail.com. Tel: +94715704783

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rubsoside, steviolbioside, dulcoside, rebaudiosides and stevioside (Starratt et al., 2002). 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 (Kinghorn, 1992). The sweet compounds pass through the digestive track without chemically breaking down, making stevia safe for diabetic and obese people.

Stevia can be propagated using the seeds, or stem cuttings. However, propagation by the seed is not efficient due to low fertility (Tadhani et al., 2006). Propagation using the seed also causes great variability on stevioside level and composition (Nakamura and Tamura, 1985). Seed germination is often poor and rates below 10% (Miyazaki and Wantabe, 1974). The stem cutting method has limitations such as low number of new plants, destruction of the donor plant and requires a significant amount of labor. Therefore, the conventional methods of cultivation and propagation of stevia are time consuming, unpredictable, unreliable and less productive.

Micropropagation or *in vitro* culture appears to be the best method to overcome those problems and has the potential to produce large quantity of stevia plantlets in a short time. Uddin et al. (2006), established *in vitro* propagation from the leaf, nodal and internodal segments of *S. rebaudiana* by using certain plant growth regulators in the medium. Stevia can form multiple shoots from the nodal explants and appears to be suitable for large- scale production. Furthermore stevia *in vitro* cultures can be grown in bioreactors and stevioside can be extracted continuously (Ramírez-Mosqueda et al., 2016).

The ability of *in vitro* micropropagation to produce high quality planting material in large quantities is hindered by their high cost of production. Hence, the price of planting material becomes high, making them unaffordable to growers. To overcome this barrier, use of the seesap (CSUP) technique has to be investigated on stevia micropropagation (Peiris et al., 2012). CSUP technique is a low cost method, where sodium hypochlorite is used to sterilize glassware and culture media. When glassware is sterilized by rinsing with 10% (v/v) sodium hypochlorite solution and the culture medium is poured into the glassware, the medium becomes sterilized and even the transferring of axenic cultures can be performed without the use of a laminar flow cabinet.

This technique can be adopted to replace the use of an autoclave and the laminar air flow cabinet in the major micropropagation activity of *in vitro* multiplication. Hence this method saves the capital cost by Rs. 1.5 to 2 Million, which will encourage entrepreneurs to have small scale laboratories (Peiris et al., 2012). In connection with that, this study was carried out to develop an efficient low-cost method of micropropagation of stevia. Hence, the objective of this research was to optimize the stevia micropropagation protocol using the low cost CSUP technique.

MATERIALS AND METHODS

All the experiments were conducted at the Plant Tissue Culture Laboratory, Faculty of Science, Horizon Campus, Malabe, Sri Lanka.

Plant material

Stevia rebaudiana plants used in this study were procured from the Sepra Natural Pvt. Ltd, Godapola, Imbulgasdeniya, Polgahawela, Sri Lanka, and maintained in the plant protected house of Faculty of Science, Horizon Campus, Malabe, Sri Lanka.

Surface sterilization

Stevia nodal segments excised from three months old mother plants were placed under running tap water for 15 to 30 min to remove the dust particles. Then, the explants were soaked in a detergent (Teepol™) for 5 min, rinsed with distilled water and soaked in 70% (v/v) ethanol for a few seconds. The explants were further sterilized by immersing in 10% (v/v) sodium hypochlorite (common house hold bleaching solution) for 10 min and rinsed with sterile distilled water three times in the laminar flow cabinet.

Sterilization of glass jars/plastic tubes and culture medium

Seesap (CSUP) technique was used for sterilization of glassware and culture media (Peiris et al., 2012) used in this study. Under this technique 10% concentration of sodium hypochlorite was identified as the suitable concentration to sterilize glassware/plastic tubes and culture media in a preliminary study.

Multiple shoot induction

The *in vitro* shoots generated in the establishment stage were transferred to semisolid MS medium in 16 treatment combinations of BAP (0, 0.5, 1.0 and 1.5 mg/L) with KN (0, 0.5, 1.0 and 1.5 mg/L) for shoot multiplication. The number of shoots and shoot length were recorded after four weeks. 20 samples were used per treatment combination and the experiment was repeated twice.

Root induction

The *in vitro* shoots were transferred onto semisolid half MS medium supplemented with nine combinations of IBA (0, 1.0 and 2.0 mg/L) with sugar (3, 4 and 5%) to study the effects on root induction. Number of roots and root length was recorded after four weeks. 20 samples were used per treatment combination and the experiment was repeated twice.

All *in vitro* cultures were kept in a growth room at 25 ± 2°C temperature with around 75% humidity and under illuminated (60 µmoles/m-^{2}/s^{-1}, 12 h/day) condition.

Acclimatization

In vitro rooted plantlets, on ½ MS medium supplemented with 3, 4 and 5 % of sugar were removed gently from the medium, washed in running tap water to remove all the traces of the rooting medium and soaked in 0.2 g/L fungicide (Daconil™) solution for 1 min. Plantlets were transferred to Jiffy™ bags for acclimatization. Survival percentage of the acclimatized plantlets was recorded after

Table 1. Effects of various combinations of BAP with KN on multiple shoot induction from nodal segment of *S. rebaudiana* after 4 weeks (28 days) of cultivation.

Treatment	Concentration of PGRs (mg/L)		Number of shoots	Length of shoots (cm)
	BAP	KN	Mean ± sd	Mean ± sd
1	0	0.5	1.40 ± 0.1[b]	1.08 ± 0.1[b]
2	0	1.0	1.80 ± 0.1[b]	1.01 ± 0.1[b]
3	0	1.5	1.00 ± 0.0[b]	0.96 ± 0.1[b]
4	0.5	0	6.20 ± 0.2[ab]	0.62 ± 0.1[b]
5	1.0	0	3.60 ± 0.3[ab]	0.43 ± 0.1[b]
6	1.5	0	2.80 ± 0.1[b]	0.64 ± 0.1[b]
7	0.5	0.5	7.60 ± 0.3[ab]	0.59 ± 0.1[b]
8	0.5	1.0	6.40 ± 0.1[ab]	0.87 ± 0.1[b]
9	0.5	1.5	7.20 ± 0.2[ab]	0.83 ± 0.1[b]
10	1.0	0.5	8.80 ± 0.1[ab]	0.72 ± 0.1[b]
11	1.0	1.0	8.20 ± 0.1[ab]	0.77 ± 0.2[b]
12	**1.0**	**1.5**	**11.80 ± 0.4[a]**	**1.09 ± 0.1[b]**
13	1.5	0.5	8.60 ± 0.3[ab]	0.58 ± 0.1[b]
14	1.5	1.0	7.20 ± 0.2[ab]	0.48 ± 0.2[b]
15	1.5	1.5	4.60 ± 0.1[ab]	0.76 ± 0.2[b]
16	0	0	1.40 ± 0.1[b]	**2.20 ± 0.1[a]**

n = 20; Values expressing the mean ± standard deviation (sd) followed by similar letters in a column do not differ significantly at $p < 0.05$.

three months.

Statistical analysis

The experiments were arranged in a Completely Random Design (CRD). Each experiment was repeated twice. All the values were expressed as the mean ± standard deviation (sd), calculated using Minitab Statistics software. Data from *in vitro* cultures were subjected to analysis using one-way analysis of variance (ANOVA) and means were compared using the Tukey's significance difference test.

RESULTS

Shoot Induction

There were significant differences in the mean values of number of shoots (P = 0.0009) and shoot length (P = 0.0009) of the 16 different combinations after 4 weeks (28 days) of culturing of *S. rebaudiana in vitro* generated micro shoots.

The highest number of shoots (11.80 ± 0.4) was observed on the MS medium supplemented with the growth regulator combination of 1.0 mg/L BAP and 1.5 mg/L KN and the least number of shoots (1.00 ± 0.0) was found on the MS medium supplemented with 1.5 mg/L KN with 0 mg/L BAP after four weeks of culture. The maximum shoot length (2.20 cm ± 0.1) was observed on the MS medium without any growth regulators (control). However, the second shoot length (1.09 ± 0.1) was recorded on the MS medium supplemented with 1.0 mg/L BAP and 1.5 mg/L KN. The least shoot length (0.43 ± 0.1) was recorded on the MS medium supplemented with 1.0 mg/L BAP with 0 mg/L KN (Table 1).

Root induction

There were significant differences in the mean values of number of roots (P = 0.015) and root length (P = 0.006) of the 9 different treatments after 4 weeks (28 days) of culturing of *S. rebaudiana in vitro* generated shoots.

The maximum number of roots (7.20 cm ± 0.4) and the longest root length (1.07 cm ± 0.1) were observed on the half MS medium supplemented with 2.0 mg/L of IBA and 5% sugar. The medium without IBA did not exhibit any root induction. The results clearly showed that the number of roots as well as the root length increased with increasing the sugar level. Out of the three different sugar percentages (3, 4 and 5%) used along with IBA, 5% sugar produced the highest shoot number and highest shoot length in both the IBA concentrations (Table 2).

Acclimatization

The results were recorded after three months of acclimatization, and the effect of different sugar concentrations used in pre-transplant stage for survival rate of the acclimatized stevia plantlets were analyzed.

Table 2. Effects of various concentrations of IBA and sugar combinations on root induction from micro shoots of *Stevia rebaudiana* after 4 weeks (28 days) of culturing.

Treatment no.	Concentration		Number of roots	Length of roots(cm)
	IBA (mg/L)	Sugar (%)	Mean ± sd	Mean ± sd
1	0	3	0.00 ± 0.0[b]	0.00 ± 0.0[b]
2	0	4	0.00 ± 0.0[b]	0.00 ± 0.0[b]
3	0	5	0.00 ± 0.0[b]	0.00 ± 0.0[b]
4	1	3	0.80 ± 0.1[ab]	0.14 ± 0.1[ab]
5	1	4	1.40 ± 0.2[ab]	0.84 ± 0.1[ab]
6	**1**	**5**	**3.80 ± 0.2[ab]**	**0.60 ± 0.1[ab]**
7	2	3	1.20 ± 0.1[ab]	0.08 ± 0.1[ab]
8	2	4	1.60 ± 0.1[ab]	0.10 ± 0.1[ab]
9	**2**	**5**	**7.20 ± 0.4[a]**	**1.07 ± 0.1[a]**

n= 20; values expressing the mean ± standard deviation (sd) followed by similar letters in a column do not differ significantly at $p < 0.05$.

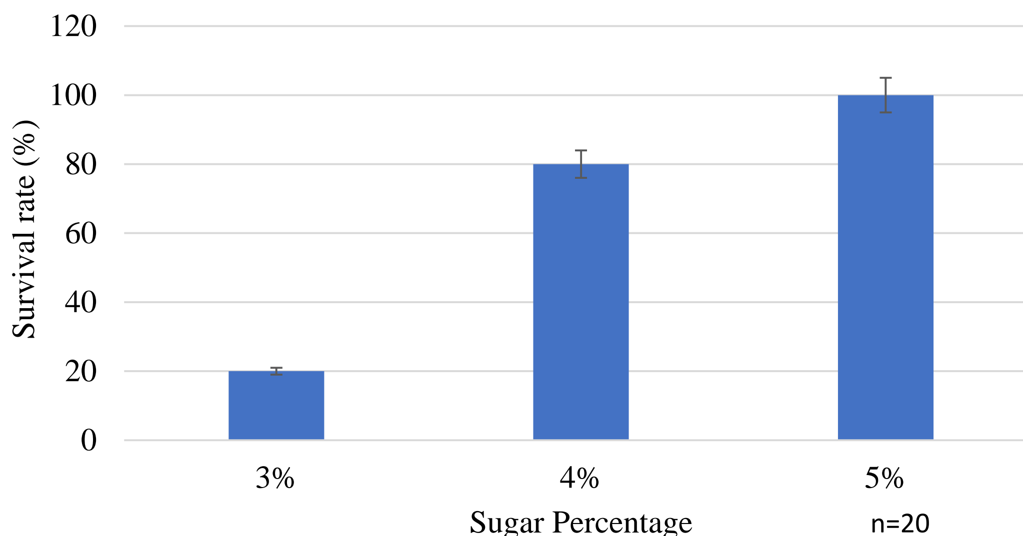

Figure 1. Survival rate of the plants after acclimatization based on the sugar concentration used to culture the plants in the pre-transplant stage in agar-gelled medium.

The results showed that the plants grown on culture medium containing 5% sucrose produced the best response and showed 100% survival rate even after 3 months of acclimatization (Figure 1). From Figure 2, it was evident that the survival rate of the stevia plants assessed after acclimatization are all directly proportional to the concentration of sugar used in the culture medium in the *in vitro* pre-transplant stage in agar gelled medium.

DISCUSSION

In the present study higher concentrations of BAP or KN resulted in less multiple shoot induction. Sivaram and Mukudan (2003) reported that the combination of low concentrations of BAP and KN (0.5 mg/L) induced high number of multiple shoots and higher concentration of BAP resulted in decreased multiple shoots formation of stevia. However, Tadhani et al. (2006) showed that the maximum number of shoots was achieved on MS medium supplemented with 0.6 mg/L of BAP. According to the results shown in Table 1, the highest number of shoots was produced by 0.5 mg/L BAP concentration out of 3 different concentration of BAP alone and 3 different concentrations of KN alone. This is the best result recorded when BAP and KN growth regulators were used independent from each other. The large number of shoots produced in the presence of BAP was due to the

(A)

(B)

(C)

(D)

Figure 2. (A) Initiation of multiple shoot formation of *S. rebaudiana;* (B) Development of multiple shoot after 4 weeks; (C) Root formation from regenerated shoot; (D) Acclimatized plantlets in Jiffy bags filled with sterilized coir dust.

fact that BAP greatly reduced apical dominance and released lateral buds from dormancy and enhanced shoot formation (George and Sherrington, 1984). Das et al. (2011) also reported the longest shoot length of stevia when KN was present in the medium. Although the maximum shoot length of this study was recorded in the control, the second maximum shoot length was recorded in growth regulator combination of 1.0 mg/L BAP and 1.5

mg/L KN where high amount of KN present in the medium.

The various concentrations in combination of the growth regulators used in this study induced the proliferation of shoots derived from the nodal segments of *S. rebaudiana.* Kinetin was less effective at inducing multiple shoots compared to BAP. According to Rafiq et al. (2007), no specific increase in multiple shoot formation occurred when the explants were cultured on KN based media. Similar results were found in *Vriesea scalaris*, where KN alone had no effect in *in vitro* multiplication (Silva et al., 2009). Nevertheless, KN can be more effective to induce multiple shoot when combined with BAP (Verma et al., 2011). According to Anbazhagan et al. (2010), the combinations of BAP and KN work well for both shoot proliferation and their elongation from the nodal explants of *S. rebaudiana.*

According to the present study, 2.0 mg/L IBA used along with 5% sugar showed the highest root induction and elongation. Verma et al. (2011) reported that half strength MS medium supplemented with 2.0 mg/L IBA proved the best with 87.8% rooting and early root initiation of *in vitro* cultures of *S. rebaudiana.* Hwang (2006) reported the maximum numbers of roots using a treatment of 1.0 mg/L IBA in the MS medium with up to 100% rooting. Tadhani et al. (2006) also obtained the highest rate of root induction in 1.0 mg/L IBA included medium. Tesfa et al. (2016), reported that increase in sucrose concentration from 0 to 50 g/L (0 - 5%) along with a definite concentration of NAA (5 mg/L), significantly increased the average root length and root number to 4.58 and 18.00 in sugarcane genotype N52, and to 4.54 cm and 21.76 in genotype N53, respectively. Tesfa et al. (2016) also showed that further increase in sucrose concentration to 70 g/L (7%) resulted in reduction in average root length and number of roots in both sugarcane genotypes. Earlier reports also confirmed that higher concentrations of sucrose in the medium have a negative impact on overall rooting due to accumulation of rooting inhibitors, the reduction of rooting promoters in the medium, and the transformation of added sugars into insoluble and storage form (Ahmed et al., 2004).

Root length increased on IBA containing medium and this was accentuated as the IBA concentration increased (Trauttman and Visser, 1990). The IBA seems to be the best auxin in *S. rebaudiana* to the initiation of the root induction. The potential of IBA in root induction has also been reported in many species (Epstein et al., 1993). IBA has been observed to induce strong rooting response and has been extensively used to promote rooting in a wide range of plant species by Rani and Rana (2010), Steephen et al.(2010) and Yadav and Singh (2011b).

Sucrose is a prime carbon source of stevia micropropagation and developing vigorous plantlets, but the performances of other disaccharides or mono-saccharide are not fully explained as far as optimum stevia tissue culture is concerned. There is a general agreement in the literature that sucrose is required in the medium for stevia tissue culture. The concentration and type of exogenous carbon sources added to medium to serve as energy and also to maintain the osmotic potential (Lipavska and Konradova, 2004). Plant cells and tissues under *in vitro* conditions are mixotrophic in nature and needs supply of external carbon source for its metabolic activities (Lipavska and Konradova, 2004).

The present study showed that the plants grown in culture medium containing 5% sucrose gave the best response and showed 100% survival rate even after 3 months of acclimatization. The success of using high concentration of sucrose for pre-transplant stage in orchid micropropagation is stated by many researchers. Collins and Dixon (1992), studied different sucrose concentrations in *in vitro* culturing and observed that for the Australian terrestrial orchid *Diuris longifolia*, 20 g/L sucrose plus charcoal had a similar rooting effect as 40 g/L sucrose without charcoal. According to Kerbauy (1993), high agar levels, together with the sucrose, promote great longitudinal root growth and lateral aerial growth, while low levels of these components favor the formation of protocorms in *in vitro* culture of *Oncidium varicosum* (Orchidaceae). Ishii et al. (1998) observed that in *Phalaenopsis* the presence of sucrose in the culture medium caused protocorm formation and its absence caused callus proliferation.

This increase in the amount of sucrose in the culture should be taken with caution and should not be progressive, because, according to Capellades et al, (1991) and Hdider and Desjardins (1994), higher sucrose concentrations in *in vitro* cultures favor carbohydrate accumulation and hinder photosynthesis.

During this study sterilized coir dust in Jiffy™ bags were used as the hardening medium in order to promote water retention since the plantlets were kept in an acclimatization chamber consist of high temperature and high humidity. Meera and Sathyanarayana (2010) reported that with sand as the hardening media, mist house had higher survival rates than greenhouse. Sand did not retain much water in greenhouse to ensure proper growth and survival compared with mist house with frequent misting. In all other cases, the survival was found to be better in greenhouse (28%) compared with mist house (23%). With frequent misting, water logging occurred around the plantlets, and they started to rot which lead to reduction in survival. This was absent in sand due to its high porosity and rate of percolation. They also reported that the survival percentage recorded with cocopeat under greenhouse was around 75.5% that was almost 60 times higher than the lowest survival recorded with 100% sand under greenhouse (1.5%).

This study proved that the CSUP technique can be successfully applied to micropropagate *S. rebaudiana* as a low cost application where equipment cost can be

Table 3. Total cost reduction with the use of CSUP method instead of autoclaving.

Conventional tissue culture	Cost (USD)
Cost of an autoclave	8543.40
If the durability is 10 years, the annual cost	854.34
Cost of electricity to autoclave 1L of medium (10 mL/tube and 4000 tubes = 40 L)	0.23
Cost of electricity to autoclave 40 L of medium	9.11
CSUP technique	
Cost of 1 L of Clorox	2.16
10% of 100mL can be used to rinse 200 tubes effectively (2000 tubes can be sterilized using 1 L of Clorox. We used 10% of Clorox solution to sterilize tubes. For our experiments about 4000 tubes were used)	
Cost of 2 L of Clorox (to sterilize 4000 tubes)	4.33
Total cost reduction	**(854.34+9.11) - 4.33 = 859.12 USD**

reduced by 97% (Peiris et al., 2012). According to the Table 3, the total cost reduction percentage was 99.5%.

Attempting to reduce cost of *S. rebaudiana* micropropagation, Sharma et al. (2013) reported 85% cost reduction for the medium by replacing laboratory reagent grade sucrose by locally available commercial sugar, bacteriological grade agar by Isabgol as the gelling agent and distilled water by tap water. In our study also sugar available at the market was used rather than the analytical grade sucrose which is another cost reduction. All of the aforementioned studies prove evidence for the credibility of the results obtained from the current research.

Conclusion

Stevia rebaudiana micropropagation protocol carried out using seesap (CSUP) low cost media sterilization showed maximum *in vitro* shoot multiplication of 11.8 shoots/shoot on the MS medium supplemented with 1.0 mg/L BAP, 1.5 mg/L KN in combination and 3% sugar at 5.8 pH. The highest number of roots and maximum root length were observed on the half MS medium supplemented with 2.0 mg/L IBA with 5% sugar combination. The plants grown in pre-transplant culture medium containing 5% sucrose showed 100% survival rate in acclimatization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors gratefully acknowledge Horizon Campus, Malabe, Sri Lanka for providing financial assistance to carry out this study. They are also grateful to Ms. Hansini Jayanetti, Technician, Horizon Campus for her technical assistance.

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