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Full Length Research Paper

Explant type and hormone regime influences somatic embryogenesis and regeneration in cassava

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Cassava (*Manihot esculenta* Crantz) is a tropical root crop that serves as a food staple and a vital income source to resource deprived farmers in the tropics. Despite its importance, cassava production and consumption is faced by a myriad of biotic/abiotic constraints. Genetic transformation which can be applied to mitigate these challenges however has as a prerequisite the availability of robust regeneration systems. This study evaluated the effect of explant type and hormone regime on somatic embryogenesis and regeneration of Kenyan cassava cultivars. The embryogenic competence of immature leaf lobe and stem explants of three cassava cultivars was determined by culturing them in MS medium supplemented with picloram (4, 6, 8 and 10 mg/l). The optimum media composition for embryo maturation, germination and plant recovery was assessed by culturing embryos in media supplemented with varying concentrations of 6-Benzylaminopurine (BAP), α–Naphthalene acetic acid (NAA) and Gibberellic acid (GA_3). Somatic embryo formation frequencies in leaf explants were significantly higher (P≤0.05) than in stem explants. Embryo formation rates were found to increase with increasing concentrations of picloram. Cultivar 08/080 gave the least response to embryogenesis while embryogenesis rates for cultivar 08/274 were similar to the control cultivar TMS 60444. Maturation and plant recovery rates differed significantly with regard to the ratios of BAP, NAA and GA_3 in the maturation media. The optimal medium for embryo germination and plant recovery was supplemented with 1 mg/l BAP, 0.02 mg/l NAA and 1.5 mg/l GA_3. This optimized regeneration protocol can be coupled with mutation breeding or genetic transformation to improve the cassava germplasm.

Key words: Cassava, explant, regeneration, somatic embryogenesis, plant growth regulator.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz, family Euphorbiaceae) is an important food security crop and a feasible feedstock for numerous industrial processes. In addition to use as food, cassava tubers are important for

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use as animal feed, for manufacturing industrial products such as an adhesive for laundry purposes, for manufacturing paper, biodegradable materials, beverages and pharmaceutical products (Anyanwu et al., 2015). Due to its increasing consumption in the starch industry and its potential use for biofuel production, the global demand for cassava is growing rapidly (Jansson et al., 2009, FAOSTAT, 2017). The main value of this tuber crop resides in its efficient carbohydrate production, ability to remain in the soil for long without decaying, and the capability of growing in marginal conditions (Alves, 2002). Though maximum yields are gotten under humid tropical conditions on loamy sandy soil, cassava can tolerate marginal/eroded soils and it easily adapts to poor acidic soils that are predominant in the tropics. Additionally, cassava has developed natural barriers against herbivores and arthropods (FAO Joint, 2018).

Cassava is clonally propagated using stem cuttings. Clonal propagation facilitates free exchange of planting material among farmers but is faced by a myriad of challenges such as susceptibility to pests and diseases, accumulation of cyanogens, post-harvest physiological deterioration and a low commercial quality and nutritional value of the starch (Piero et al., 2015). Macro-propagation is constrained by the accumulation of viral and bacterial diseases, unavailability or limited access to planting materials and low multiplication rates which reduce crop productivity. Improvement of the cassava germplasm by traditional breeding methods has been hampered by the non-availability of necessary genes in the germplasm, high heterozygosity, allopolyploidy, low fertility as well as unsynchronized flowering of cassava making it difficult and time-consuming. Genetic transformation techniques that can be applied to complement conventional breeding are hindered by the requirement of a reproducible transformation and regeneration system. In cassava, somatic embryogenesis is the most frequently used regeneration system for the recovery of transgenic events (Chavarriaga-Aguirre et al., 2016). Though efficient, responses to somatic embryogenesis greatly vary among genotypes, and not all cassava varieties are amenable to this morphogenesis process (Marius et al., 2018). Somatic embryogenesis is also affected by explant type and the composition of the culture medium among other factors (Shen et al., 2015). This therefore necessitates optimization of the different regeneration parameters to suit each cultivar of interest.

The morphogenic response of plants *in vitro* is highly depended on the balance between auxin and cytokinin hormones in the media (Schaller et al., 2015). Somatic embryogenesis and *in vitro* regeneration can therefore be improved by identifying an optimum hormonal balance for regeneration media (Wongtiem et al., 2011). This study determined the effect of genotype, explant type and hormone regime on somatic embryogenesis and shoot regeneration of selected Kenyan cassava cultivars. We found out that the tested cassava cultivars were amenable to regeneration via somatic embryogenesis though the rates varied among the cultivars.

MATERIALS AND METHODS

Plant material and culture conditions

Three selected cassava cultivars, namely TMS 60444, 08/080 and 08/274 were obtained from the Kenya Agricultural and Livestock Research Organization (KALRO)- Mtwapa, Kenya. TMS 60444 is a model genotype in cassava regeneration and transformation and was used as a control in the study because of the excellent regeneration capacity of its embryogenic tissues (Zainuddin et al., 2018). *In vitro* cultures were established by culturing surface sterilized cuttings (5 cm long) on cassava micro-propagation medium (CMM) containing MS salts with vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.3% gelrite; pH 5.8 and were sub-cultured every four weeks. All cultures except for callus induction were maintained in the growth chamber at 25±2°C, 16 h /8 h light/dark cycle. Cassava mother stock and regenerated plants were grown in a glasshouse at 27±1°C.

Effect of explant type on callus induction and somatic embryogenesis

Immature leaf lobes (ILL) and stem internodes (both 5 to 10 mm long) from 8 weeks old *in vitro* plantlets were excised and cultured in callus initiation media for 8 weeks in the dark at 25±2°C. Callus were sub-cultured onto fresh media after every 2 weeks. The callus induction media was composed of MS salts, Gamborg B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol, 0.2% sucrose, 0.5 mg/l CuSO4, 50 mg/l casein hydrolysate) supplemented with picloram at concentrations 4, 6, 8 and 10 mg/l. The type of callus, number of explants producing callus and direct somatic embryos were recorded after 8 weeks of culture.

Maturation of somatic embryos

Callus (8 weeks old) were transferred to MS medium supplemented with various hormone combinations of α-naphthalene acetic acid NAA, 6-benzylaminopurine (BAP) and gibberellic acid (GA_3), The media were abbreviated M, M1, M2, M3 and M4 (Table 1). The number of cotyledonary embryos formed from each callus was recorded following 4 weeks of culture. The percentage maturation was the number of callus that produced cotyledonary embryos as a percentage of the total number of callus transferred to maturation media. The mean number of cotyledonary embryos was assessed by determining the average number of cotyledonary embryos produced from each callus.

Embryo germination and plant recovery

Callus harboring cotyledonary embryos and shoots were aseptically transferred to hormone free MS media supplemented with 0.8% activated charcoal for 14 days. Embryos with defined root and shoot apices were transferred and maintained on CMM for 4 weeks. The number of germinated plantlets/callus cluster was recorded.

Acclimatization

Plantlets with defined roots and shoots were transferred to small pots filled with sterile peat moss for four weeks in the glass house.

Table 1. Effect of explant type on the rate (%) of cassava callus induction in MS media supplemented with picloram concentrations.

Genotype	Auxin concentration							
	4 mg/l Picloram		6 mg/l Picloram		8 mg/l Picloram		10 mg/l Picloram	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
08/274	87.3±1.3[a]	90.2±1.2[a]	88.1±1.2[a]	91.1±1.1[a]	89.5±1.3[a]	92.7±0.9[a]	90.3±1.6[a]	92.5±1.3[a]
08/080	87.1±2.5[a]	86.3±1.2[a]	89.2±1.8[a]	88.1±1.7[a]	89.5±1.0[a]	92.2±1.1[a]	89.9±1.9[a]	95.5±1.4[a]
TMS60444	81.2±0.3[a]	92.0±0.9[a]	82.9±0.8[a]	93.1±0.5[a]	84.5±0.8[a]	94.1±0.7[a]	86.0±0.8[a]	95.8±0.7[a]

Values are means (±standard error). Values followed by different superscripts in same row are significantly different at P≤0.05 by Duncan's multiple range test.

After the lapse of four weeks, the surviving plants were transferred to larger pots filled with a mixture of peat moss and soil (50/50 % v/v). After four weeks, surviving plants were transferred to soil. The ability of regenerated cassava plants to acclimatize and efficiently establish in the glasshouse was assessed by measuring the increment in height during each stage of acclimatization and the number of plants that survived for up to 3 months after transplanting to soil.

Study design and statistical analysis

For callus induction, ten explants were cultured on a 90 mm petri dish and a total of 60 explants were tested for each treatment; the experiment was repeated thrice. The frequencies for callogenesis and embryogenesis were calculated as the number of callus or embryos formed as a percentage of the total number of explants cultured. Frequencies of callus induction, somatic embryogenesis and shoot formation were subjected to analysis of variance (ANOVA) using SPSS statistics program (SPSS software for Windows release 10.0; SPSS Inc., Chicago, IL) and differences among treatment means were separated by Duncan's multiple-range test (DMRT) at a 95% confidence level (P≤0.05). All quantitative data values were expressed as Mean ± SEM (n=3).

RESULTS

Effect of explant source on callogenesis and somatic embryogenesis

Immature leaf lobes (ILL) took an average of 10 days to initiate callus while stem explant took up to 15 days. Callus formation in immature leaf lobes (Figure 1A) started off by the formation of a swollen mass of tissue at the cut edges of the leaf (Figure 1B) then spread into the entire leaf. Callus from meristematic stem segments were mainly loose, friable, non-embryogenic and white in color (Figure 1C) while callus from immature leaf lobes were mainly translucent, gelatinous and highly embryogenic (Figure 1D). Although no significant differences were noted in the ability of stem and leaf explants to form callus (Table 2), significant differences were noted in their ability to form organized embryogenic structures (Figure 2). Callus from leaf explants gave significantly higher embryogenesis frequencies than the stem explants in all the tested cultivars. An increase in the concentration of picloram led to an increase in the frequency of callus formation and embryogenesis in all the cultivars tested. Embryogenesis frequencies for ILL in cultivars 08/274, TMS 60444 and 08/080 ranged from 62 - 84, 70 - 83 and 46 - 63%, respectively. Embryogenesis frequencies for stem explants ranged from 38 - 44, 30 - 39 and 28 - 29%, respectively. Among the three cultivars tested, 08/274 gave the best response for somatic embryo formation using both leaf lobes and stem internodes (Figure 2).

Effect of varying plant growth regulator combinations on embryo maturation

Results on somatic embryo maturation and number of cotyledonary embryos per callus showed that both parameters varied across each media formulation and cultivar (Table 3). Embryogenic callus obtained from stem explants turned green in maturation media but neither formed distinct cotyledons nor plantlets (Figure 1E). Immature leaf lobe derived embryos matured to form either two distinct cotyledons, one cotyledon or fused cotyledons, but germination was attained in all the three cotyledon morphologies. Cotyledonary embryos either formed cotyledonary leaves (Figure 1F) or a collar like cotyledon, which ultimately formed a horn like structure (Figure 1G). Calli cultured on M and M1, both of which had high BAP concentration (3 mg/l) were found to generally produce the fused type of cotyledonary embryos (Figure 1F). Most of the cotyledonary embryos from these media formulations did not, however, convert to complete plantlets hence significantly lower (P≤0.05) maturation frequencies were obtained from the two combinations (62 and 63% respectively, Table 2). The two hormone combinations produced an average of 5 and 6 shoots per callus respectively (Table 3). Media M3 and M4 both of which had relatively low BAP concentrations (1 mg/l) and high GA_3 (1.5 mg/l) concentrations gave embryo conversion rates of 69% (Table 3). For these two media formulations, averages of 14 and 16 shoots were recovered respectively (Table 4). It was also observed that cotyledonary embryos from media formulations M3 and M4 either had only one cotyledon or two but none was fused (Figure 1H).

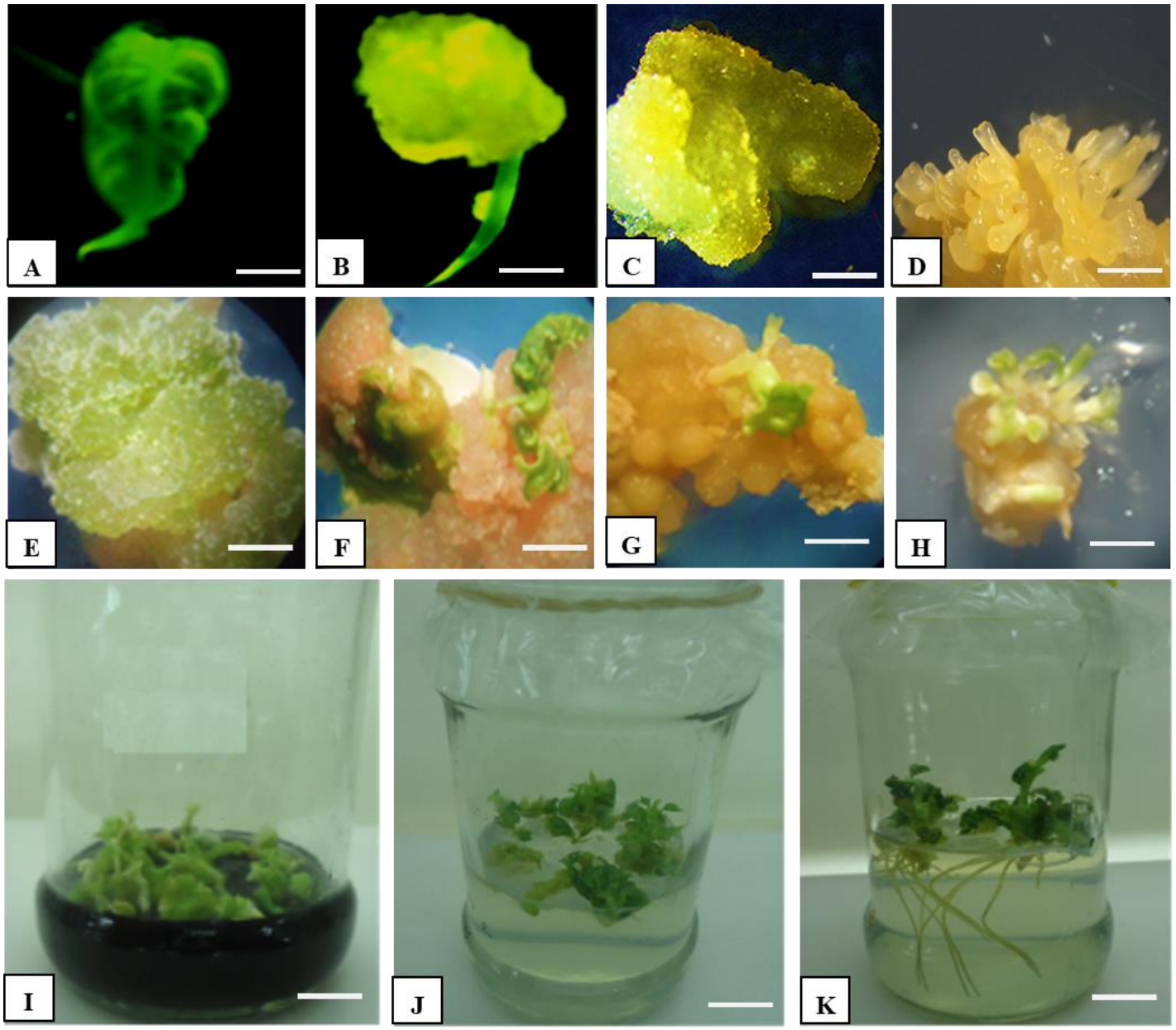

Figure 1. A: Immature leaf lobe on day 0 of callus induction; B: Callus on day 7 of callus induction; C: Callus from a stem explant; D: Callus from a leaf explant; E: Stem explant callus on maturation medium; F: Callus matured in media M; G: Callus with a horn-like cotyledonary structure; H: Callus matured in media M4; I: Desiccation of shoots in activated charcoal; J: Cotyledon germination in hormone free MS media; K: Rooted embryos in germination media; Scale bars: (A, B) 0.25 cm; (C, D, E, F, G, H) 0.5 cm; (I, J) 1.0 cm.

Table 2. Types and concentrations of growth regulators used for embryo maturation.

Media	Growth regulator concentration (mg/l)		
	BAP	NAA	GA_3
M	3	0.02	0.5
M1	3	0.01	1
M2	2	0.03	0.5
M3	2	0.01	1.5
M4	1	0.02	1.5

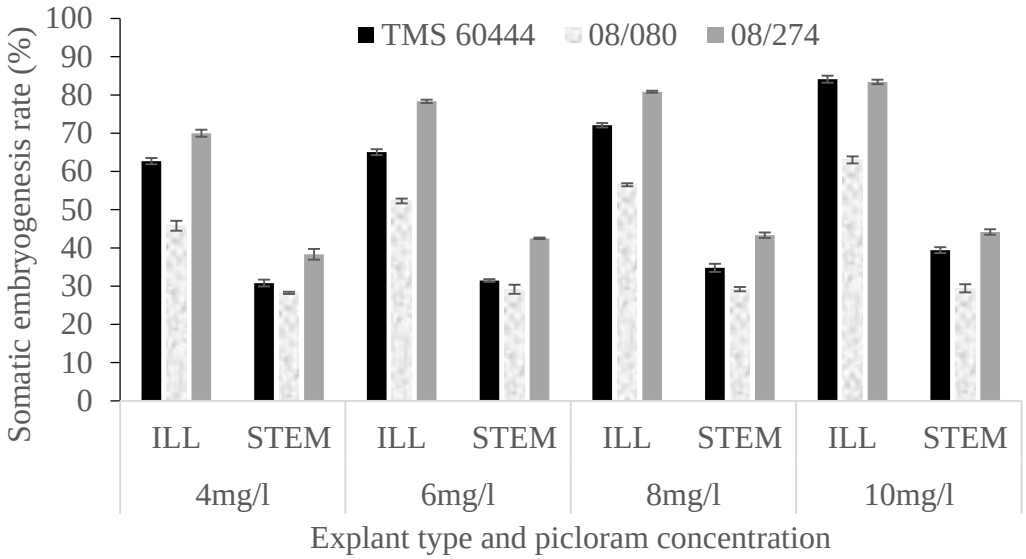

Figure 2. Effect of explant type on cassava embryogenesis in MS media supplemented with varying concentrations of picloram.

Table 3. Effect of phyto-hormone combinations on maturation of cassava embryogenic callus.

Media	Percentage maturation	Number of cotyledonary embryos/callus
M	62.60±0.96[a]	4.87±0.41[a]
M1	63.67±0.97[ab]	6.49±0.22[a]
M2	65.42±0.95[b]	9.94±0.19[b]
M3	69.04±0.89[c]	14.01±0.55[c]
M4	69.58±0.89[c]	15.83±0.38d

Values are means (±standard error). Values followed by different superscripts in same column are significantly different at P≤0.05 by Duncan's multiple range test.

Embryo germination and plantlet acclimatization

Embryo germination started off by the gradual opening of cotyledonary leaves and horns from where a shoot emerged (Figure 1I). Shoots (Figure 1J) of all cultivars successfully established roots (Figure 1K) on cassava basic medium. Regenerated plants were phenotypically similar to the mother stock plants in the glass house and their growth rate was normal. Well-developed cassava plantlets measuring 8 to 10 cm (Figure 3A) were able to establish in peat moss. During the 1-month period, an increase in the height of cassava plants up to an average of 15 cm was observed (Figure 3B). Gradual removal of the polythene bag by first punching holes then removing it after a month was found optimal for efficient cassava acclimatization (Figure 3C). Growth of the cassava plants in a mixture of peat moss and soil for 1 month led to further plant elongation (Figure 3D, 30 cm in height). Plantlets from all the three cassava cultivars were able to efficiently establish in the glasshouse. Three months after transplanting cassava to soil, the plants were found to elongate further to a height of 60 to 90 cm (Figure 3E). In our study, all cassava cultivars attained a survival rate of 60%. Overall, the regeneration efficiency of cassava cultivar 08/080(48.1%) was significantly lower (P≤0.05) than that of cultivars 08/274(75.1%) and TMS 60444(76.3%).

DISCUSSION

Auxins are significant plant growth regulators for stimulating the embryogenic competence of somatic tissues (Gaj, 2004; Lincy et al., 2009). Different explants of the same plant species, however, respond differently to the same hormone treatment. In the present study, immature leaf lobes cultured in picloram gave a better response for somatic embryogenesis as compared to stem explants in the three cassava cultivars tested. A similar response was reported in *Quercus robur* L. where leaf explants gave an embryogenesis frequency of 16% while stem internode segments gave an embryogenesis

Table 4. Effect of phyto-hormone combinations on maturation of cassava embryogenic callus per cultivar.

Cultivar	Media	Percentage maturation	Number of cotyledonary embryos/callus
08/080	M	58.70±0.74^{a}	3.1±0.51^{a}
	M1	61.58±0.80^{a}	5.3±0.46^{ab}
	M2	63.32±0.85^{ab}	6.2±0.43^{ab}
	M3	67.85±0.79^{bcd}	10.1±0.30^{cd}
	M4	69.05±0.83^{cd}	12.3±0.19^{de}
08/274	M	61.56±0.81^{a}	6.3±0.40^{abc}
	M1	61.64±0.77^{a}	7.8±0.34^{bc}
	M2	64.10±0.82^{abc}	11.8±0.26^{de}
	M3	69.52±0.78^{cd}	15.2±0.14^{ef}
	M4	69.62±0.84^{d}	17.5±0.05^{f}
TMS 60444	M	67.54±0.98^{bcd}	5.2±0.49^{ab}
	M1	67.80±0.86^{bcd}	6.4±0.37^{abc}
	M2	68.84±0.84^{cd}	11.8±0.23^{de}
	M3	69.76±0.77^{d}	16.7±0.10^{f}
	M4	70.08±0.98 ^{d}	17.7±0.49^{f}

Values are means (±standard error). Values followed by different superscripts in same column are significantly different at P≤0.05 by Duncan's multiple range test.

rate of 4% in media containing NAA (Cuenca et al., 1999). This trend is also consistent with the report by Xu et al., (2014) who noted that the response of *Solanum nigrum* leaf explants cultured in auxin media was better than that of stem explants cultured in the same medium. Differences in the response of different explants to somatic embryogenesis could be attributed to their differences in morphological structure and stages of maturity (Schädel et al., 2010). The flat shape of leaf explants as opposed to the round shape of stem explants allowed for a bigger leaf surface area to be in contact with the media hence a better embryogenesis response. Additionally, the differential response by the two explants could be attributed to fact that leaves are less lignified as compared to stem explants making them more responsive to regulatory hormones and easily programmed to undergo dedifferentiation (San-José et al., 2010; Schädel et al., 2010; Twumasi et al., 2009). This study noted that when stem segments were cut and poked, they produced latex that possibly interfered with the formation of somatic embryos.

Indirect somatic embryogenesis in cassava previously relied on the use of the auxin 2,4-dichlorophenoxy acetic acid (2,4-D) for initiation of embryogenic competence on different tissues (Danso et al., 2010). This has however been found to lead to low rates of primary embryogenesis and poor plant recovery rates owing to lack of root primordium especially when the culture period in callus induction media is prolonged (Raemakers et al., 1993). To enhance somatic embryogenesis system in cassava, it is therefore important to investigate on the potential of other auxins such as picloram or dicamba as well as jasmonates and brassinosteroids especially in cassava landraces whose embryogenic ability is unknown. We investigated the optimum concentration of picloram for somatic embryogenesis in cassava and noted that embryogenesis frequencies increased with increasing concentrations of picloram from 4 to 10 mg/l. The culture of immature leaf explants in 10 mg/l picloram was found optimal for maximum somatic embryogenesis.

High auxin concentrations evoke callogenesis and embryogenesis but impede embryo germination and shoot regeneration. Consequently, most studies use a high auxin/ cytokinin ratio to evoke callus formation after which the auxin level is reduced while increasing the cytokinin level to stimulate embryo germination and shoot formation. According to Uzelac et al., (2007), the poor capacity of somatic embryos to mature and germinate limits the application of somatic embryogenesis for crop improvement in a number of species.

Embryo maturation was successfully achieved in all the tested maturation media formulations. Differences in embryo germination and plant recovery rates were however recorded between the various plant growth regulator combinations tested. Media M3 and M4 were found to be better suited for maximal plant recovery in cassava. Embryo maturation was characterized by the formation of cotyledonary embryos which differentiated into either cotyledonary leaves or a collar like cotyledon that ultimately formed a horn like structure. Alterations in auxin and cytokinin ratios are sufficient to control plant morphogenesis. In *Pinus caribaea*, it was found necessary to have a 1:2 NAA and BAP ratio to regenerate plants (Akaneme and Eneobong, 2008).

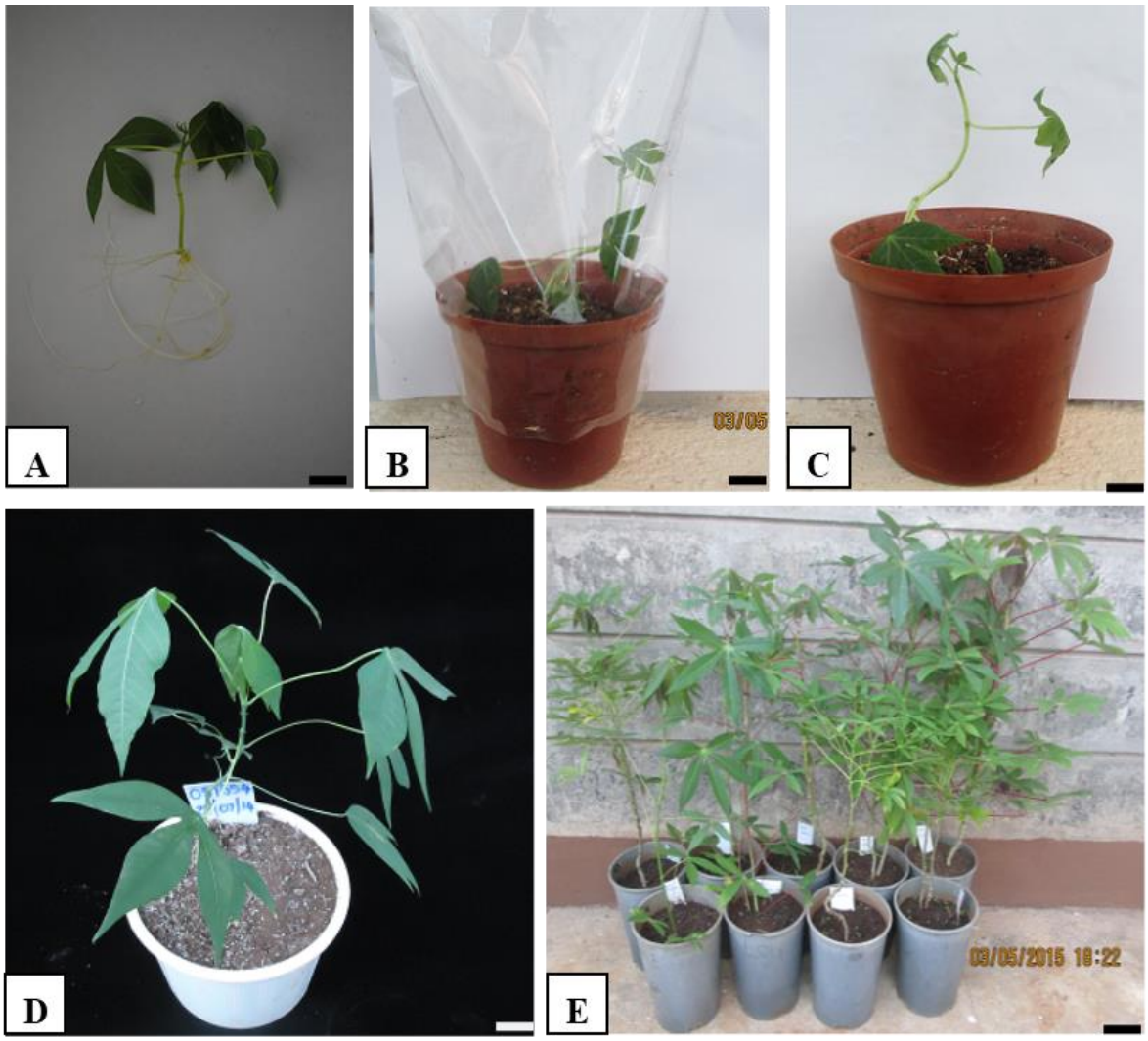

Figure 3. Acclimatization of in vitro cassava plantlets; A: Washed *in vitro* plantlet ready for hardening; B: Regenerated plantlets grown in peat moss covered with a polythene bags; C: Regenerated plantlet in (a) after gradual removal of the polythene bag; D: Regenerated plantlet grown in peat moss: soil mixture; E: Mature plants grown in soil. Scale bars: (A, B, C) 0.25 cm; (D, E) 1.0 cm.

According to Moura, (2009), the low rate of somatic embryo conversion to plantlets has hindered the improvement of *Acrocomia aculeate* using bio-technological techniques. To improve embryo conversion rates, GA_3 is usually added into such systems. In this study, an increase in plant recovery rates was observed with increase in the concentration of GA_3 used. Xu et al., (1997) and Yang and Choi (2000) also gave reports of significant stimulatory effects of GA_3 in cultures of *Sesamum indicum* and in *P. ginseng,* respectively.

Conclusion

Cassava regeneration via somatic embryogenesis can be coupled with genetic transformation and mutation breeding to mitigate the challenges associated with the application of conventional breeding for the improvement of the cassava genome. The regeneration pipeline must hence be optimized to suit each cultivar of interest. This study presents a somatic embryogenesis based cassava regeneration system capable of producing shoots within 5 to 6 months.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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

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***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

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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^{TM} 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 $p < 0.05$.

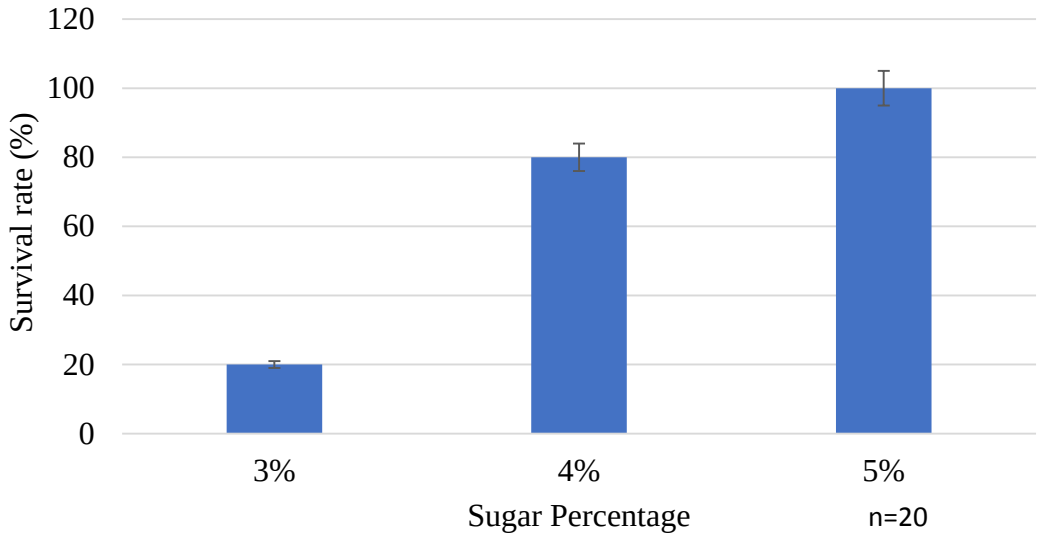

Figure 1. Survival rate of the plants after acclimatization based on the sugar concentration used to culture the plants in the pre-transplant stage in agar-gelled medium.

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.

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