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RESEARCH ARTICLE

Plant Tissue Culture

Micropropagation of *Stevia rebaudiana* (Bertoni) Bertoni using nutrient water of *Cocos nucifera* var. *aurantiaca (*King coconut) as a natural growth enhancer

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Abstract: *Stevia rebaudiana* (Bertoni) Bertoni is a herb producing zero-calorie diterpene glycosides in its leaves, which are extensively used as a substitute for sugar since they are about 200 – 400 times sweeter than sucrose. It is an economically important plant in the food and beverage industry around the world. The drawbacks associated with the conventional propagation through seeds and cuttings impede its mass production. Thus, effective *in vitro* micropropagation is advantageous on an industrial scale. Partial media replacement was done to the Murashige and Skoog (MS) medium with water from immature (6 – 7 months from flowering) *Cocos nucifera var. aurantiaca* (King coconut). Sterilized young nodal cuttings cultured on the MS medium with 10 % (v/v) coconut water (CW), without any hormones, induced 93.3 % bud break or shoot initiation from the nodes with a mean shoot length of 41.86 ± 0.85 mm. Increasing the CW to 30 % with 0.2 mg/L 6-benzylamino purine (BAP) produced the highest mean number of shoots (9.44 ± 0.51) and the highest number of leaves (8.28 ± 0.81) in three weeks. Modified rooting media incorporating only macronutrients, 0.2 mg/L of indole-3-butyric acid (IBA) and 5 % CW without micronutrients showed the earliest rooting (6.6 days). The highest number of root initiations (13.5) was with ½ MS + 0.2 mg/L IBA + 5 % CW. Regenerated plantlets were successfully hardened and acclimatized in glass jars with compost and sand (1:3 ratio) covered with polypropylene caps under greenhouse conditions. Our results suggest that *Stevia* can be propagated effectively with CW incorporated media and in the future, this could be used in small-scale commercial cultivations.

Keywords: Acclimatization, coconut water, nodal segments, rooting, shooting, *Stevia rebaudiana*.

INTRODUCTION

Stevia rebaudiana (Bertoni) Bertoni is a member of the family Asteraceae that produces zero-calorie diterpene glycosides in its leaves, which can be used as a substitute for sucrose (Megeji *et al.*, 2005; Goyal *et al.*, 2010). According to the reports, sweetness in *Stevia* comes from six compounds: stevioside, rebaudiosides A, D and E and dulcosides A and B (Kohda *et al.*, 1976; Kennelly, 2001) where stevioside acts as the prominent sweetener (Chang *et al.*, 1983). Studies have indicated that these glycosides are 200-400 times sweeter than sucrose (Wölwer-Rieck, 2012; Gupta *et al.*, 2013; Czinkóczky *et al.*, 2018; Chughtai *et al.*, 2020). The plant shows zero glycemic index since the human body is unable to breakdown these glycosides (Brandle *et al.*, 1992; Chughtai *et al.*, 2020). Although, most artificial sweeteners are known to have neurological or renal side effects, *Stevia* is known to have no side effects and is recommended for diabetes patients and has been extensively tested on animals (Megeji *et al.*, 2005). Additionally, it possesses anti-fungal and anti-bacterial properties, and can safely be used in herbal medicine, tonics for diabetic patients, and

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daily usage since mild *Stevia* leaf offers excellent relief for stomach problems (Momtazi-Borojeni *et al.*, 2017). *Stevia* has recently attained better awareness owing to its superior sweetness and curative values for restraining the deposition of fat and lowering blood pressure in humans (Singh *et al.*, 2005; Kalpana *et al.*, 2009; Chughtai *et al.*, 2020). Although the plant is native to Paraguay and Southern Brazil, it is now cultivated on a commercial scale for food, beverage, and pharmaceutical products in other countries such as Japan, Taiwan, Korea, Thailand, and Indonesia (Perera *et al.*, 2014). However, it will become a major source of high potency sweetener for the growing natural food market in the future.

Conventional propagation through seeds and cuttings has become unproductive due to inefficient sexual reproduction from self-incompatibility and insufficient pollination (Yadav *et al.*, 2011; Khalil *et al.*, 2014). Cross-pollination promotes heterogeneity in *Stevia* resulting in variability in chemical composition (glycoside) in plant parts (Sakaguchi *et al.*, 1982; Loc *et al.*, 2005; Ramírez-Mosqueda *et al.*, 2016). In addition, seeds lose viability within a short period and the presence of immature embryos result in germination problems (Khalil *et al.*, 2014). Vegetative propagation is limited by the smaller number of plants that can be obtained simultaneously from a single plant due to pathogen accumulation in the tissues (Sakaguchi *et al.*, 1982). Thus, under this scenario, stem cuttings and seed germination are not considered as effective methods to cater to the demand for commercial cultivation.

Micropropagation using plant tissue culture technology is a better option since it is capable of producing large numbers of genetically similar, disease-free plants in a short time under limited space. It is a conventional method of *in vitro* propagation where plants are grown in artificial nutrient media consisting of essential macronutrients, micronutrients, vitamins, amino acids, hormones, and sucrose. *Stevia* has been previously micropropagated with direct organogenesis through shoot tips (Ibrahim *et al.*, 2008; Seema *et al.*, 2011; Pawar *et al.*, 2015), leaves (Jain *et al.*, 2009; Kalpana *et al.*, 2010), nodal segments (Ma *et al.*, 2008; Seema *et al.*, 2011; Pawar *et al.*, 2015), and thin layer sections of hypocotyl (Ramírez-Mosqueda *et al.*, 2016).

When moving from traditional practices to novel techniques, such as plant tissue culture, the synthetic chemicals used in media preparation are expensive. The present study suggests the use of naturally available substitutes for synthetic chemicals in the tissue culture medium.

Coconut water (CW) is known to be a good source of nutrients, consisting of the unique chemical composition of sugar, vitamins, minerals, amino acids, and phytohormones (Yong *et al.*, 2009). It has been reported in many studies as an alternative source of nutrients for media preparation in plant tissue culture (Al-Khayri *et al.*, 1992; Dornelas *et al.*, 1994). Cytokinin is known to be the significant and most useful component in CW (Kende *et al.*, 1997) and together with auxins play a major role in plant morphology by controlling the growth of shoots, roots and overall growth of the plant. Though few studies have used CW in *Stevia* micropropagation (Ahmed *et al.*, 2016; Herath *et al.*, 2017), the nutrient composition of CW varies with climatic, environmental and geographical regions (Yong *et al.*, 2009). King coconut (*Cocos nucifera var. aurantiaca*) was recognized as an intermediate type of coconut among Sri Lankan varieties. According to Perera *et al.* (2014), king coconut belongs to the category with highest bunches per tree (annual average bunches is 16.5). Here, this variety was selected to extract CW due to its higher distribution around the country and nutritional composition (Herath *et al.*, 2017).

In this study, we investigated the effectiveness of using CW to replace synthetic chemicals (micronutrients, vitamins, and amino acids) in *in vitro* cultures of *S. rebaudiana.* CW was incorporated from the culture initiating step until rooting.

MATERIALS AND METHODS

Plant material Preparation

Shoots were taken from three-month-old *S. rebaudiana* plants grown in the greenhouse. Explants with 3 - 4 nodes (6-10 cm) were washed in running tap water for 10 -15 min and sequentially rinsed with liquid detergent (Teepol 1 % (v/v)) for 3-5 min to remove superficial dust particles, microorganisms and their spores. Explants were transferred to a laminar flow hood, washed with 5 % NaOCl with Tween 20® for 5 min and washed three times with sterile double distilled water. The same procedure was practised for another 5 min, followed by three rinses with sterile distilled water.

Preparation of CW and MS media

Young nuts (*Cocos nucifera var. aurantiaca)* at age of 6 - 7 months after flowering were collected from Kandy. The nuts were washed with tap water thoroughly to remove dust and dirt from the outside, and surface

sterilized with 1 % Teepol (v/v) followed by a wipe with 70 % ethanol (v/v) before transfer into the laminar flow hood. The nuts were cut inside the laminar flow hood to collect the nutrient water. This solution was not autoclaved, and freshly mixed with pre-autoclaved MS media at the time of media preparation to avoid the destruction of thermolabile compounds which are unique and vital for CW.

Full MS (Murashige *et al.*, 1962) media composed of macronutrients, vitamins and amino acids was mixed with 30 g/L of sucrose (commercial sugar). Sequentially, different levels of hormones (BAP and IBA) were added according to different plant development stages. The pH of the medium was adjusted to 5.8 ± 0.2 with 1 N NaOH and 1 N HCl, before adding 0.8 % Bacto agar as the gelling agent. The media were autoclaved in Erlenmeyer flasks at 1.06 kg/cm^2 and 121 °C for 20 min, mixed with the different levels of fresh CW, and poured into containers in the sterile bench.

Shoot initiation and multiplication

Nodal explants (1-1.5 cm) with a single axillary bud were cultured vertically under aseptic conditions on full MS (Murashige *et al.*, 1962) media fortified with different levels of CW (0, 10, 20, 30, and 40 %) (v/v), and the phytohormone BAP (0, 0.2, 0.5 mg/L) for shoot initiation and multiplication (Table 1). The experiment was arranged in a complete randomized design (CRD) with ten replicates for each treatment. Cultures were incubated at a constant temperature of 25 ± 1 °C with a 16 h photoperiod (2000 lux). Final data were recorded after three weeks on the number of explants showing shoot initiation, the number of days for shoot initiation, the average number of shoots per culture, the average length of shoots per culture and the average number of leaves per culture to identify best-performing media. Subcultures were done every three weeks after collecting the data. Nodal segments from the proliferated shoots were subcultured again in a medium which gave the highest shoot multiplication for further shoot production.

Rooting

Multiple shoots regenerated from the nodal segments were separated into individual shoots and cultured on MS medium (Full MS and ½ MS) with different concentrations of indole butyric acid (IBA) and CW. Different IBA levels (0, 0.2 mg/L) were used to promote rooting. Basal media were full strength MS and ½ strength MS with the incorporation of macro-elements and different concentrations of CW (0, 5, 10, 20 %). Since CW is a good source of micro-elements and vitamins, they were not added to the rooting media. The experiment was arranged in a (CRD) with ten replicates for each treatment.

Acclimatization

After 4 wks, well-rooted plantlets were separated from the cultures and sub-cultured on ½ MS media with 10 g/L of sucrose and without any growth hormones for 1 week. Before transferring to the soil, all the MS media around the roots were removed by rinsing gently with warm water under aseptic conditions. The plantlets were transferred to different hardening media inside glass bottles with compost and sand mixed in different ratios (compost : sand in 2:1, 1:1, 1:2 and 1:3) and covered with a transparent polythene cap for another 1 week. They were then kept in a propagator with 100 % relative humidity by spraying with water. The relative humidity was gradually reduced, and the light intensity gradually increased. After another 2 wks, the plantlets were transferred to larger pots. Survival rate was observed every week.

Data Analysis

The data were analysed by Analysis of Variance (ANOVA) and significant differences among the treatments determined with Tukey multiple range test at 5 % level of significance using the Minitab 17.0.1 statistical package.

RESULTS AND DISCUSSION

Number of days to initiate shoots and % of bud break

Shoots were initiated in all the treatments within 4 to 8 days showing its higher bud break potential as a herbaceous species. In the absence of CW and BAP, the explants required the maximum number of days to initiate shoots (around 7-8 days). However, a small amount of CW (10 %) made no significant change at $p < 0.05$ (7.50 ± 0.33 days) in the number of days, but showed the highest percentage of response in shoots (93.33 %). The rate was higher than that obtained with the media with BAP, showing that a small incorporation of CW can induce bud break regardless of the presence of artificial cytokinin (BAP) (Table 1). Another fact identified was that 10 % CW with 0, 0.2 and 0.5 mg/L of BAP gave the highest rates of bud breaks (93.33 %, 90.67 % and 89.33 %, respectively) showing that 10 % of CW is best to induce bud break in *Stevia.* Further, the results suggest that with a higher concentration of CW (40 %) and

BAP (0.5 mg/L), bud break was faster (4.16 ± 0.35 ds). However, at this concentration, the number of explants developing shoots declined to 70 - 72 % (Table 1). Altogether, with CW, more than 50 % of the explants initiated shoots, in its absence, it was 49 %, either with (0.2, 0.5 mg/L) or without BAP (Table 1). Although the percentage of explants initiating shoots was highest in the absence of BAP, its presence in the medium sustained shoot initiation at over 80 % (Table 1). Even though the present study reported a positive response with CW, studies by Ahmed *et al.* (2016); Wahyono *et al.* (2021) and Asmono *et al*. (2017) explained the inefficiency of CW in shoot induction of *Stevia*; the lowest duration was taken by the media that excluded CW.

In this study, CW from young nuts was selected, since they have more nutrients than mature nuts. Previously, autoclaved CW was used in experiments (Al-Kayri *et al.,* 1992; Dornelas *et al*., 1994; Ahmed *et al.*, 2016), while we used fresh CW and observed no media contamination. The cytokinin zeatin and the auxin IAA present in CW, which are known to be heat-sensitive, would have their efficiency altered if autoclaved (Yong *et al.*, 2009). Further, in contrast to the previous findings by Ahmed *et al.* (2016), Asmono *et al*. (2017) and Wahyono *et al.* (2021), we could observe positive results with CW for *Stevia*. It may be due to preserving nutrients that are not being destroyed by autoclaving. The quality of the CW or the nutritional composition depends on the maturity, variety, location, soil conditions, climate conditions, and other environmental conditions (Hall *et al.*, 2000). Therefore, nuts in the same maturity stage were collected from the same ecological zone to avoid the influence on the nutritional composition.

Vegetative growth (number of shoots, average length of shoots and average number of leaves per culture)

Vegetative growth determines the amount of active compounds that can be extracted from the *Stevia* plant. Hence, commercially, vegetative production is very important. In *Stevia,* the highest number of shoots were observed in the media with 20 % and 30 % of CW with 0.2 mg/L BAP (9.00 and 9.44) and the results are significantly higher ($p < 0.05$) than those of all the used concentrations (Table 1). A sudden drop in shoot number to 2.28 with 40 % of CW, shows the inhibitory effect of CW at higher concentrations even though BAP was present at lower concentration (0.2 mg/L). The results suggest that there is a considerable effect from CW on shoot multiplication. In the absence of BAP, shoot number was reduced to 2 to 3 (Table 1). A contrasting observation was that in the absence of CW, shoot production was higher in the treatments T14 and T15 (5.78 and 5.47, respectively). However, the presence of BAP (0.2 and 0.5 mg/L) has retrieved that effect of CW. Hence, the optimum conditions were identified as little incorporation of BAP (0.2 mg/L) with 20-30 % of CW.

Previously, Ahmed *et al.* (2016) obtained highest shoot multiplication with 10 % CW with BAP (2.0 mg/L), Kinetin (0.5 mg/L) and NAA (0.1 mg/L). However, Asmono *et al.* (2017), Herath *et al.* (2017), and Naranjo *et al.* (2016) found contrasting results with no significant growth in *Stevia* with CW or in combination with BAP, while higher concentrations inhibited the growth. Even though the findings of Ahmed *et al*. (2016) are compatible with our results, where a small supplementation of CW enhanced the shoot multiplication whilst increased concentration inhibited the growth, they have applied around 4-8 % of CW to enhance vegetative growth.

The average length of *Stevia* shoots were taken as a parameter since higher internodal distance is advantageous in subculturing as well as in initiation of new shoots by bud break. The highest length was observed with 10 % CW without BAP (41.86 mm) and in the media with only 10 % CW and 0.2 mg/L BAP (34.99 mm) showing that a lower amount of CW shows the highest growth rate even if the shoot number is lower (2.52). At the same time, shoot growth was significantly lower ($p < 0.05$) in the media with highest amount of BAP (0.5 mg/L) (11.38 mm) and the result was the same at 40 % CW. These results indicated that, when the number of shoots increase, leaves also increase. The highest number of leaves was observed as 8.28 in the media with 30 % CW and 0.2 mg/L BAP; the same media showed the highest number of shoots (Table 1). In the absence of CW, the cultures developed the least number (4 to 5) of leaves. Similar to our results, studies on *Passiflora* species by Dornelas *et al.* (1994) found that MS + 2.0 mg/L, and BA + 10 % CW induced the highest bud break while growth was suppressed with higher levels of CW. *Gloriosa superba* L. and *Dendrobium fimbriatum* Hook also have shown improved results compared to those without CW (Roy *et al.*, 2003; Ibrahim *et al.*, 2008).

In an experiment with olive, Peixe *et al.* (2007) showed the effect of CW in combination with BAP was similar to the more expensive cytokinin, zeatin. In *G. superba*, the addition of 15 % (v/v) CW to MS basal medium with BA, NAA and activated charcoal increased the number of shoots in *in vitro* cultures (Ibrahim *et al.*, 2008). Shoot induction and multiplication in *Curcuma zedoaria* Roxb. was also successful with 20 % CW (v/v) in combination with BA and kinetin (Roy *et al.*, 2003). In addition, CW has been successfully used for *in vitro* propagation of passion fruit (Hall *et al.*, 2000), coffee

(Ismail *et al.*, 2003) and orchids (Santos-Hernández *et al.*, 2005). Similar to our results with *Stevia*, CW alone was not sufficient to promote satisfactory shoot multiplication in all the above mentioned species; a blend of CW with BAP was necessary (Peixe *et al.*, 2007).

Callus development in *in vitro* cultures is undesired since nutrients in the culture medium are diverted to this undifferentiated mass of cells at the expense of shoot and leaf development. In this study, cultures with callus at the base of the explant retarded the development of shoots (Figure 1c). High levels of CW (20 %, 30 %, and 40 %) in combination with 0.5 mg/L BAP induced callusing in *Stevia* after three weeks of culture (Table 1). In the process of keeping the clonality of a species, avoiding callus-phase is advantageous. Thus, it is important to determine the optimum concentrations of CW and BAP for efficient micropropagation of *Stevia* without the proliferation of calluses. In the process of somatic embryogenesis, the induction of callus is an important stage. However, a study by Naranjo *et al.* (2016) described the inefficiency and the inhibiting activity of CW in the somatic embryogenesis of *Stevia*.

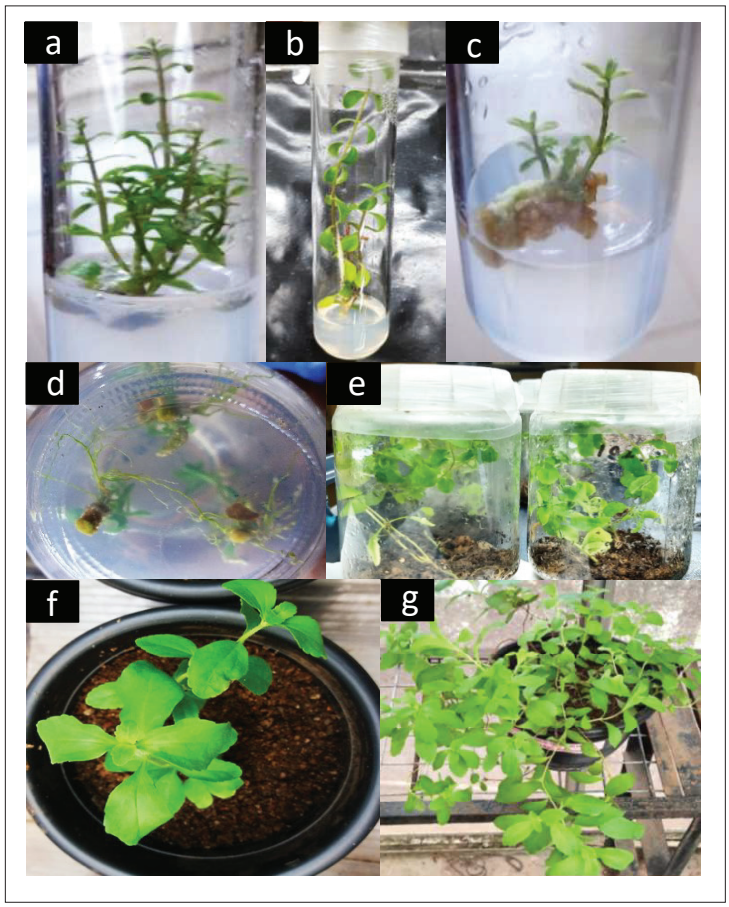

Figure 1: Different stages of in vitro growth of the *Stevia* plant (a). shoot multiplication (T7 – 30 % CW+0.2 mg/L BAP), (b). highest shoot length (T5 - 10% CW+0.2 mg/L BAP), (c). cuttings with callus initiation at the base showed retarded growth (T12 - 40% CW+0.5 mg/L) (d). rooting in media with ½ MS+ 5% CW+ 0.2 mg/L IBA, (e). acclimatization of plants under in vitro conditions, (f). *Stevia* plants after training to the natural environment, (g). fully grown plant after 2 months

Table 1: Effects of different concentrations of coconut water (CW) in MS medium with cytokinin (BAP) on in vitro shoot proliferation from nodal segments of *Stevia*. Data were recorded after three weeks of culture (-: no callus, +: very little callus, ++: moderate callus, +++: profuse callus)

Experiment	Amount of CW (%) (v/v) with full MS	BAP (mg/L)	% of bud break	Number of days for shoot initiation	Average number of shoots per culture	Average length of shoots per culture (mm)	Average number of leaves per culture	Callus growth at the base of the shoots (after 3 weeks)
T1	10	0	93.33	7.50 ± 0.33^{ab}	2.52 ± 0.65^{def}	41.86 ± 0.85^{a}	5.29 ± 0.42^{efgh}	-
T2	20	0	70.67	6.82 ± 0.48^{abcd}	2.78 ± 0.69^{def}	22.37 ± 0.46^{d}	6.82 ± 0.48^{bcd}	-
T3	30	0	54.67	6.06 ± 0.89^{def}	3.00 ± 0.33^{cdef}	19.91 ± 0.82^{e}	7.93 ± 1.13^{ab}	+
T4	40	0	68.00	5.28 ± 0.42^{fg}	2.80 ± 0.50^{def}	27.00 ± 0.59^{c}	6.06 ± 0.89^{cdefg}	++
T5	10	0.2	90.67	7.11 ± 0.31^{abc}	6.00 ± 0.88^{b}	34.99 ± 0.66^{b}	6.37 ± 0.25^{cdef}	-
T6	20	0.2	82.67	6.65 ± 0.39^{bcd}	9.00 ± 0.25^{a}	22.22 ± 0.92^{d}	7.96 ± 0.68^{ab}	+
T7	30	0.2	69.33	6.37 ± 0.25^{cde}	9.44 ± 0.51^{a}	21.40 ± 0.99^{de}	8.28 ± 0.81^{a}	++
T8	40	0.2	49.33	5.16 ± 0.16^{fgh}	2.28 ± 0.26^{ef}	15.50 ± 0.66^{g}	5.17 ± 0.17^{fgh}	++++
T9	10	0.5	89.33	7.34 ± 0.23^{abc}	4.44 ± 0.51^{bcde}	17.71 ± 0.83^{f}	6.65 ± 0.39^{bcde}	+
T10	20	0.5	86.67	5.10 ± 0.83^{fgh}	4.80 ± 0.64^{bcd}	16.82 ± 0.67^{fg}	7.11 ± 0.32^{abc}	+++
T11	30	0.5	72.00	4.60 ± 0.16^{gh}	5.39 ± 0.92^{bc}	16.80 ± 0.61^{fg}	5.10 ± 0.83^{fgh}	+++
T12	40	0.5	70.67	4.16 ± 0.35^{h}	1.92 ± 0.36^{f}	12.47 ± 0.34^{h}	4.70 ± 0.92^{gh}	++++
T13	0	0	48.00	7.77 ± 0.29^{a}	3.65 ± 0.10^{bcde}	21.33 ± 0.89^{de}	5.41 ± 0.21^{defgh}	-
T14	0	0.2	49.33	5.41 ± 0.20^{efg}	5.78 ± 0.84^{b}	18.07 ± 0.84^{f}	4.60 ± 0.17^{h}	++
T15	0	0.5	49.33	5.07 ± 0.69^{fgh}	5.47 ± 0.81^{b}	11.38 ± 0.63^{h}	5.07 ± 0.69^{fgh}	+++

Numbers represent the mean ± SE (standard error). Means with different letters are significantly different (Tukey, $p \leq 0.05$)

Table 2: Effect of different concentrations of coconut water (CW) in MS medium with auxin (IBA) on *in vitro* rooting of *Stevia.* Data were recorded after four weeks of culture

Basal media (MS)	Amount of CW (%)	IBA concentration (mg/L)	Days for root initiation	Mean number of roots
Full	0	0	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
Full	0	0.2	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
½	0	0	20.83 ± 0.40^{a}	2.75 ± 0.61^{d}
½	0	0.2	20.16 ± 0.75^{a}	8.33 ± 1.33^{b}
Full	5	0	6.66 ± 0.51^{d}	9.83 ± 0.52^{b}
Full	10	0	20.00 ± 0.54^{a}	3.00 ± 0.79^{d}
Full	20	0	18.00 ± 1.09^{b}	1.33 ± 0.52^{d}
Full	5	0.2	20.58 ± 0.49^{a}	3.16 ± 0.68^{d}
Full	10	0.2	20.91 ± 0.66^{a}	1.00 ± 0.00^{ef}
Full	20	0.2	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}
½	5	0	20.75 ± 0.60^{a}	2.75 ± 0.61^{d}
½	10	0	20.75 ± 0.61^{a}	6.25 ± 0.68^{c}
½	20	0	20.16 ± 0.75^{a}	3.33 ± 0.41^{d}
½	5	0.2	12.75 ± 1.47^{c}	13.53 ± 0.51^{a}
½	10	0.2	20.25 ± 0.88	1.51 ± 0.55^{f}
½	20	0.2	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}

Numbers represent the mean ± SE (standard error). Means with different superscripts shows significantly different values (Tukey, $p \leq 0.05$)

Rooting

The number of days to induce roots ranged from 6 to 20 days. Earliest rooting was observed after six days in a medium with MS + 5 % CW, which also produced the maximum mean number of roots (9.83 ± 0.52) (Table 2). The combination of ½ MS with 5 % CW and 0.2 mg/L IBA induced roots after 12 days (Table 2). The induction of roots was suppressed with the increasing levels of CW (20 %) in the medium (Table 2). The presence of CW with ½ MS medium did not promote early rooting.

According to the previous studies, different auxins have promoted root initiation and development of *Stevia*. Addition of 1.0 mg/L NAA with full MS achieved 63.2 % rooting within 12.3 days (Pawar *et al.*, 2015). Root growth of 4.4 cm was achieved in ½ MS with 1 mg/L IBA (Seema *et al.*, 2011). The full MS medium with 0.25 mg/L IAA produced 100 % rooting (7.6 roots per explant) within 3 weeks (Yücesan *et al.*, 2016), while ½ MS with 0.5 mg/L IBA produced 92 % rooting (Puneet *et al.*, 2018). Full MS with 0.2 mg/L IBA produced 93.33 % rooting (Majumder *et al.*, 2016), while full MS with 1.0 mg/L IBA or 1.0 mg/L NAA produced 66.67 % rooting (Ahmed *et al*., 2016). CW has been used as a media enhancer for rooting in limited species (Roy *et al.*, 2003). Even though the researchers have studied shoot proliferation of *Stevia* with CW (Ahmed *et al*., 2016; Asmona *et al*., 2017; Wahyono *et al*., 2021), they have not applied CW for root induction or root growth. According to Yong *et al.* (2009), CW from immature nuts contains 150 nM of IAA, which is an important auxin for root formation. Since the CW in the present study was not autoclaved, heat-sensitive IAA may be retained in the media in its natural form and induce roots.

Aman *et al.* (2013) observed that the rooting response decreases with the addition of IAA, NAA and IBA to full MS-medium. Full strength MS + 20 % CW + 0.2 mg/L IBA did not produce roots; indirectly, high nutrients in the culture medium encouraged callus growth at the base of the shoots which suppressed the development of roots. Recent studies have confirmed that a low concentration of MS salts without PGRs contributed to 90-100 % rooting of *S. rebaudiana* (Seema *et al.*, 2011; Gantait *et al.*, 2015; Ramírez-Mosqueda *et al.*, 2016). The above results indicate that *Stevia* is a plant, which needs a smaller amount of nutrients for its growth *in vitro* and the addition of minimum CW can stimulate the growth of shoots as well as roots. It was noted that growth

parameters were highly influenced by the addition of CW since their performance was very high when compared with controls without CW.

Acclimatization

The prerequisites for successful acclimatization were healthy *in vitro* plantlets with thick stems (high stem diameter), a large number of leaves and well-developed roots. Early subcultures of the shoots should be selected to induce roots since the survival of later subcultures was poor. Media composed of ½ strength MS without any hormones and reduced sugar content was used to encourage photosynthesis. The rooted plantlets were transferred to ½ strength MS media with a sugar content of 10 g/L and then shifted to soil media.

Accordingly, of the four treatments, compost: sand in 1:3 ratio showed the highest survival rate (93.33%) while increase of compost resulted in a reduced level of plant survival. Compost : sand in 2:1, 1:1, and 1:2 ratios showed a gradual increase in survival after three weeks (40.0 %, 60.0 %, and 66.7 %, respectively). The results suggest that under greenhouse conditions, *Stevia* requires well-drained soil to survive. When considering the survival of the *in vitro* cultured plants, hardening appears to be crucial and the most difficult part.

Morpho-physiological studies have noted the importance of controlling the water loss for the survival of cultured plants since they consist of little epicuticular wax and guard cells that are highly variable in morphology and size (Ziv *et al.*, 1987), and lacked starch grains in their cells (Ibrahim *et al.*, 2008). During hardening, the appearance of starch grains precedes the normalization of mesophyll cells. Hence, maintenance of microclimatic conditions is a must for a higher survival rate. The internal environment of the propagators, covered with polythene was gradually changed by increasing the temperature and light intensity (moving plantlets from culture room to greenhouse) and decreasing the humidity by opening the polythene. In a previous study of *in vitro* grown Carnation plantlet, the reduced humidity inside the culture tubes and higher agar concentration induced the stomatal development by increasing the survival percentage (Ziv *et al.*, 1987). When calluses were present at the base, plantlets did not survive. *Stevia* is a delicate plant and needs to be carefully monitored through the process of hardening. Under good conditions, plantlets can be hardened in six weeks.

CONCLUSION

Nodal cuttings of *S. rebaudiana* cultured on MS medium with 10 % CW induced 93.3 % bud break. Increasing the coconut water to 30 % with 0.2 mg/L BAP produced the highest mean number of shoots (8.44) and the highest number of leaves (8.28) in three weeks. Modified rooting media incorporating only MS macronutrients, IBA and 5 % CW, without micronutrients, showed earliest rooting (6.6 days). The highest number of root initiation (13.5) was with ½ MS+ 0.2 mg/L IBA and 5% CW. Regenerated plantlets were successfully hardened and acclimatized in glass jars with compost and sand with 3:1 ratio under greenhouse conditions. Our results suggest that *Stevia* plants can be propagated effectively with CW incorporated media and can be potentially adapted for small to medium scale commercial cultivation.

Conflicts of interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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