

Article

An efficient protocol for *in vitro* regeneration of *Stevia rebaudiana*

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Abstract: An efficient high frequency plant regeneration protocol through direct organogenesis was developed for *Stevia rebaudiana*. Shoot tips containing axillary buds were used as an explant and inoculated on Murashige and Skoog's (MS) medium containing 3% (w/v) sucrose, 0.6% (w/v) agar supplemented with various concentrations of benzy-ladenine (BA), kinetin (Kn) and thidiazuron (TDZ).. BAP proved to be a better choice than Kn and the maximum number of shoots (3.75) was obtained on 2.0 mgL⁻¹ BAP concentration. Considering all parameters, combination of BAP and Kn gave comparatively better performance than single BAP or Kn. TDZ was effective for multiple shooting. Though, 1.5 mgL⁻¹ TDZ gave the best number of shoots (14.5), but 1.0 mgL⁻¹ TDZ gave best performance in response to all parameters under study. The highest number of shoots was obtained in 60 mL⁻¹ coconut water, but 40 mL⁻¹ coconut water gave the best result to all parameters. Root induction was tested by using two auxins namely NAA and IBA at different concentrations (1.0, 1.5, 2.0 mgL⁻¹) on the MS medium. IBA at 1.0 mgL⁻¹ increased the rooting response (66.67%), number of roots (7.0) and root length (2.9 cm). Higher concentration of IBA and NAA (2.0 mgL⁻¹) showed poor results of rooting response (33.33%).

Keywords: *in vitro* regeneration, *Stevia rebaudiana*

1. Introduction

Stevia rebaudiana is one of the 154 species in the genus of *Stevia*. It is a herbaceous perennial shrub belonging to the sunflower family Asteraceae which is indigenous to Brazil and Paraguay (Uddin *et al.*, 2006; Alhady, 2011). It is commonly known as candy leaf, sweet leaf, sweet herb or honey leaf which is estimated to be 300 times sweeter than sugar cane (Chalapathi and Thimmegowda, 1997). *S. rebaudiana* has also been used as a medicinal plant to cure many diseases like obesity, hypertension, heartburn, hypoglycemia and to lower the uric acid levels (Ahmed *et al.*, 2007). *Stevia* has a potential commercial value. So, with the approval of FDA committee for consumption of *Stevia* as a food supplement for sweetening, it is commercially cultivated in Brazil, Uruguay, Central America, Israel, Thailand, Australia, Japan, Korea and China. *Stevia* largest producer is China with about 13, 400 ha of planted area and about 40 000 tons of *Stevia* leaves every day. In recent years, *Stevia* products find widespread use in the food industry (Savita *et al.*, 2004; Midmore and Rank, 2006). Conventionally, it is propagated by seeds or stem cuttings. Propagation through seed is not adequate owing to low seed germination percentage. The stem cutting method has limitations such as low number of new plants and destruction of the donor plant (Razak *et al.*, 2014). To overcome these obstacles, tissue culture techniques is commonly used as a viable alternative for the production of disease free plants within a shorter period of time with less cost (Satpathy and Das, 2010). The hormonal balance between auxins and cytokinins can regulate the formation of roots, shoots and callus tissue *in vitro* (Papry *et al.*, 2015). Furthermore, the hormones auxins and cytokinins have a multitude of complex interactions, which control plant development.

2. Materials and Methods

The research work was conducted at laboratory of the Department of biotechnology and physics with a common help of central laboratory, Bangladesh Agricultural University, Mymensingh during the period from July to November, 2015. Shoot tips were used as explants for shoot regeneration. All the explants were collected from 4-6 month old yard grown plants from physics department of BAU. Five experiments with different treatments were carried out in this research. Single or combined effect of cytokinin (BAP or Kn), effect of thidiazuron, effect of coconut water concentrations on shoot proliferation and effect of auxin (NAA and IBA) on root induction of *Stevia rebaudiana* was observed in these experiments.

2.1. Preparation of MS medium

MS medium was used to detect the effect of hormone and coconut water concentration on the shoot and root development of natural sweetener containing herb *Stevia rebaudiana*

Following procedure was maintained to prepare 1000ml of MS media with care-

- a) The appropriate amount of each of stock solutions (macrosalts, microsals, Fe-EDTA, vitamins, Myo-inositol)
- b) 500 ml distilled water was added in the flask to dissolve all the ingredients on a heater cum magnetic stirrer.
- c) 30 gm sucrose was added to this solution and agitated gently to dissolve completely.
- d) Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.
- e) The whole mixer was then made up to 1L with adding distilled water.
- f) The pH of the liquid medium was adjusted to 5.75-5.80 with the aid of 0.1N HCl or 0.1 N NaOH, wherever it necessary. This operation was done using a digital pH meter.
- g) 0.6% agar was added to the liquid medium to make solid medium. The culture medium was dispensed into culture vial (20 ml/vial)
- h) Medium was finally sterilized by autoclaving.

2.2. Sterilization

Contamination can be occurred by air, explants, contaminated vials or workers. Elimination of microbiological organisms for aseptic condition is mandatory, to conduct a successful *in vitro* regeneration process. Both MS were poured in 25 ml small sterilized vials which was autoclaved at 121°C at 1.16 kg/cm for 20 minutes for sterilization. Sterilization of glassware and other instruments achieved by following approaches: (i) dry heat, (ii) flame sterilization, (iii) autoclaving, (iv) wiping with 70% ethanol. Further sterilization was done under aseptic conditions in a laminar air flow cabinet.

2.2.1. Surface sterilization of plant material

The explants were cut into small pieces (about 1.5 cm long) and initially washed under running tap water for 6-7 mins. Explants were rinsed with distilled water containing few drops of savlon and tween-20 with continuous shaking for 10-15 seconds and washed thoroughly with distilled water. They were then taken under laminar air flow cabinet to soak them with 70% alcohol for 10 seconds and washed 3-4 times with autoclaved distilled water. Finally, explants were immersed in 0.1% commercial HgCl₂ solution in combination with 2-3 drops tween-20 for 4-5 minutes with continuous shaking followed by adding sterilized distilled water 6-7 times to drain out last particle of HgCl₂.

2.3. Explant inoculation technique

The explants were inoculated on MS medium fortified with cytokinins (BAP, Kinetin and TDZ in single or in combination) for shoot induction and auxins (NAA and IBA) for root induction. The pH of the media was adjusted at 5.75-5.80 before gelling the medium with 0.6% agar. The cap of vial was held by little finger and was flame sterilized. During inoculation special care was taken so that the explants must touch the medium equally and not dept into the medium.

2.4. Incubation

After inoculating the explants onto culture media, cultures were incubated on culture rack at 25 ± 2⁰ C constant temperature under the white 25 ± 2⁰ C. The photoperiod was maintained as 16 hours light and 8 hours dark.

2.5. Collection of data

Data were collected on the effect of different treatments on direct shoot and root regeneration. For shoot induction, data were recorded in terms of days required for shoot initiation, percentage of shoot regeneration, shoot length (cm) and number of shoots per explants. The days required for shoot initiation, percentage of rooted shoots, number of roots formed, average root length (cm) were determined after 3 weeks of culture on the rooting medium.

2.6. Statistical analysis

The data for the character under present study were statistically analyzed wherever applicable. The experiment was conducted in growth room and arranged in completely randomized design (CRD). The analysis of variance of different characters was performed and means were compared by the Duncan's multiple range test (DMRT).

3. Results and Discussion

3.1. Effect of cytokinin (BAP or Kn) on shoot proliferation of *Stevia rebaudiana* after 4 week

3.1.1. Number of shoots explant⁻¹

The significant variation data of shoot regeneration have been presented in Table 1 and Figure 1 where it was found that the treatment 2 mgL⁻¹ BAP produced the highest number of shoot explant⁻¹ (3.750) followed by 3 mgL⁻¹ BAP (3.25). On the other hand, the treatment T₀ (control) showed the lowest number of shoot explant⁻¹ (1.664) which also differed significantly from all other treatments. To a certain extent, with increasing concentration of BAP and kinetin, an increase in number of multiple shoots was observed. Higher concentrations of BAP in the medium resulted in a reduced number of shoots. By comparing all parameters of all treatments, 2 mgL⁻¹ BAP gave best performance. This result partially supported Mehta *et al.*, (2012). Between the two hormone (BAP and Kn), BAP induced higher number of shoots than Kn. Among the different treatment of Kinetin, the lowest number of shoots (2.332) was achieved from 1 mgL⁻¹ Kn which was closely related to BAP (1 or 2 mgL⁻¹) and Kn (2 or 4 mgL⁻¹). Mathur and Begum (2015) showed enhanced shoot proliferation on MS medium containing BAP and Kn in the range 1.0-5.0 mgL⁻¹. BAP at its 3.0 mgL⁻¹ concentration evoked best response. According to Pawar *et al.*, (2015) the higher number of shoots per explants (eight) was obtained from nodal explants as compared to shoot tip (six) on the medium i.e. (MS + 2 mgL⁻¹ BAP). Kavitha *et al.*, (2012) reported that shoot tip explants cultured on MS medium supplemented with Kn (3.0 mgL⁻¹) produced the maximum number of shoots (12.6 ± 0.68) per explants. This indicates that these explants contain sufficient endogenous level of auxins or capable of its de novo synthesis which can induce shoot formation even in a medium containing cytokinin alone (Julliard *et al.*, 1992).

Cytokinins, especially BAP, are reported to overcome apical dominance, release lateral buds from dormancy and promote shoot formation (George, 1993). Superiority of BAP in inducing multiple shoot formation has also been reported for a number of plants e.g. *Tridax procumbens* (Sahoo, Chand 1998), *Cypripedium flavum* (Yan *et al.*, 2006) and *Medicago truncatula* (Neves *et al.*, 2001). Kn alone was not very efficient in inducing shoot multiplication in the present experiments. Low rate of multiplication in medium containing Kn has been observed in a number of plants e.a. *Bambusa balcooa* (Mudai and Borthakur, 2009), *Ocimum gratissimum* (Gopi *et al.*, 2006) and *Mentha arvensis* (Chishti *et al.*, 2006).



Figure 1. No. of shoots and length of shoots at 2 mgL⁻¹ BAP after 21 days.

Table 1. Effect of BAP or Kn on shoot proliferation of *Stevia rebaudiana*.

BAP / Kinetin concentrations (mgL ⁻¹)	Days to shoot initiation	Number of shoots vial ⁻¹	Shoot regeneration (%)
0	3.332 d	1.664 e	70.00 b
BAP 1.0	4.250 c	2.500 cd	80.00 a
BAP 2.0	4.750 bc	3.750 a	80.00 a
BAP 3.0	5.500 a	3.250 ab	80.00 a
BAP 4.0	5.000 ab	2.664 bcd	60.00 b
Kn 1.0	4.664 bc	2.332 d	60.00 b
Kn 2.0	5.250 ab	2.500 cd	80.00 a
Kn 3.0	5.500 a	3.000 bc	80.00 a
Kn 4.0	5.500 a	2.500 cd	40.00 c
LSD_(0.05)	0.629	0.578	3.13
CV (%)	10.05	16.71	3.53
Level of significance	**	**	**

In a column figures having similar and no letter(s) do not differ significantly at 5% level whereas figures with dissimilar letter(s) differ significantly as per DMRT at same level.

DMRT= Duncan's Multiple Range Test; LSD= Least significant difference and CV= Coefficient of variation; **= Significant at 1% level of probability

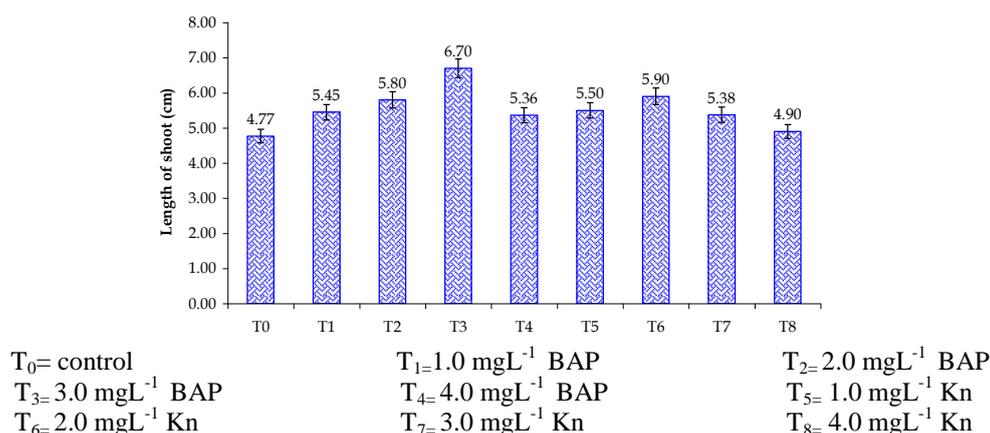
3.1.2. Shoot regeneration

Shoot regeneration was significantly influenced by the effect of different concentrations of BAP or Kinetin where it varied from 40 to 80% (Table 1). From the Table 1, it was found that the treatment BAP (1, 2, 3 mgL⁻¹) and Kn (2-4 mgL⁻¹) showed same regeneration percentage of shoot (80.00%). On the other hand, treatment 4 mgL⁻¹ Kn produced the lowest regeneration of shoot (40%) which was statistically differed from all other treatment of the study. According to Mathur and Begum (2015), 2 mgL⁻¹ BAP gave the highest response (80%).

3.1.3. Length of shoot

Statistically significant variation was observed among different treatments on the length of plantlets. The length of plantlets varied from 6.7cm to 4.768 cm (Figure 2). The longest length of shoots (6.7 cm) was recorded in 3 mgL⁻¹ BAP among all the treatments of both BAP and Kn. 2 mgL⁻¹ Kn gave longest shoot (5.9 cm) among all the different concentration of Kn treatment and had statistically identical shoot length with 2 mgL⁻¹ BAP. The shortest length of shoot (4.768 cm) was observed in control (0 mgL⁻¹ hormone) which was statistically similar to 4 mgL⁻¹ Kn (4.90). Rest of the treatments had also four statistically identical shoot length which was observed in both case of BAP (1,4 mgL⁻¹) and Kn (1,3 mgL⁻¹). According to Hassanen and Khalil (2013) shoot length decreased by increasing Kn concentrations above 2.0 mgL⁻¹. Superiority of BAP over kinetin was also demonstrated by several workers (Van-Eck *et al.*, 1990; Sen *et al.*, 1991; Mishra *et al.*, 1995).

Shekhawatat (2012) reported that, although the number of shoots induced was considerably higher, stunted growth was observed at higher concentrations of BAP (3-5 mgL⁻¹). In most cases, BAP was found to be essential for growth and better than Kn for shoot induction (Tawari *et al.*, 2010). Shahriyar *et al.*, (2015) found the lowest percentage of shoot multiplication was 63% and length was 3 cm obtained in Murashige and Skoog +0.3 mg/l gibberellic acid within 10-15 days.

**Figure 2. Effect of BAP or Kn on shoot proliferation of *Stevia rebaudiana*.**

3.1.4. Days to shoot initiation

The number of days required for shoot initiation varied significantly. There was a significant difference within the medium, for all observations. The most early shoot initiation was observed in 4.25 days from MS + 1 mgL⁻¹ BAP medium among all the treatments of different concentration of BAP or Kn except control. The highest days required for shoot initiation was found 5.5 days from three similar hormone concentration (3 mgL⁻¹ BAP and 3-4 mgL⁻¹ Kn) which was statistically closely related to 4 mgL⁻¹ BAP and 2 mgL⁻¹ Kn.

Although, BAP (2 and 3 mgL⁻¹) and Kn (2 and 3 mgL⁻¹) gave highest shoot regeneration, 2 mgL⁻¹ BAP gave the best performance by taking into account all other parameters like percentage of shoot regeneration (80%) along with average number of shoots per explants (3.750), average length of shoot (5.80 cm) and days required to shoot regeneration (4.75 days).

3.2. Combined effect of cytokinins (BAP and Kn) on shoot proliferation of *Stevia rebaudiana* after 4 week

In the combined effect of treatments, the percentage of shoot regeneration, days required for shoot initiation, number of shoot per explant and length of shoot showed statistically significant.

3.2.1. Shoot regeneration

The highest percentage of shoot regeneration (100%) was recorded in on MS medium supplemented with 1 mgL⁻¹ BAP+ 1 mgL⁻¹ Kn. Statistically similar shoot regeneration percentage (83.33%) was observed in 0.5 mgL⁻¹ BAP in combination with 0.5-2 mgL⁻¹ Kn. Lowest shoot regeneration percentage (50%) was obtained in 1 mgL⁻¹ BAP in combination with 3 mgL⁻¹ Kn which was statistically differed from other treatments.

3.2.2. Number of shoots

The highest number of shoots per explants (3.6) was found in 0.5 mgL⁻¹ BAP + 2 mgL⁻¹ Kn which was closely related to 1 mgL⁻¹ BAP + 1 mgL⁻¹ Kn and 1.0 mgL⁻¹ BAP + 2.0 mgL⁻¹ Kn (Table 2). This result was supported by the finding of Alhady *et al.*, (2011) who obtained the maximum number of proliferated shoots on MS medium supplemented with 2.0 mgL⁻¹ BAP + 0.5 mgL⁻¹ Kn. However, MS medium supplemented with Kn resulted in elongated shoots. Statistically identical number of shoots per explant was obtained in T₁, T₂, T₄, T₅ which gave 2.8, 3.0, 2.75 and 2.75 number of shoot respectively. The reason for higher number of shoots per explants might be due to uptake of nutrients easily of explant. This result was partially supported Mehta *et al.*, (2012) who obtained best response was on medium containing 0.5 mgL⁻¹ BAP + 0.5 mgL⁻¹.

Table 2. Effect of treatment combinations of different BAP and Kn concentrations on shoot proliferation of *Stevia rebaudiana*.

BAP + Kinetin concentrations (mgL ⁻¹)	Days to shoot initiation	Number of shoots vial ⁻¹	Shoot regeneration (%)
0	3.500 g	1.500 d	66.67 c
0.5 + 0.5	3.800 fg	2.800 bc	83.16 b
0.5 + 1.0	4.200 efg	3.000 bc	83.33 b
0.5 + 2.0	4.800 cde	3.600 a	83.33 b
0.5 + 3.0	5.500 c	2.750 bc	66.67 c
1.0 + 0.5	4.500 def	2.750 bc	66.67 c
1.0 + 1.0	5.167 cd	3.333 ab	100.0 a
1.0 + 2.0	6.750 b	3.250 abc	66.67 c
1.0 + 3.0	7.663 a	2.663 c	50.00 d
LSD_(0.05)	0.797	0.542	6.447
CV (%)	13.4	16.29	7.46
Level of significance	**	**	**

In a column figures having similar and no letter(s) do not differ significantly at 5% level whereas figures with dissimilar letter(s) differ significantly as per DMRT at same level.

DMRT= Duncan's Multiple Range Test; LSD= Least significant difference and CV= Coefficient of variation; **= Significant at 1% level of probability

3.2.3. Days to shoot initiation

The days required for shoot appearance was statistically significant ranged from 7.663 to 3.500 days (Table 2). The reason for varied duration to shoot appearance might be due to genetical make up of the genotypes. The minimum number of days required for shoot initiation was observed in control (T₀). The highest days required for shoot initiation was recorded in 1 mgL⁻¹ BAP+ 3 mgL⁻¹ Kn. The days required for shoot initiation was increased with the increase of hormone concentration.

3.2.4. Shoot length

The longest shoot length (6.444 cm) was observed in 0.5 BAP in combination with 0.5 mgL⁻¹ Kn (Table 2 and Figure 3) which was followed by 1 mgL⁻¹ BAP + 1 mgL⁻¹ Kn (5.86 cm). The lowest shoot length (4.43 cm) was recorded at 0 mgL⁻¹ BAP + 0 mgL⁻¹ Kn which was preceded by 1 mgL⁻¹ BAP + 3 mgL⁻¹ Kn (4.80 cm). In the present study, gradual increase in shooting frequency, number of shoots per explant was observed with increasing concentrations of BAP in combination with Kn. In a nutshell, it can be concluded that combination of 0.5 mgL⁻¹ BAP and 2 mgL⁻¹ Kn showed the best performance to all parameter. This study also similar to the result of Pawar *et al.*, (2015). Mehta *et al.*, (2012) reported that the maximum number of shoots (3.42±0.58) was developed on MS media fortified with 0.5 BAP±2.0 Kn. According to Sridhar and Aswath (2014) the maximum regeneration frequency (70%), shoot number (5.0) and shoot length (4.4 cm) were observed in the combination of 2.0 mgL⁻¹ BAP + 0.5 mgL⁻¹ Kn + 0.1 mgL⁻¹ IAA. It can be concluded that with the increase of cytokinin hormone combination, number of shoot increased and length of shoot decreased. At higher concentration of hormone combination, it may be stunted growth of shoot.

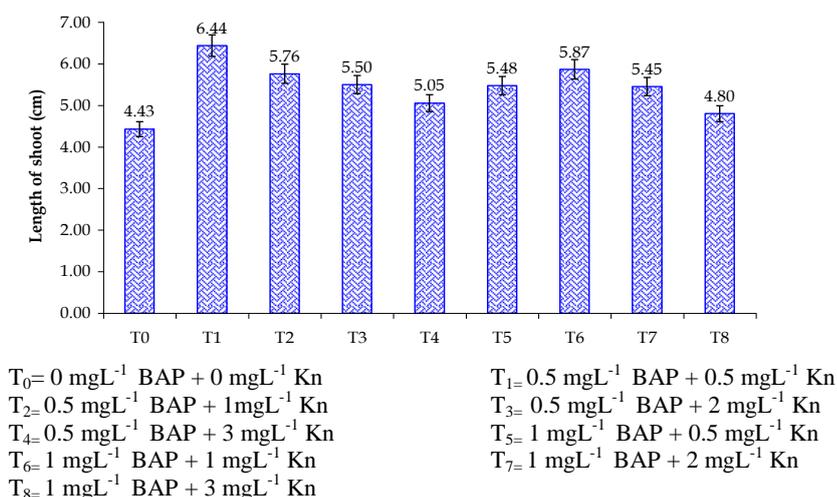


Figure 3. Effect of treatment combinations of different BAP and Kn concentrations on shoot proliferation of *Stevia rebaudiana*.

3.3. Effect of different concentrations of TDZ on direct shoot regeneration of *S. rebaudiana* after 3 week

3.3.1. Shoot regeneration percentage

The explants were inoculated with different concentrations of TDZ (0.5, 1.0, 1.5, 2.0 mgL⁻¹) in combination with 1.0 mgL⁻¹ BAP to observe their effect on shoot development and multiplication. The highest percentage (75%) of explant showing shoot was recorded in TDZ at 0.5 mgL⁻¹ which was similar to TDZ 1.0 and 0 mgL⁻¹ (75%). The lowest percentage of explants (50%) showing shoot was recorded in 1.5 and 2.0 mgL⁻¹ TDZ (Table 3). TDZ is a phenylurea type plant growth regulator, which was used earlier as a cotton defoliant (Arndt *et al.*, 1976). Later, it was believed to exhibit strong cytokinin-like activity almost similar to that of N6-substituted adenine derivatives (Mok *et al.*, 1982; Gyulai *et al.*, 1995). It has been shown to induce shoot proliferation (Niewkerk *et al.*, 1986).

Table 3. Effect of different concentrations of TDZ on shoot proliferation of *Stevia rebaudiana*.

TDZ concentrations (mgL ⁻¹)	Days to shoot initiation	Number of shoots vial ⁻¹	Shoot regeneration (%)
0	6.332	2.665 e	75.00 a
0.5	6.665	7.332 d	75.00 a
1.0	7.000	11.33 b	75.00 a
1.5	6.500	14.50 a	50.00 b
2.0	6.500	8.500 c	50.00 b
LSD_(0.05)	0.836	0.836	4.172
CV (%)	8.22	6.12	4.17
Level of significance	NS	**	**

In a column figures having similar and no letter(s) do not differ significantly at 5% level whereas figures with dissimilar letter(s) differ significantly as per DMRT at same level.

DMRT= Duncan’s Multiple Range Test; LSD= Least significant difference and CV= Coefficient of variation; **= Significant at 1% level of probability and NS= not significant

3.3.2. Days required for shoot appearance

The days required for shoot appearance differs non-significantly (Table 3). The explants treated with 1 mgL^{-1} TDZ took highest number days for shoot appearance (7.00 days) followed by 0.5, 1.5 and 2.0 mgL^{-1} TDZ. But TDZ 0 mgL^{-1} (control) took lowest number days for shoot appearance (6.332 days).

3.3.3. Number of shoot per explant

Noticeable variation was observed among the treatments in respect of number of shoot per explants (Table 3). The highest number of shoots per explants (14.0) was recorded in 1.5 mgL^{-1} TDZ followed by 1.0 mgL^{-1} , 2.0 mgL^{-1} TDZ (Figures 4 and 5). The TDZ concentration more than 1.5 mgL^{-1} reduced the number of shoot per explants. The lowest number of shoots were obtained from T_0 (control contained only 1 mgL^{-1} BAP). The higher concentrations of TDZ (2.0 mgL^{-1}) resulted depletion in shoot regeneration. Yucesan *et al.*, (2007) reported that higher concentration of TDZ reduced number of shoots per explants. This was due to the adverse effect of higher concentration of TDZ. But in both parameters, values of shoot number parameter started to decline after the third level.



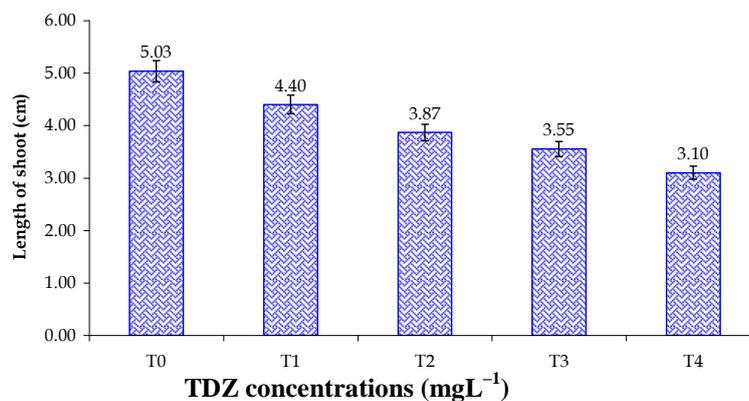
Figure 4. Shoot multiplication at $0.5, 1.0, 1.5 \text{ mgL}^{-1}$ TDZ respectively after 21 days.



Figure 5. No. of shoots at 1.5 mgL^{-1} TDZ.

3.3.4. Shoot length

Different levels of TDZ had significant effect on length of shoot (Figure 6). The longest length of shoot (5.033 cm) was observed in control containing 1 mgL^{-1} BAP without TDZ. Length of shoot reduced with the increase of TDZ concentration. The longest shoot (5.03 cm) was measured in control (T_0) followed by TDZ 1 mgL^{-1} (4.4 cm). The shortest length of shoot (3.1 cm) was noted in TDZ 2.0 mgL^{-1} . It is clear that an increase in TDZ concentration could promote the rate of shoot proliferation but there was a certain limitation. Moreover, the rising of shoot number might affect the length since the nutrients taken up were distributed to more shoots (Huy and Xuan-Mai, 2014).



T₀: 0 mgL⁻¹ TDZ
T₃: 1.5 mgL⁻¹ TDZ

T₁: 0.5 mgL⁻¹ TDZ
T₄: 2.0 mgL⁻¹ TDZ

T₂: 1.0 mgL⁻¹ TDZ

Figure 6. Effect of different concentrations of TDZ on shoot proliferation of *Stevia rebaudiana*.

3.4. Effect of Coconut water on shoot induction of *S. rebaudiana* after 3 week

Coconut water is the colorless liquid endosperm of green coconuts (*Cocos nucifera*). Coconut water is the more complex combination of compounds than coconut milk (Molnar *et al.*, 2011). To measure the effect of coconut water, explants were cultured on MS medium with four different concentrations: 20 mL⁻¹, 40 mL⁻¹, 60 mL⁻¹ and 80 mL⁻¹. Diphenylurea, a growth factor found in coconut water, exhibits cytokinin-like responses (Gnasekaran *et al.*, 2010).

So, as a source of cytokinin, 20-80% coconut water was added to the medium. For number of shoot, the appropriate doses of coconut water for *Stevia* was 60 mL⁻¹ in MS medium which gave maximum number of shoot (7.332 cm) (Figure 7). Statistically similar number of shoot was obtained from 40 mL⁻¹ and 80 mL⁻¹ coconut water. The lowest number of shoots per explant (1.66) was recorded in control. Control gave the longest length of shoot (5.33 cm) (Table 4). By comparing all the parameter of the coconut water treatment, the appropriate doses of coconut water for *Stevia* was 40 mL⁻¹ in MS medium which gave longest length of shoot (5.10 cm), 4.75 number of shoot and required 5.7 days to shoot initiation (Table 4).

Highest percentage of shoot regeneration was obtained from 20 and 40 mL⁻¹ coconut water which were similar to each other (Table 4). Kuraishi & Okumura (1961) reported the cytokinin activity in fresh coconut water. This class of plant growth regulator has the main function to stimulate cell division, subsequently to morphogenesis. Some natural cytokinins have been isolated from coconut water, such as zeatin and N⁷-diphenyl urea (George, 2008), but levels of cytokinins and their specificity in coconut water are still unclear.

Table 4. Effect of different concentrations of coconut water along with 1.0 mgL⁻¹ BAP + 0.1 mgL⁻¹ NAA on shoot proliferation of *Stevia rebaudiana*.

Coconut water (ml/L)	Days to shoot initiation	Number of shoots vial ⁻¹	Length of shoot (cm)	Shoot regeneration (%)
0	4.332 d	1.664 d	5.300 a	60.00 b
20	5.250 c	3.000 c	4.300 b	80.00 a
40	5.750 bc	4.750 b	5.100 a	80.00 a
60	6.332 ab	7.332 a	4.032 c	60.00 b
80	6.668 a	5.332 b	3.632 d	60.00 b
LSD(0.05)	0.695	0.712	0.252	6.704
CV (%)	9.15	12.02	4.2	7.35
Level of significance	**	**	**	**

In a column figures having similar and no letter(s) do not differ significantly at 5% level whereas figures with dissimilar letter(s) differ significantly as per DMRT at same level.

DMRT= Duncan's Multiple Range Test; LSD= Least significant difference and CV= Coefficient of variation; **= Significant at 1% level of probability



Figure 7. No. shoots and length of shoots at 60 mL⁻¹ after 3 week

Peixe *et al.*, (2007) also showed the positive effect of coconut water *in vitro* culture as substitute of Zeatin which is an important organic compound used in the process of micropropagation. This means that the supplementation of culture medium with coconut water can be beneficial to growth and morphogenesis of tissues, not only due the mineral nutrition that it provides but also because it is a source of natural growth regulators. The beneficial effect of coconut water was clearly observed on plant growth parameters such as shoot length, number of shoot (Table 4).

3.5. Effect of auxin (NAA and IBA) on root induction of *S. rebaudiana*

Formation of plantlet which was the aim of *in vitro* regeneration includes shoot formation followed by root initiation. Auxins (NAA) have characteristic feature of promoting cell division, stem elongation and rooting (Razdan, 2003). The regenerated shoots were implanted onto MS medium fortified with varying concentration of NAA (1.0, 1.5, 2.0 mgL⁻¹) and IBA (1.0, 1.5, 2.0 mgL⁻¹). Assessment of root formation was studied through the parameters percent of root initiation, days to root initiation and number of root formation per shoot and length of root. 4-cm-and-above shoots were transferred to rooting media in order to help the plants take up nutrients more efficiently (Table 5 and Figure 8). It was revealed from table 11 that, the MS medium without auxin was not useful for rooting. However, The highest percentage of root formation (66.67%) was found in NAA (1,1.5 mgL⁻¹) and 1.0 mgL⁻¹ IBA, where lowest percentage of root formation (33.33%) was observed in 1.5 mgL⁻¹, 2.0 mgL⁻¹ IBA and 2.0 mgL⁻¹ NAA which are same to one another (Table 5).

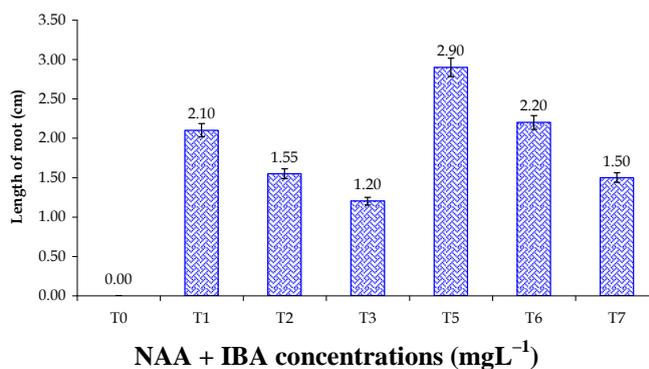
Different concentrations of NAA and IBA significantly affected the length of roots and number of roots. Both NAA and IBA at 1.0 mgL⁻¹ concentration gave the best response (66.67%) in case of number of roots and length of roots (Figure 8). Between these two rooting hormones (IBA and IAA), IBA produced the higher number of roots (7.0) per shoot. MS media supplemented with 1 mgL⁻¹ NAA were found to be the best for rooting in terms of rooting percentage (66.67 %) and days to root initiation (9.5). Moreover, the longest root (2.9 cm) was recorded in 1mgL⁻¹ IBA followed by 1.5 mgL⁻¹ IBA and the shortest root (1.2 cm) was recorded in 2 mgL⁻¹ NAA (Figure 6). The highest number of root (7.0) was recorded in MS medium supplemented with 1.0 mgL⁻¹ IBA followed by 1.0 mgL⁻¹ NAA (Figures 9 and 10). The lowest number of root was found in both 2 mgL⁻¹ NAA and IBA. No root was found at both 0 mgL⁻¹NAA and IBA.

Table 5. Effect of different concentrations of NAA or IBA on root formation of *Stevia rebaudiana*

NAA / IBA concentrations (mgL ⁻¹)	Days to root initiation	Number of roots shoot ⁻¹	Root formation (%)
0	0.000 e	0.000 e	0.000 c
NAA 1.0	9.500 d	4.500 b	66.67 a
NAA 1.5	10.50 c	3.500 c	66.67 a
NAA 2.0	12.00 b	2.000 d	33.33 b
IBA 1.0	10.50 c	7.000 a	66.67 a
IBA 1.5	12.00 b	3.000 c	33.33 b
IBA 2.0	16.00 a	2.000 d	33.33 b
LSD_(0.05)	0.561	0.692	3.625
CV (%)	3.64	14.37	5.52
Level of significance	**	**	**

In a column figures having similar and no letter(s) do not differ significantly at 5% level whereas figures with dissimilar letter(s) differ significantly as per DMRT at same level.

DMRT= Duncan's Multiple Range Test; LSD= Least significant difference and CV= Coefficient of variation; **= Significant at 1% level of probability.



T₀=0 mgL⁻¹ NAA + 0 mgL⁻¹ IBA
 T₁=1 mgL⁻¹ NAA + 0 mgL⁻¹ IBA
 T₂=1.5 mgL⁻¹ NAA + 0 mgL⁻¹ IBA
 T₃=2.0 mgL⁻¹ NAA + 0 mgL⁻¹ IBA
 T₄=0 mgL⁻¹ NAA + 1 mgL⁻¹ IBA
 T₅=0 mgL⁻¹ NAA + 1.5 mgL⁻¹ IBA
 T₆=0 mgL⁻¹ NAA + 2 mgL⁻¹ IBA

Figure 8. Effect of different concentrations of NAA or IBA on root formation of *Stevia rebaudiana*

The maximum days for root appearance were recorded on 2 mgL⁻¹ IBA. The minimum days required for root initiation was observed in 1 mgL⁻¹ NAA. Root induction gradually decreased with increasing concentrations of auxin of both NAA and IBA. NAA was less effective for root formation and promoting root extension. A significant difference was found on root length at different concentrations and combinations of growth regulators. There were noticeable differences in the morphology of roots from two different hormone treatments. IBA tended to develop thick and hairy fibrous roots while NAA gave rise to less hairy and thin roots. MS liquid medium containing 1.0 mgL⁻¹ IBA produced a greater number of healthy and sturdy roots than those cultured with NAA (Nagesh, 2008).



Figure 9. No. roots and length of roots at 1 mgL⁻¹ NAA after 3 week



Figure 10. No. roots and length of roots at 1 mgL⁻¹ IBA after 3 week

4. Conclusions

Present studies were conducted with a view to evaluate the effect of different concentration of cytokinin hormone (BAP, Kn, Thidiazuron), coconut water on shoot regeneration and the effect of different concentration of auxins (NAA and IBA) on root formation. Micropropagation technology of *Stevia rebaudiana* has been briefly analyzed. Thus, this protocol could be helpful to establish and cultivate stevia as a promising newly introduced non-caloric sweetener and anti-diabetic medicinal plant in Bangladesh.

Conflict of interest

None to declare.

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