Development of a protocol for *in vitro* regeneration of *Stevia rebaudiana* Bertoni

ABSTRACT

The experiment was conducted to find out an effective hormonal combination and concentration for *in vitro* regeneration of *Stevia rebaudiana* Bertoni, a medicinally important, zero-calorie value, sweet tasted and an antidiabetic herb. Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of BAP and NAA were used for callus induction and shoot regeneration using shoot tip as explant. The highest shoot regeneration percentage (83.33%) was obtained from the MS medium supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA followed by 72.92% of shoot regeneration from MS medium supplemented with 3.0 mg/l BAP + 3.0 mg/l NAA. The lowest percentage (29.17%) of shoot regeneration was observed in MS medium with 0 mg/l BAP + 1.0 mg/l NAA. Significant numbers of shoots were proliferated in the present study through an intervening callus phase showing normal phenotypes. The micro-cuttings of *in vitro* proliferated shoots were inoculated on MS medium supplemented individually with 0, 1.0, 6.0 mg/l IBA in each of 16 treatments for root initiation. Among the treatments used for root initiation, the highest percentage (75 %) of root formation was observed in MS medium supplemented with 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA followed by 71.43 % in MS medium with 6 mg/l IBA + 3.0 mg/l BAP + 3.0 mg/l NAA. In contrast, the lowest percentage (14.28 %) of root formation was obtained in MS medium supplemented with 6 mg/l IBA + 0 mg/l BAP + 1.0 mg/l NAA. For maximum output , full strength MS media supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA ,in combination with full strength MS media supplemented with 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA should be used for micropropagation at commercial level.

Key words: *Stevia rebaudiana*, *in vitro* regeneration, plant growth regulator, MS media.

INTRODUCTION

Stevia rebaudiana Bertoni belongs to the family Asteraceae. It is one of 154 members of the genus Stevia and one of only two species that produce sweet steviol glycosides. Dry leaves of *Stevia rebaudiana* are 300 to 450 times more sweeter than sucrose (Chang *et al.,* 2007; Yu and Shi, 2009; Silvia and Luciana, 2013) and also contain flavonoids, vitamins, tannins, phytosterols, minerals, alkaloids, hydroxycynnamic acid, essential oils and other miscellaneous compounds with antimicrobial as well as antioxidant properties (Tadhani and Subhash, 2006; Darabpour *et al.,* 2010; Muanda *et al.,* 2011; Mondaca *et al.,* 2012; Rieck, 2012; Galbis *et al.,* 2014). Consumption of dry leaves of *Stevia rebaudiana* Bertoni positively influences the human health by regulating hypotension, enhancing antiglycaemic activity, preventing dental cavity, decreasing heart rate or improving digestion and gastrointestinal functioning (Lee *et al.,* 2001; Midmore and Rank, 2002; Jeppesen *et al.,* 2003; Gupta *et al.,* 2013). With the current demand for food supplements having low carbohydrate, minimum calorie and low sugar content large scale production of *Stevia rebaudiana* Bertoni is needed. But seeds of *Stevia rebaudiana* Bertoni show a very low germination percentage (Felippe and Lucas, 1971; Monteiro, 1980; Toffler and Orio, 1981), and vegetative propagation through cuttings is limited by the small number of individuals (Sakaguchi and Kan, 1982). The conventional approaches to stevia mass propagation or multiplication are less effective, impulsive, time-consuming, and unreliable. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition (Nakamura and Tamura, 1985). Commercial cultivation requires a homogeneous range of improved plants; however, plants germinated from seeds exhibit variability. Field conditions exhibit significant variation due to external environmental factors, including plant pathogens, temperature, drought, and waterlogging, resulting in differences in composition and sweetening levels (Nakamura and Tamura, 1985).

Considering the prospects of commercial propagation of Stevia, the present investigation was commenced to develop a simple, rapid, and economical, reproducible and high frequency regeneration protocol from shoot tips of *Stevia rebaudiana* Bertoni to be used in large scale propagation. This experiment also focused on optimizing suitable concentrations and combinations of commonly available plant growth regulators for effective proliferation of shoots and roots.

MATERIALS AND METHODS

The experiment was conducted during the period from January 2023 to November 2023 in the Plant Tissue Culture laboratory, Department of Biotechnology, Bangladesh Agricultural University (BAU), Mymensingh. Shoot tips of healthy, vigorous *Stevia rebaudiana* Bertoni field grown plants were used as explant. Explants were washed in running tap water and then treated with 2% savlon with constant shaking for 5-6 minutes and washed thoroughly with distilled water. Then the explants were taken under laminar air flow cabinet. The surface sterilization of the explants were done with 0.1% mercuric chloride solution and 1-2 drops of Tween-20 to remove the superficial dust particles

as well as fungal and bacterial spores for 5 minutes under aseptic condition followed by washing 5-6 times with sterilized distilled water and finally shoot tips were inoculated aseptically for *in vitro* propagation on culture medium. MS (Murashige and Skoog, 1962) medium was used as culture medium and sixteen different combinations of NAA and BAP were used (Table 1) for shoot induction (Table 1).

Table 1: Treatments with different concentrations of BAP and NAA for shoot initiation

Treatment	Concentration of growth regulator (BAP+NAA) mg/l	Treatment	Concentration of growth regulator (BAP+NAA) mg/l
T1	0+0	T9	2+0
T2	0+1	T10	2+1
T3	0+2	T11	2+2
T4	0+3	T12	2+3
T5	1+0	T13	3+0
T6	1+1	T14	3+1
T7	1+2	T15	3+2
T8	1+3	T16	3+3

The culture vessels with inoculated explants were incubated both in dark and light (the photoperiod was maintained as 16 hours light and 8 hours dark) in a temperature controlled growth room (25 ± 1°C). Subculture was performed within same concentration at regular interval until explants showed sufficient shootlet formation. When the plantlets showed proper growth they were rescued aseptically from the culture vessels and were separated from each other and again subcultured on culture vessels with freshly prepared root induction medium containing IBA (0, 1, 6 mg/l) along with sixteen shoot proliferation treatments for consistent growth both shoots and roots. To investigate the effect of different treatments on *in vitro* regeneration of *Stevia rebaudiana*, data were collected on number of shoots per culture, shoot length, percentage of shoot regeneration, days to shoot initiation, number of roots per plantlet, percentage of root induction and days to root initiation. Then percentage of shoot regeneration and root induction was calculated on the basis of following formula:

$$\text{Percentage of shoot regeneration} = \frac{\text{Number of explants showing shoot}}{\text{Number of inoculated explants}} \times 100$$

$$\text{Percentage of root induction} = \frac{\text{Number of shoots with root}}{\text{Number of inoculated shoots}} \times 100$$

The recorded data were statistically analyzed using Microsoft Statistical (MSTAT) programme and Microsoft Excel wherever applicable. The experiment followed Completely Randomized Design (CRD) design and data for the characters under the present study were statistically analyzed following analysis of variance (ANOVA) technique. The analysis of variance was performed and means were compared by Least Significant Difference (LSD) test at 5% level of probability for results (Gomez and Gomez, 1984) and the mean differences were adjusted by DMRT (Ducan's Multiple Range Test) and the ranking was indicated by letters.

RESULTS AND DISCUSSION

Effect of BAP and NAA on shoot regeneration in *Stevia rebaudiana* Bertoni

Shoot initiation of *Stevia rebaudiana* was significantly influenced by different concentrations of BAP and NAA. After inoculation of explant in the culture medium, shootlets were proliferated. The highest number of shootlets per culture (4.170) was observed in 1.0 mg/l BAP + 2.0 mg/l NAA supplemented MS medium whereas explants inoculated in MS medium without growth regulator did not proliferate any shootlets until 30 days after inoculation (Table 2).
Laribi *et al.* (2012) found in their experiment that BAP (1 mg l^{-1}) and IAA (0.25 mg l^{-1}) combination was superior for multiple shoot bud induction (4.25 shoots). BAP was identified as the most effective cytokinin for promoting multiple shoot regeneration in the experiment of Thiyagarajan and Venkatachalam (2012). Our experiment uses combination of NAA and BAP, that's why the shoot regeneration is satisfactory. Shulgina *et al.* (2021) experimented on Stevia considering impacts of both PGPRs and light effect on regeneration *in vitro*, they found that *S. rebaudiana* microshoots, cultured on MS media containing 1.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} IAA with red monochrome light treatments, increased the multiplication coefficient by 30% compared with controls (white light, media without PGRs).
Uddin *et al.* (2006) observed multiple shoots from modified MS medium with 1.0 mg/l BAP. Sivaram and Mukundan (2003) used BA (2 mg/l) and IAA (1 mg/l) to regenerate shoots from shoot tips, nodal and leaf segments of *Stevia rebaudiana* Bertoni. Sritongkum (1995) found that MS medium supplemented with 12.0 mg/l Kinetin was good for shoot multiplication from shoot tips.
The highest shoot regeneration percentage (83.33%) was obtained when the MS medium was supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA followed by 72.92% of shoot regeneration from 3.0 mg/l BAP and 3.0 mg/l NAA. The lowest percentage (29.17 %) of shoot regeneration was found in 0 mg/l BAP + 1.0 mg/l NAA (treatment 2) and MS medium without growth regulator did not show any response. The data revealed that percentage of shoot regeneration gradually increased with increasing concentrations of NAA (Fig. 1).

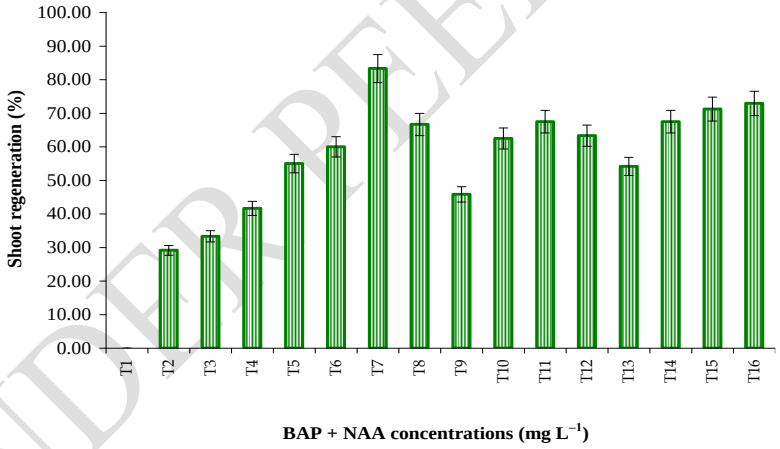

Figure 1 : Effect of different concentrations and combinations of BAP and NAA on percentage of shoot regeneration in *Stevia rebaudiana* Bertoni

Ali *et al.* (2010) observed the maximum shoot formation when BAP was used in combination with kinetin. Sridhar and Aswath (2014) also observed the best result with the combination of BAP and Kinetin for shoot regeneration.
The longest shoot (6.53 cm) was produced while MS medium was supplemented with 1.0 mg/l BAP + 2.0 mg/l NAA and the shortest shoot (1.28 cm) was produced in MS medium with 0 mg/l BAP + 1.0 mg/l NAA (Figure 2). The shortest time (10 days) required for shoot initiation when the explants were cultured in MS medium with 1.0 mg/l BAP + 2.0 mg/l NAA whereas the longest time (32 days) was observed when the culture medium was supplemented with 0.0 mg/l BAP + 1.0 mg/l NAA (Table 2). It was found that small amount of BAP in combination with increasing amount of NAA (0, 1.0, 2.0 and 3.0 mg/l respectively) increased percentage of shoot regeneration and shoot length

in *Stevia rebaudiana* Bertoni at a certain limit and then decreased the percentage of shoot regeneration and shoot length gradually (Figure 1 and 3). This may happen as most of the plant hormones are functional at very low concentrations and a balance of BAP and NAA promotes the highest shoot regeneration.

Table 2: Effect of different treatments on various shoot characteristics of *Stevia rebaudiana*

Treatments	No. of shootlets culture^{-1}	Shoot regeneration (%)	Shoot length (cm)	Days required for shoot initiation
T1	**0.000 l**	**0.000 l**	**0.000 l**	-
T2	1.430 k	29.17 k	1.280 k	**32.00 a**
T3	1.670 j	33.33 j	2.410 j	28.00 b
T4	2.000 i	41.67 i	3.120 i	26.30 c
T5	2.780 g	55.00 g	4.180 g	25.00 d
T6	3.000 f	60.00 f	4.680 f	19.20 gh
T7	**4.170 a**	**83.33 a**	**6.530 a**	**10.00 j**
T8	3.330 d	66.66 d	4.790 f	21.50 f
T9	2.140 h	45.83 h	3.960 h	25.10 d
T10	3.130 e	62.50 e	4.970 e	18.50 hi
T11	3.330 d	67.50 d	5.030 de	22.00 f
T12	3.180 e	63.33 e	5.110 d	**17.50 i**
T13	2.860 g	54.17 g	4.210 g	24.20 de
T14	3.330 d	67.50 d	4.790 f	23.50 e
T15	3.460 c	71.25 c	5.510 c	19.70 g
T16	**3.640 b**	**72.92 b**	**6.180 b**	18.40 hi
LSD (0.05)	**0.1179**	**1.374**	**0.1179**	**1.01**
CV (%)	**2.57**	**1.51**	**1.67**	**2.93**
Level of significance	**	**	**	**

****=** Significant at 1**%** level of probability

Figure 2: Plate 1-2: Shoot proliferation in Stevia rebaudiana in MS medium supplemented with of 1.0 mg/l BAP + 1.0 mg/l NAA

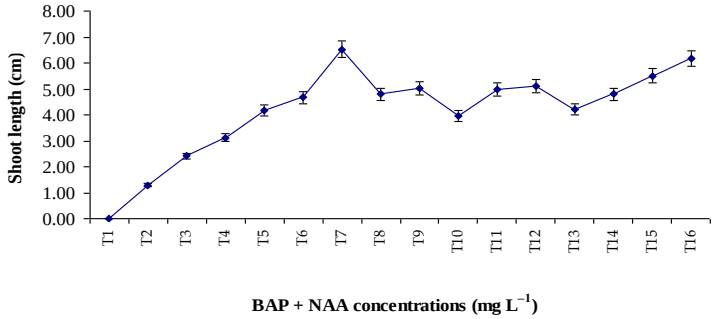

Figure 3 : Effect of different concentrations of BAP and NAA on shoot length of *Stevia rebaudiana*

Root formation in regenerated shoots

Each Single shootlet of *in vitro* proliferated shoots were carefully isolated and inoculated on MS medium supplemented individually with 0, 1.0, 6.0 mg/l IBA in each of 16 treatments for root initiation. No root formation was observed while IBA concentration was 0 and 1.0 mg/l. Among the treatments used for root initiation the highest percentage (75 %) of root formation was observed with MS medium + 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA followed by 71.43 % when using MS medium + 6 mg/l IBA + 3.0 mg/l BAP + 3.0 mg/l NAA. In contrast, the lowest percentage (14.28 %) of root formation was obtained in MS medium + 6 mg/l IBA + 0 mg/l BAP + 1.0 mg/l NAA (Table 3 and Figure 4). Uddin *et al.* (2006) recorded more than 90% rooting in modified MS medium with 1.5 mg/l IBA. The lowest number of days (9.30) were required for root induction with MS medium + 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA. This may be due to selection of appropriate growth regulator with suitable concentration.

Table 3. Effect of different concentrations of BAP, NAA and IBA on root initiation of *Stevia rebaudiana*

Concentration of BAP, NAA and IBA (mg L^{-1})	Root formation (%)	No. of roots plantlet^{-1}	Days for root induction
0 + 0 + 0	0.000 l	0.000 m	0.000 m
0 + 1.0 + 6.0	**14.28 k**	**0.710 l**	21.20 a
0 + 2.0 + 6.0	16.67 j	0.830 k	20.10 b
0 + 3.0 + 6.0	20.00 i	1.000 j	19.50 b
1.0 + 0 + 6.0	33.33 h	1.670 i	17.50 cd
1.0 + 1.0 + 6.0	50.00 e	2.500 f	15.20 fgh
1.0 + 2.0 + 6.0	**75.00 a**	**3.750 a**	9.300 l
1.0 + 3.0 + 6.0	58.34 d	3.000 d	14.20 hi
2.0 + 0 + 6.0	45.72 f	2.370 g	16.20 ef
2.0 + 1.0 + 6.0	57.14 d	2.860 e	15.10 gh
2.0 + 2.0 + 6.0	58.57 d	2.920 de	14.00 ij
2.0 + 3.0 + 6.0	48.57 e	2.500 f	16.50 de
3.0 + 0 + 6.0	42.86 g	2.140 h	17.90 c
3.0 + 1.0 + 6.0	50.00 e	2.500 f	16.00 efg
3.0 + 2.0 + 6.0	66.67 c	3.330 c	13.00 j
3.0 + 3.0 + 6.0	**71.43 b**	**3.570 b**	11.30 k
LSD (0.05)	**1.374**	**0.1179**	**1.01**
CV (%)	1.86	3.14	4.09
Level of significance	**	**	**

**= Significant at 1% level of probability

Figure 4: Plate 3- 4: Root formation in proliferated shoots of *Stevia rebaudiana* using MS medium + 6 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l NAA

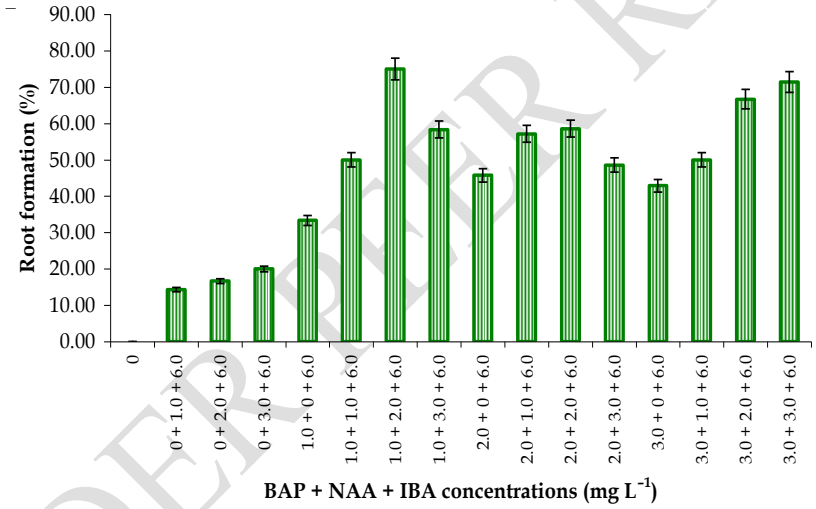

Figure 5: Percentage of root formation in regenerated shoots of *Stevia rebaudiana* using different concentrations and combinations of BAP + NAA + IBA (mg L^{-1}).

In contrast, the maximum number of days (21.20) were required for root induction when using MS medium + 6 mg/l IBA + 0 mg/l BAP + 1.0 mg/l NAA. No root formation was observed in MS medium without growth regulator.

Similar types of results were found by earlier workers in the same species (Sivaram and Mukundan, 2003; Ahmed *et al.*, 2007; Mitra and Pal, 2007). However Tadhani and Subhash (2006) reported 0.1 mg/L IBA was the best concentration for rooting.

Razak et al. (2014) reported that maximum number of roots (30.12 ± 2.1 roots per explants) was obtained on a MS medium containing 1.0 mg L-1 IBA. Our experiment used combination of IBA, BAP and NAA that ensured best root regeneration under existing circumstances. Kumari and Chandra (2015) found in their experiment that among different

concentrations of IBA, IAA and NAA, 1 mg L^{-1} IBA was proved most effective with maximum numbers (7.6 ± 0.3) of roots which supports findings of our experiment.

CONCLUSION

The use of phytohormones in tissue culture is a crucial step in the process. In our experiment, we used several combinations of NAA, BAP and IBA for regeneration of stevia from shoot tips. After callus production from explant, we have divided the experiment in two phases; shoot elongation from callus and root initiation in shootlets. 16 combinations of treatments for shoot initiation and 16 combinations of treatments for root initiation was used. Among this, MS medium supplemented with 1.0 mgL^{-1} BAP + 2.0 mgL^{-1} NAA could be recommended for multiple proliferation of shoot from callus. MS medium supplemented with 6.0 mgL^{-1} IBA+ 1.0 mg/l BAP + 2.0 mg/l NAA could be used for root formation.

The result of the experiment clearly support the possibility of mass propagation *Stevia rebaudiana* Bertoni by adopting above mentioned *in vitro* technique. Thus, *in vitro* propagation can become an important alternative to conventional breeding procedures for *Stevia rebaudiana* Bertoni.

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