



A Novel Protocol for *Stevia rebaudiana* (Bert.) Regeneration

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Abstract: *Stevia rebaudiana* Bertoni has sweet substances (stevioside) in its leaves that are free of calories and their consumption is beneficial for diabetic patients and is also helpful in high blood pressure also. Because of low capability in seed germination, tissue culture is an appropriate method for propagation of this plant. In the current study, optimization of stevia *in vitro* cultivation via direct organogenesis with different explants, light intensities and plant hormones has been examined. These treatments included BAP (at 0.5, 1, 1.5 and 2mg/l) in combination with 2,4-D, IBA and NAA (each with concentrations of 0.1, 0.2 and 0.5mg/l) and different light intensities (Dark, 2000, 4000 and 6000 lux). MS was utilized as a basal medium. Results indicated the highest rate of organogenesis (85%) occurred on the axillary buds explants with a medium containing 1.5mg/l BAP + 0.1mg/l NAA under 6000 lux light intensity. Also, the highest range of primary organ per explant (42) with 0.3cm length was achieved at this condition. The most efficient medium for rhizogenesis i.e. 100% root production along with the highest root number (11 with approximately 7.13cm length) was obtained in presence of activated charcoal and 1mg/l of IBA. At the end of rhizogenesis experiments, the plantlet length and node multiplicity were 12.8cm and 7 respectively. Greenhouse cultivation of these plantlets was successful.

Keywords: Stevia, Organogenesis, Axillary bud, IAA, IBA, BAP and NAA.

1. Introduction

Stevia (Stevia rebaudiana Bertoni) is a perennial plant, which belongs to Asteraceae family. This plant endemically grows in southern America, Amambay high areas and suburbs of Mandi River (a zone between Brazil and Paraguay (Soejarto *et al.*, 1983). In addition to pharmaceutical benefits, this sweet plant has been used to sweeten beverages and food for centuries (Priyanka and Banerjee, 2009). Sweetener substances (especially stevioside) in stevia leaves are 30 times stronger than sucrose (Bhosle, 2004). Since stevioside is not absorbable in human body, it will not be translated in terms of calories (Tanaka, 1982); so it could be a suitable item to replace artificial sweeteners such as aspartame, sodium saccharin and cyclamate.

Being non-fermentable and high-temperature stability at 100-centigrade degree are other interesting characteristics of this plant. Because of presence of

flavonoid and phenolic compounds in stevia, this plant has also antioxidant and antimicrobial virtues (Uddin *et al.*, 2006), which are useful in cancer and heart disease treatment (Jeppesen *et al.*, 2003). Stevia is sensitive to cold and drought and requires sufficient water and moderate temperature. Its shoot is delicate and fragile with small ellipsoid leaves that have alternate phyllotaxis. It has also an expanded root system (Entrepreneur, 2004). Stevia propagates by slips and seed, nevertheless, its seed has low capability in germination (Felippe *et al.*, 1971; Felippe and Lucas, 1971, Toffler & Orio, 1981). Moreover, its multiplication by seed does not let homogenous population production and subsequently affect its commercial characteristics such as variation in sweetening rate and secondary metabolites ratio (Tamura *et al.*, 1984; Nakamura and Tamura, 1985). On the other hand, the propagation of this plant by slips cannot be a professional method, as it is revealed that

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the plants arising from shoot cutting are low in stevioside content (Sakaguchi & Kan, 1982). Thus, tissue culture technique is considered as a proper approach to stevia propagation (Handro, 1977; Bondarev *et al.*, 1998, 2002).

Recent reports have shown that plant population produced by direct organogenesis from shoot meristem and leaf explants are homogenous (Tamura, 1984; Miyagawa, 1986). Therefore, genetically identical plants could be provided via regeneration in large scales. The first researches on stevia tissue culture brought in mass propagation of stevia through cultivation of shoot apex meristem in a medium containing high level of kinetin (10mg/l) and light intensity of 5000 lux. Afterward, a range of further experiments on stevia tissue culture are carried out such as application of shoot primordial explants with a light intensity of 6000 lux (Akita *et al.*, 1994), shoot apex, nodal and leaf explants along with a light at $80\mu\text{mol}/\text{m}^2\text{s}^{-1}$ (Sivaram & Mukundan, 2003), nodal explants treated by 2500 lux of light (Rafiq *et al.*, 2007) and a leaf direct organogenesis with a light intensity of $40\mu\text{mol}/\text{m}^2\text{s}^{-1}$ (Sreedhar *et al.*, 2008). Other researchers (Ahmed *et al.*, 2007; Debnath, 2008) have also reported using nodal explants without considering the importance of light intensity. Literature review reveals stevia rhizogenesis firstly occurred through shoot explants in MS medium contained 0.1mg/l NAA (Tamura *et al.*, 1984). Then, Ferreira & Handro (1988) reported stevia rhizogenesis by utilization of MS medium including BAP along with IBA. Additionally, Sivaram & Mukundan (2003) achieved 100% of rooting in $\frac{1}{2}$ MS with IBA. Rafiq *et al.*, (2007) explained that maximum root production would be obtained in MS medium containing 0.5mg/l NAA. At the same time, Ahmed & Salahin (2007) attained 97% stevia rooting in MS medium along with 0.1mg/l IAA in their experiments. Successful root production attempts in $\frac{1}{2}$ MS + IBA, and MS + 2mg/l IBA are already reported (Sreedhar *et al.*, 2008; Ibrahim *et al.*, 2008). Ibrahim *et al.*, (2008) expressed that among NAA, IBA and IAA, IBA is the best substance for stevia rooting. But, Jain (2009) has shown MS medium with 0.5mg/l NAA as the most efficient medium for stevia rooting.

To improve stevia tissue culture, in the present study complementary examinations were conducted with different explants (shoot, leaf, and axillary bud) subjected to various light intensities and plant growth regulators in MS medium.

2. Materials and methods

2.1 Experimental conditions

Direct Organogenesis experiments (in two different steps including shoot and root induction) were accomplished for stevia regeneration. Different explants from shoot, leaf and axillary buds of stevia were cultivated in Murashige and Skoog (MS), as the basal

medium, under different light treatments including dark, 2000, 4000 and 6000 Lux, for several consecutive generations. Organogenesis induction medium was prepared using BAP (in 0.5, 1, 1.5 and 2mg/L levels) in combination to three kinds of auxin (IBA, 2,4-D, NAA; each at 0.5, 1, 1.5 and 2mg/L) classified as three tests; following mentioned as the first (BAP + 2,4-D), second (BAP + IBA) and third (BAP+ NAA) tests. The basal medium without hormones was observed as control. Ten numbers of each explant were placed in separate Petri dishes followed with incubation in a germinator with 2000, 4000 and 6000 lux light intensities and $25 \pm 1^\circ\text{C}$ (16L/8D) for 5 weeks. After induction of primary organs, the samples were subcultured and remained in the same conditions as above for one month. Subsequently, the new plant organs arising from the primary explants were evaluated.

2.2 Histological studies

After organogenesis experiments, for validation of displayed direct organogenesis (with no callus phase) an axillary bud cultivated on the medium through tissue culture was selected for histological studies. A mixture of formalin (90ml) + acetic acid (5ml) + ethanol (5ml) was used as a fixation solution. Dehydration procedure was performed using different degrees of ethanol (70, 80, 90 and 100%). Samples were embedded in paraffin (with a melting degree of 85°C). The sectioned samples were $40\mu\text{m}$ in diameter. To stain for light microscopy, methylene blue and carmine were applied.

2.3 Root induction

Root induction experiments has been performed in MS medium with IAA, IBA and NAA treatments (all at 0, 1 and 2mg/L concentrations) with or without activated charcoal (AC). The medium without hormones was considered as control. Cultivated samples were kept in a growth chamber under $25 \pm 1^\circ\text{C}$ (16L/8D) and 6000 lux light for one month. After that, to assess the best medium for rhizogenesis, the numbers and length of produced roots on each explant were measured. In addition, the plantlet length and number of produced nodes, if any, were also recorded.

2.4 Hardening and transferring to greenhouse

Intact *in vitro* plantlets were washed to eliminate the medium adhering to the roots. Then the plantlets were transferred to plastic pots containing pit-mass and were placed in the phytotron under high humidity (60-70%). After one week, plantlets were transferred into larger pots in the greenhouse.

2.5 Experimental Design and Statistical Analysis

The organogenesis experiments were carried out as a completely randomized design in form of factorial rest with 4 replications. Data were statistically analyzed by using the SAS package (version 8).

3. Results

3.1 Shoot induction experiments

Analysis of variance showed the effects of used treatments on shoot production, the mean of primary organ numbers per explants and the mean number of them, which grew, were significant ($P \leq 0.01$). No organogenesis observed in the control medium. Results indicated that the most effective concentrations of BAP on all characters in the first (BAP + 2,4-D) and third test (BAP + NAA) were 2, 1.5mg/L respectively; whilst in the second test (BAP + IBA), the BAP concentration at 1.5mg/L caused the highest values for the parameters (Tables 1-3). Among concentrations of 0.1, 0.2 and 0.5mg/L for 2,4-D, IBA and NAA, the least level (0.1mg/L) showed to be most effective for the shoot induction parameters. Results illustrated that among all applied light intensities, 4000 lux in the first and second tests and 6000 lux in third examination had the most positive effects on the measured characters.

In the first test (BAP + 2,4-D), peak value for the organogenesis percentage (77%) occurred from the buds explants at 2mg/l BAP + 0.1mg/l 2,4-D and 4000 lux; no organogenesis was observed on the leaves or shoots explants (Table 1). The highest values for the number of primary organs/explants (22) and number of appeared organs, which successfully grew (15), also belonged to this treatment.

In the second test (BAP + IBA), the highest organogenesis percentage (44%) was observed on the buds explants at 1.5mg/l BAP+ 0.1mg/l IBA and 4000 lux, no organogenesis happened on the leaves explants (Table 2). The maximum organogenesis percentage which occurred on the shoots explants was still ignorable (6%) in comparison to those of buds explants. The highest values for the number of primary organs/explants (24) and number of appeared organs, which successfully grew (20), were also related to this treatment.

In the third test (BAP + NAA), the uppermost organogenesis percentage (85%) was seen on the axillary buds explants at 1.5mg/l BAP + 0.1mg/l NAA and 6000 lux; The utmost values for the number of primary organs/explants (48) and number of appeared organs which successfully grew (40) also belonged to this treatment (Table 3; Fig. 1). In this test, direct organogenesis occurred on all three kinds of explants in terms of axillary buds, shoots and leaves. But, the values of this parameter were merely 12 and 5% for leaves and shoots explants, respectively. Histological studies confirmed development of new regions of meristemoids on the axillary buds explants (Fig. 2). As it is shown in Fig. 2, association of vascular elements and new meristematic regions were continuously established.

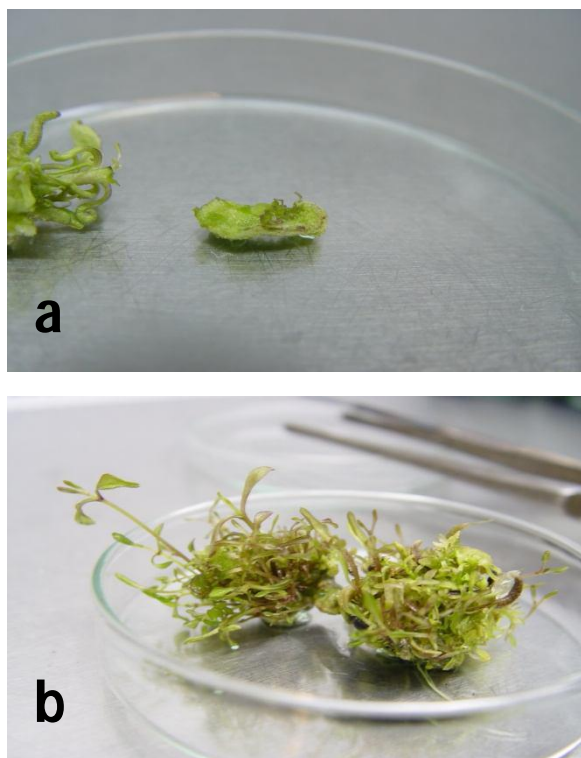


Fig. 1. Shoot induction on stevia explants. a) Organogenesis on stevia leaf explants in the third test (BAP + NAA); b) Organogenesis on stevia axillary bud in the third test (BAP + NAA).

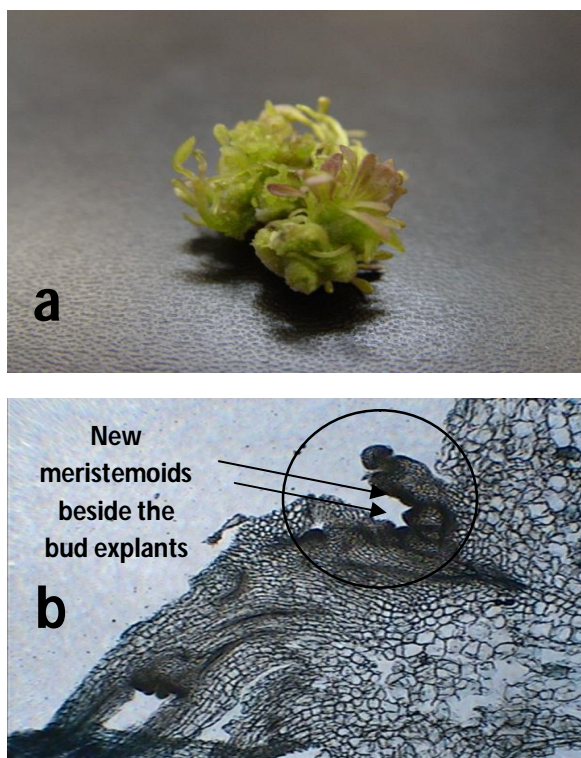


Fig. 2. Stevia bud explants and formation of new meristemoid regions on these explants; a) An axillary bud explant with new organs before sectioning; b) Longitudinal section of axillary bud explants along with appearing and growing of new meristemoids, x400.

Table 1. Effects of different levels of hormones, light and explants on shoot induction after 4 weeks treatment in the first test.

S. No.	Treatments	Shoot induction (%)	The number of primary organs/explants*	The number of appeared organs which grew*
1	CONTROL	0	0	0
2	0.5(BAP)+(0.1) 2,4-D,2000LUX,AX.bud	2.88	4.5	4.5
3	0.5(BAP)+(0.1) 2,4-,4000LUX,AX.bud	10.75	7.25	7.25
4	1(BAP)+(0.5)2,4-D,4000LUX,AX.bud	15	8.5	8.25
5	1.5(BAP)+(0.1)2,4-D,2000LUX,AX.bud	8	8.25	5.25
6	1.5(BAP)+(0.5)2,4-D,4000LUX,AX.bud	13	12	9.25
7	1.5(BAP)+(0.5)2,4-D,6000LUX,AX.bud	30.75	7.25	7.25
8	2(BAP)+(0.1)2,4-D,2000LUX,AX.bud	20	5	5
9	2(BAP)+(0.1)2,4-D,4000LUX,AX.bud	5.25	5.75	5.75
10	2(BAP)+(0.1)2,4-D,4000LUX,AX.bud	76.5	22	15.25
11	2(BAP)+(0.5)2,4-D,2000LUX,AX.bud	5	7.75	7.75

*Values are mean \pm standard error of four replicates with ten cultures per replicate.

Table 2. Effects of different levels of hormones, light and explants on shoot induction after 4 weeks treatment in the second test.

S. No.	Treatments	Shoot induction (%)	The number of primary organs/explants*	The number of appeared organs which grew*
1	CONTROL	0	0	0
2	0.5(BAP)+(0.1) IBA,2000LUX,AX.bud	9.5	3.75	3.75
3	1(BAP)+(0.1) IBA,2000LUX,AX.bud	10.75	6	4.5
4	1(BAP)+(0.1) IBA,4000LUX,AX.bud	10	8	7.75
5	1.5(BAP)+(0.1) IBA2000LUX,AX.bud	5	5	5
6	1.5(BAP)+(0.1) IBA,4000LUX,AX.bud	43.75	24.5	20
7	1.5(BAP)+(0.1) IBA,6000LUX,AX.bud	35.5	9	8.75
8	2(BAP)+(0.1) IBA,2000LUX,shoot	5	2	2
9	2(BAP)+(0.1) IBA,4000LUX,shoot	6.25	1.75	1.75
10	2(BAP)+(0.1) IBA,4000LUX,Ax.bud	8.25	4.5	4.5
11	2(BAP)+(0.2) IBA,4000LUX,shoot	4.5	1.5	1.5
12	2(BAP)+(0.2) IBA,4000LUX,Ax.bud	24	13	10.75
13	2(BAP)+(0.5) IBA,2000LUX,Ax.bud	7.5	5.5	5.5

* Values are mean \pm standard error of four replicates with ten cultures per replicate.

Table 3. Effects of different levels of hormones, light and explants on shoot induction after 4 weeks treatment in the third test.

S. No.	Treatments	Shoot induction (%)	The number of primary organs/explants*	The number of appeared organs which grew*
1	CONTROL	0	0	0
2	0.5(BAP)+(0.1) NAA,4000LUX,AX.bud	15.25	7.25	7.75
3	0.5(BAP)+(0.1) NAA,6000LUX,AX.bud	22.25	23.25	15
4	0.5(BAP)+(0.2) NAA,2000LUX,shoot	10	2	2
5	1(BAP)+(0.1) NAA,6000LUX,AX.bud	8	8	8.25
6	1(BAP)+(0.2) NAA,4000LUX,AX.bud	5	6.75	6.75
7	1(BAP)+(0.5) NAA,4000LUX,AX.bud	5	13	12.5
8	1(BAP)+(0.5) NAA,6000LUX,AX.bud	10.75	10	9
9	1.5(BAP)+(0.1) NAA,2000LUX,AX.bud	31	15	14
10	1.5(BAP)+(0.1) NAA,2000LUX,leaf	18.5	3.25	3.25
11	1.5(BAP)+(0.1) NAA,4000LUX,AX.bud	10.75	15	13.25
12	1.5(BAP)+(0.1) NAA,4000LUX,leaf	2.25	3.25	3.25
13	1.5(BAP)+(0.1) NAA,6000LUX,AX.bud	85	48	40.25
14	1.5(BAP)+(0.1) NAA,6000LUX,leaf	9.25	3	3
15	1.5(BAP)+(0.2) NAA,2000LUX,AX.bud	34	9	8
16	1.5(BAP)+(0.2) NAA,4000LUX,AX.bud	37.5	13.5	13
17	1.5(BAP)+(0.2) NAA,4000LUX,leaf	12.5	4	4
18	1.5(BAP)+(0.2) NAA,6000LUX,AX.bud	55.25	23	20.5
19	1.5(BAP)+(0.2) NAA,6000LUX,leaf	11.88	12.75	8
20	2(BAP)+(0.1) NAA,2000LUX,AX.bud	12.5	7.25	13.75
21	2(BAP)+(0.1) NAA,4000LUX,AX.bud	45	16.25	13
22	2(BAP)+(0.1) NAA,6000LUX,AX.bud	5	24.25	17.25
23	2(BAP)+(0.2) NAA,4000LUX,leaf	2.75	2.25	2.25
24	2(BAP)+(0.5) NAA,2000LUX,shoot	5	4.75	4.75
25	2(BAP)+(0.5) NAA,6000LUX,AX.bud	8	7	7
26	2(BAP)+(0.5) NAA,6000LUX,leaf	22.25	4.75	4.75

*Values are mean \pm standard error of four replicates with ten cultures per replicate.

3.2 Root induction

Results indicated the effects of hormonal treatments and activated charcoals (AC) were significant on the stevia root induction ($P \leq 0.01$). Results showed that among applied auxin (IAA, IBA and NAA), IBA (1mg/L) led to producing the highest percentages of root (100%), and the highest number of root/plantlets (11), all in AC medium (Fig. 3 & Table 4). In addition to the root induction characters, plant growth parameters i.e. the length of grown plantlets and number of nodes were also at the highest values in this medium (in turn -12.8cm & 7).

Results revealed that the medium containing AC had the highest effects on both plantlets growth and root production parameters for all measured parameters, i.e. 100% rhizogenesis, 9.13cm for the root length, 11 for the number of root/plantlets, 12.8cm for the length of plantlets and 7 nodes (Table 4). In the absence of AC, the maximum percentage of root production (53%) happened in the medium containing 1mg/L NAA.

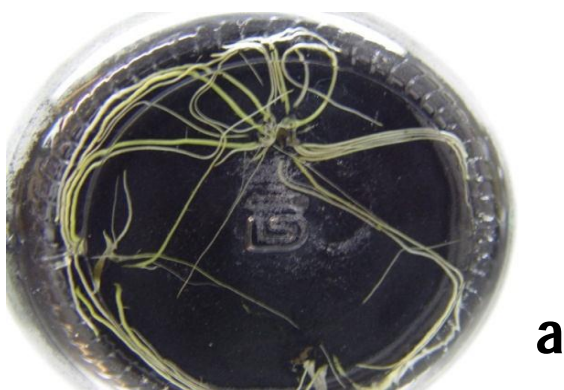


Fig. 3. Root induction on the produced plantlets of stevia in the medium with (a) and without (b) AC.

To sum up, 90% of rooted plantlets were successfully established in pots containing pit mass and vermiculate (3:1 ratios) and hardened in the greenhouse (Fig. 4).



Fig. 4. *In vitro* produced plantlets grown in the greenhouse.

Table 4. The effects of activated charcoal and different hormones on root induction and plant growth parameters after 4 weeks.

S. No.	Treatments	Rhizogenesis (%)	The number of root/explants*	The length of roots*	The length of grown plantlets*	The number of nodes*
1	0mg/L IBA without AC	17.33	1.73	2.4	4.3	2.67
2	0mg/L IBA with AC	29.33	3.6	4.44	5.97	3.67
3	1mg/L IBA without AC	29.33	4	2.67	0.87	0.87
4	1mg/L IBA with AC	100	11	9.13	12.8	7.27
5	2mg/L IBA without AC	34.67	3.67	2.33	0.87	1.4
6	2mg/L IBA with AC	53.33	5.07	8.93	11.13	7.07
7	0mg/L IAA without AC	17.33	1.73	2.4	4.3	2.67
8	0mg/L IAA with AC	29.33	3.6	4.44	5.97	3.67
9	1mg/L IAA without AC	22	3.33	4.43	4.87	3.47
10	1mg/L IAA with AC	53.33	5.87	6.6	8.23	4.93
11	2mg/L IAA without AC	44	4.93	4.07	5.73	5.2
12	2mg/L IAA with AC	47.33	3.07	4.74	8	5.13
13	0mg/L NAA without AC	17.33	1.73	2.4	4.3	2.67
14	0mg/L NAA with AC	29.33	3.6	4.44	5.97	3.67
15	1mg/L NAA without AC	52.67	8.6	3.4	2.6	3.2
16	1mg/L NAA with AC	64	4.73	9.77	10.6	6
17	2mg/L NAA without AC	30.67	3.4	3	0.93	1.2
18	2mg/L NAA with AC	87	4.33	5.87	6.23	5.07

*Values are mean \pm standard error of four replicates with ten cultures per replicate.

4. Discussion

Generally, there are widespread ranges of dividing potential, asexual reproduction capacities and organ production in plants. Regeneration and plant propagation is the base of tissue culture methods. On the other hand, the quantity and quality of regeneration potential and organogenesis depend on the origin of explants (Jarrett *et al.*, 1980). Differences in regeneration in different organs were reported by many researchers such as Wheeler (1995), Dale and Hampson (1995). In the light of literature, it is cleared that despite various studies on stevia tissue culture using different parts of this plant as the explants, though the capability of the axillary bud, shoots and leaves have not compared at the same experiments. In the present research, stevia axillary bud was recognized as the most efficient organ for its direct organogenesis in comparison to that in its shoot and leaf explants. Probably axillary buds comprising primary meristem beside the node region have a high competence for organogenesis in hormonal medium. Histological studies of the axillary bud after organogenesis confirmed the appearance of new meristemoid regions indicating its potential to form new organs.

In this relate, the best condition was 1.5mg/l BAP + 0.1mg/l NAA and 6000 lux for light which resulted in the uppermost organogenesis percentage (85%) along with the highest values for the number of primary organs/explants (48) and number of appeared organs (40) which successfully grew. In the previous works (such as Sivaram & Mukundan, 2003; Ahmed *et al.*, 2007) 21 and 10 were the number of organs/ explants in axillary buds or shoots respectively.

In tissue culture, significantly cardinal effects of plant growth regulators (PGRs) on plant metabolism have been widely proved; because any shift in plant metabolism and protein synthesis establishes a new fate and destination for plant tissues. As it is known, plant development routes are controlled with several gene networks. It is assumed that proper conditions in tissue culture probably activate homeotic genes such as KNOX genes, which are responsible for remaining meristematic situation of shoot cells. Alternatively, any plant growth substances might activate different routes and genes toward organogenesis. Plasticity nature of plants let them to adjustment with environment condition by changing metabolism, growth and evolutionary reactions. The purpose of the first step in this research was shoot induction on stevia explants. BAP -because of its well-known effective role in shoot production in tissue culture was selected in constant level with combination to varieties of Auxins. Cytokinins lead to cell division, organs elongation, controlling organs development, organogenesis etc. On the other hand, auxins have also considerable functions on induction of cell division, cell elongation, callus

formation and root production (Arteca, 1996). Efficient level of BAP for shoot induction depends on its combination to other hormones (especially auxins) and laboratory conditions. The present results indicated that the concentration of 1.5mg/L BAP could be an effective level in stevia organogenesis. This result is not consistent with reports of Ibrahim *et al.*, (2008); Debnath (2008). In their experiments, 2mg/L BAP was reported as the best concentration to produce the highest range of shoots on stevia buds. On the other hand, in agreement with our results, Ahmed *et al.*, (2007) also expressed 1.5mg/L BAP would be a proper level for stevia organogenesis, but they used BAP in combination to Kin but not auxins. In any case, in all of our experiments, the means of primary and grown organs were much higher than of those obtained in the previous works. Any minute changes in laboratory conditions and materials even distilled water and different light intensities might make a difference in the final results.

Among applied auxins in the current experiments (2,4-D, IBA, NAA), in terms of shoot or root induction (with no AC) in stevia, NAA was recognized as the most effective auxin. Furthermore, the least utilized level of this auxin exhibited the best shoot or root organogenesis. According to these results, apparently there might be a negative correlation between auxin effects and its levels; in other words, the least concentration of auxin, the most effective of auxin on the stevia organogenesis which was evident. The current experiments showed that produced roots by NAA were shorter and thicker with a lower growth rate in comparison to those produced by other auxins.

Nevertheless, using AC in stevia root induction medium altered the position toward IBA, as in this case 1mg/L of IBA caused 100% rhizogenesis along with the highest values for other measured parameters such as the number of root/explants (11), length of roots (9.13cm), length of grown plantlets (12.8) and number of nodes (7). It is known that activated charcoal eliminates toxic materials that are effluxed from plant tissues and plays a beneficial role in culture conditions with media darkening and pH adjusting. This positive effect of AC probably derived from its effective characters for binding and absorbing phenols or other factors, which can prevent regeneration of explants and root production. AC has a lot of pores that numerous materials were absorbed by those. Thus, the positive effect of activated charcoal was apparent in the root production and plantlet growth process of stevia. In AC medium the highest rate of plantlet growth and rhizogenesis also occurred.

The current results demonstrated that the root production (29%) also occurred even in a free auxin medium (control) but containing AC. At this condition, besides, AC caused to increase the roots elongation, plantlets growth and number of nodes. This suggests

other benefits of AC in stevia organogenesis even in the absence of PGRs. Medium without hormones are most secure and safe to prevent callus production in *in vitro* produced organs.

Ferreira & Handro (1988) approached rhizogenesis in a medium containing both BAP and IBA and no AC. But they used another medium to cover root elongation. Sivaram & Mukundan (2003) attained 100% root production in a half MS medium along with IBA and reported the occurrence of plantlet elongation in this medium.

Rhizogenesis experiments showed that applied media covered appropriate components to induce not only stevia root production but also shoot elongation in a brief time. It is of its benefit to eliminating subculturing, growth in a new medium and also decreasing the high rate of costs.

Another examined item in the present work was light intensity. Light has a major role in regeneration as an environmental factor and determinative element for shoot induction. PGRs such as BAP have shown various morphogenesis effects. They generally direct to accelerate in number of nodes and meristematic tissues differentiation. So, interference between PGRs and light might promote to internal cytokinin increasing or internal auxin decreasing such as IAA (Aksenova *et al.*, 1994). This may occur in intracellular level through redistribution of cortical microtubules, which are affected by light phytochromes (Waller & Nick, 1997). To the best of our knowledge, there are not still firm reports about the possible effects of different light intensities on stevia regeneration. In present research, diverse light intensities (2000, 4000 and 6000 lux) were examined to find the best one in stevia propagation. Among them, the light intensity of 6000 lux exhibited the maximum effect on increasing stevia shoot production. It is also suggested that observed differences among light intensity effects might be related to the auxin varieties and levels.

References

- [1]. Ahmed, M.B., Salahin, M., Karim, R., Razvy, M.A., Hannan, M.M., Sultana, R., Hossain, M. and Islam, R. (2007). An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni) in Bangladesh. *Am-Euras. J. Sci. Res.*, 2(2): 121-125.
- [2]. Arteca, R. (1996). *Plant Growth Substances: Principles and Applications*. New York: Chapman & Hall, 332 pp.
- [3]. Bondarev, N.I., Nosov, A.M., Kornienko, A.V. (1998). Effects of exogenous growth regulators on callusogenesis and growth of cultured cells of *Stevia rebaudiana* Bertoni. *Russian Journal of Plant Physiology*, 45(6): 770-774.
- [4]. Bondarev, N., Reshetnyak, O., Nosov, A. (2002). Features of development of *Stevia rebaudiana* shoots cultivated in the roller bioreactor and their production of steviol glycosides. *Planta Medica*, 68: 759-762.
- [5]. Felipe, G.M., Lucas, N.M.C. (1971). Estudo da viabilidade dos frutos de *Stevia rebaudiana* Bert. *Hoehnea*, 1:95-105.
- [6]. Felipe, G.M., Lucas, N.M.C., Behar, L., Oliveira, M.A.C. (1971). Observações a respeito da germinação de *Stevia rebaudiana* Bert. *Hoehnea*, 1:81-93.
- [7]. Ferreira, C.M., Handro, W. (1988). Production, maintenance and plant regeneration from cell Suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. *Plant Cell Report*, 7:123-126.
- [8]. Handro, W., Hell, K.G., Kerbauy, G.B. (1977). Tissue culture of *Stevia rebaudiana*, a sweetening plant. *Planta Medica*, 32: 115-117.
- [9]. Ibrahim, I.A., Nasr, M.I., Mohammed, B.R., El-Zefzafi, M.M. (2008). Plant growth regulators affecting *in vitro* cultivation of *Stevia rebaudiana*. *Sugar Tech*, 10(3): 254-259.
- [10]. Jeppesen, P.B., Gregersen, S., Rolfsen, S.E., Jepsen, M., Colombo, M., Agger, A., Xiao, J., Kruhøffer, M., Orntoft, T., Hermansen, K. (2003). Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. *Metabolism*, 52: 372-378.
- [11]. Miyagawa, H., Fujioka, N., Kohda, H., Yamasaki, K., Taniguchi, K., Tanaka, R. (1986). Studies on the tissue culture of *Stevia rebaudiana* and its components; (II). Induction of shoot primordia. *Planta Medica*, 52: 321-323.
- [12]. Akita, M., Shigeoka, T., Koizumi, Y., Kawamura, M. (1994). Mass propagation of shoots of *Stevia rebaudiana* using a large scale bioreactor. *Plant Cell Reports*, 13 (3-4): 180-183.
- [13]. Debnath, M. (2008). Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. *Journal of Medicinal Plants Research*, 2(2): 45-51.
- [14]. Nakamura, S., Tamura, Y. (1985). Variation in the main glycosides of *Stevia* (*Stevia rebaudiana* Bertoni). *Japanese Journal of Tropical Agriculture*, 29: 109-115.
- [15]. Aksenova, N.P., Konstantinova, T.N., Sergeeva, L.I., Macháčková, I., Golyanovskaya, S.A. (1994). Morphogenesis of potato plants *in vitro*. I. Effect of light quality and hormones. *Journal of Plant Growth Regulation*, 13: 143-146.
- [16]. Jain, P., Kachhwaha, S., Kothari, S.L. (2009). Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* (Bert.) Bertoni by using high copper levels in the culture medium. *Scientia Horticulturae*, 119(3): 315-319.

- [17]. Rafiq, M., Dahot, M.U., Mangrio S.M., Naqvi, H.A and Qarshi, I.A. (2007). *In Vitro* Clonal Propagation and Biochemical Analysis of Field Established *Stevia rebaudiana* Bertoni. *Pakistan Journal of Botany*, 39(7): 2467-2474.
- [18]. Sakaguchi, M., Kan, T. (1982). Japanese research on *Stevia rebaudiana* (Bert.) Bertoni and stevioside. *Ciencia e cultura - Sociedade Brasileira para o Progresso da Ciencia*, 34:235–248.
- [19]. Sivaram, L., Mukundan, U. (2003). *In vitro* culture studies on *Stevia rebaudiana*. *In vitro Cellular and Developmental Biology - Plant*, 39: 520-523.
- [20]. Soejarto, D.D., Kinghorn, A.D., Farnsworth, N.R. (1982). Potential sweetening agents of plant origin. III. Organoleptic evaluation of *Stevia* leaf herbarium samples for sweetness. *Journal of Natural Product*, 45: 590-599.
- [21]. Sreedhar, R.V., Venkatachalam, L., Thimmaraju, R., Bhagyalakshmi, N., Narayan, M.S., Ravishankar, G.A. (2008). Direct organogenesis from leaf explants of *Stevia rebaudiana* and cultivation in bioreactor. *Biologia Plantarum*, 52 (2): 355-360.
- [22]. Tamura, Y., Nakamura, S., Fukui, H., Tabata, M. (1984). Clonal propagation of *Stevia rebaudiana* Bertoni by stem-tip culture. *Plant Cell Report*, 3:183–185.
- [23]. Tanaka, O. (1982). Steviol-glycosides: New natural sweeteners. *TrAC Trends in Analytical Chemistry*, 1:246–248.
- [24]. Toffler, F., Orio, O.A. (1981). Acceni sulla pin ata tropicale ‘Kaa-he-e’ ou ‘erba dolce’. *Rev. Soc. Sci. Aliment.*, 4:225–230.
- [25]. Uddin, M.S., Hossain Chowdhury, M.S., Haque Khan, M.M., Uddin, M.B., Ahmed, R., Azizul, Baten (2006). *In vitro* Propagation of *Stevia rebaudiana* Bert in Bangladesh. *African Journal of Biotechnology*, 5(13):1238-1240.
- [26]. Waller, F., Nick, P. (1997). Response of actin microfilaments during phytochromes-controlled growth of maize seedlings. *Protoplasma*, 200: 154-162.