Journal of Plant Process and Function, Vol. 7, No. 28, Year 2019

Tissue culture and production of volatile secondary metabolites from callus of *stevia rebaudiana* at different c 4ombination of plant growth regulators

Farahrouz Zahri[1,2], Masomeh Abedini[2], Seyed Mehdi Razavi[1]*

[1]Dept. of Biology, Faculty of Science, University of Mohaghegh Ardabili, Ardabil, Iran
[2]Dept. of Biology, Faculty of Basic Science, Payam Noor University, Tehran, Iran
(Received: 08/12/2017-Accepted: 29/09/2018)

Abstract

Stevia rebaudiana **Bert (Asteraceae) is a herbaceous plant with sweet leaves, which are the source of sweetener products. The present study focuses on effects of different combination of plant growth regulators on callus induction, callus cell growth and regeneration and volatile constituents from callus of stevia in tissue culture condition. The results of present study have shown that callus formation promoted in all of media contained phytohormones of different auxin/cytokinin based combinations. However, NAA/BA combinations are significantly more effective in callus initiation and the highest amount of callus was obtained in medium supplemented with 1.5:2 mg/L of NAA/BA. It was revealed that shoot induction in callus occurred at hormonal treatments as IBA/BA and IBA/KIN (0.5:1.5 mgl^{-1}). High rooting obtained in media supplemented with NAA/KIN (1.5:0.5 mgl^{-1}). The results of GC-MS analyses of volatile constituents of calli showed that the callus oil was obtained in media supplemented with NAA/Kin (1.5:0.5 mgl^{-1}). The NAA/Kin treatment with 1:0.5 mgl^{-1} concentration was dominated with squalene (40.50%) as main compound. The callus formed in media contained NAA/BA was enriched with Tricosane (38.57%). In addition, phytol (11.69%, 6.92%) was regarded as major constituent of the oil obtained from cultures supplemented with IBA/Kin and IBA/BA, respectively. It was concluded that there was a considerable differences in the essential oil profile of callus induced from different hormonal combinations. It is thought that various hormonal treatments may result in changes in metabolic pathways.**

Keywords:, Phytol, Plant growth regulators, *Stevia Rebaudiana*, Tissue culture, Tricosane

Introduction

Stevia rebaudiana Bert (Asteraceae) is a tender perennial herbaceous plant indigenous to parts of Brazil and Paraguay. It is widely grown for its sweet leaves, which are the source of sweetener products known generically as stevia and sold under various trade names. The plant commonly known as candy leaf, sweet leaf, or sugar leaf in all over of the world. The active compounds are various steviol glycosides (mainly stevioside and rebaudioside), which are 250–300 times as sweet as sugar (Abdullateef *et al.,* 2011). *S. rebaudiana* is used for it's medicinal properties and nutritional purposes as sweetener in teas and foods (Frederico *et al*., 1996). Although stevia is useful for all people but diabetes, phenylketonuria patients and obese people who want to lose weight, it is recommended to others to use its sweetening potential (Goyal *et al.,* 2010). Moreover, Patel *et al*. (2009) claimed since Stevia leaves contain protein, fiber, carbohydrates, some vitamins and minerals, it could be used for therapeutic use on humans such as high blood pressure, infection, hyperactivity and obesity. It is also useful for people craving for carbohydrates and alcohol and tobacco. Because of the high sweet production, natural and calorifree properties of stevia, it can be used as a natural and more powerful alternative sweetener to other synthetic ones. Stevioside is regarded as main sweetener constituent of the plant. The compound has no side effects and also recommend for people with diabetes (Goyal *et al*., 2010). Due to high commercial and pharmaceutical value of *S. rebaudiana*, there is an increasing demand for it the recent years. Hence, the production of this plant in some countries is such that its commercial planting in China, Taiwan, Thailand, Korea, Japan, India and Malaysia have been increased (Rathore *et al*., 2014).

S. rebaudiana are found in the wild in semiarid habitats ranging from grassland to mountain terrain, do produce seeds, but only a small percentage of the seeds germinate. Planting cloned stevia is a more effective

*Corresponding Author, Email: razavi694@gmail.com

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

method reproduction. A number of studies have also shown that the best way to reproduce of this plant is micropropagation through tissue culture. This method can produce uniform plants which produce stable glucoside products during their vegetative in terms of quality, while the plants that were multiplied by seeding and cuttings had lost their qualitative features and their glucoside production decreased (Janarthanam *et al*., 2010). A literature review showed that various researches have been conducted on establishment of callus culture (Das *et al.*, 2006; Uddin *et al*. 2006; Gupta *et al*. 2010), different organ micropropagation (Rafiq *et al*., 2007; Das *et al*., 2011), shoot regeneration (Singh *et al.,* 2011) and somatic embryogenesis (Bespalhok-Filho and Hattori 1997) of *S. rebaudiana.* The results of the Karuppusamy (2009) research indicated in vitro plant cell cultures have potential for commercial production of secondary metabolites. It was also observed that the concentration of growth regulators in the medium induces the cell growth and production of secondary metabolites ginsenoside in the suspension cultures (Vanisree *et al*., 2004). The present study focuses on effects of different combination of plant growth regulators on callus induction, callus cells growth and shape, shoot and root regeneration, volatile constituents from callus of stevia in tissue culture condition.

Materials and Methods

Plant materials and callus formation: *S. rebaudiana* var. Bertoni plants were purchased from the National Botanical Garden of Iran National Arboretum of Tehran. The plant leaves about 0.5 cm in length were used as explants to induce callus. In this regard, the explants firstly washed under running water for 20 minutes and then sterilized using 1.5% sodium hypochlorite contained two drops of Tween 20 for two minutes. Then, the explants washed with distilled water and 70% ethanol, respectively and washed finally again with sterile water. After decontamination process, explants were cultured in different MS media supplemented with 2% sucrose, 0.8% agar and various hormonal combination of NAA (Nephtalen Acetic Acid) /KIN (Kinetin), NAA/BA (6-Bennzylaminupurin), IBA (Indole Buteric Acid)/KIN, IBA/BA, 2,4-D (2,4-Dichlorophenoxy Acetic Acid)/KIN and 2,4-D/BA at the auxin concentration of 0.5, 1, 1.5, 2 mgl^{-1} and cytokinin concentration of 0.5, 1, 1.5, and 2 mgl^{-1}, respectively, and non-combinational hormonal of auxin NAA,IBA,2,4D and cytokinin KIN, BA with a concentration of 1 mgl^{-1} separately, in a Laminar flow using sterile materials and tools. All of cultures were transfered to growth chamber at 25 °C and a photoperiod 16/8 hrs. light/dark with 1000 lux light.

After 40 days, callus growth was evaluated in each culture by fresh weight measurement.

Callus cells morphology: Morphology of the callus cells in different treatments were assessed by fixation of calli on different slides that photographed using a light microscope (LM Motic BA400 model attached to the camera Moticam 3000) at magnification of 10. Then the size of the cells was measured using 47/1 Image J software (Ferreira and Rasband, 2012).

Plant regeneration: 40-day old calli were passaged on new MS medium supplemented with 2% sucrose, 0.8% agar and combinations of hormones NAA/KIN, NAA/BA, IBA/KIN and IBA/BA with 0.5 mgl^{-1} auxin and 1.5 mgl^{-1} cytokinin concentrations for shoot induction.

The generated shoots (5-10 mm) were excised and individually transferred to sterile pteri dishes with half-strength MS medium contained 2% sucrose, 0.8% agar and combinations of hormones NAA/KIN, NAA/BA, IBA/BA and IBA/KIN with a concentration of 1.5 mgl^{-1} of auxin into 0.5 mgl^{-1} of cytokinin and 2 mgl^{-1} of auxin into 0.5 mgl^{-1} of cytokinin for rooting. Rooted plants were transferred to pots containing 3:1 mixture of sterile soil and vermiculite for further acclimation and growth in the greenhouse (16/8 hrs photo- period, 16-24ºC and 80-90% humidity).

Extraction and analysis of Volatile Secondary Metabolites: Essential oils of callus obtained from treatments of hormonal combinations that produced the maximum callus with concentrations of 1:1 (40g callus) were hydrodistillated using a Mini-Clevenger type apparatus for 3 hrs. The essential oils were dried over anhydrous sodium sulfate and stored at 4°C in dark, before analyzing by gas chromatography-mass spectrometry (GC-MS).

The oils analyzed by GC-MS. The analysis was carried out on a Agilent 7890A GC/MS instrument equipped with a HP-5MS column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The oven temperature was programmed to increase from 50ºC to 320ºC at a rate of 4 ºC/min and finally held for 7 mins.; transfer line temperature was 250ºC. Helium was used as the carrier gas at a flow rate of 0.8 ml min^{-1} with a split ratio equal to 1/60. The quadrupole mass spectrometer scanned over the 35-465 amu with an ionizing voltage of 70 eV and an ionization current of 150 µA.

For quantitation (area%), the GC-FID analyses of the oil were conducted using a Agilent 7890A instrument equipped with a HP-5 fused silica column (30 m × 0.25 mm, film thickness 0.25 µm). Nitrogen was used as the carrier gas at the constant flow of 1 mL/min; the split ratio was the same as it was for the GC-MS. The oven temperature was raised from 50ºC to 320ºC at a rate of 4ºC/min and held for 7 mins. The injector and detector (FID) temperatures were kept at 250ºC and 280ºC, respectively. Semiquantitative data obtained from FID area percentages without the use of correction factors**.** To obtain the same elution order as with GC-MS, simultaneous auto-injection performed on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds calculated from FID chromatograms.

Identification of essential oil components:

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

Table1. Analysis of variance for *Stevia rebaudiana* callus fresh weight

Source of variation	Degree of freedom	Mean of Squares					
		NAA/KIN	NAA/BA	IBA/KIN	IBA/BA	2,4-D/KIN	2,4-D/BA
Auxin effect	3	2.21**	3.49**	1.999**	1.171**	0.372**	0.894**
Cytokinin effect	3	0.49**	0.48**	0.765**	1.030**	0.98**	0.451**
Interaction of Auxin/ Cytokinin	9	0.325**	0.247**	0.166**	0.241**	0.110**	0.038**
Error	32	0.015	0.20	0.16	0.36	0.020	0.008

**** indicate significant at 1% levels of probability**

Table 2. Effect of auxins and cytokinins based hormonal combinations on *Stevia rebaudiana* callus induction

Row No	Auxin / cytokinin (mgl^{-1})	NAA/KIN (mgl^{-1})	NAA/BA (mgl^{-1})	IBA/KIN (mgl^{-1})	IBA/BA (mgl^{-1})	2,4-D/KIN (mgl^{-1})	2,4-D/BA (mgl^{-1})
		Callus Weight (g)					
1	0/0	-	-	-	-	-	-
2	0.5/0.5	0.87 ± 0.08^{g}	0.91 ± 0.06^{g}	0.51 ± 0.06^{i}	1.41 ± 0.12^{e}	1.12 ± 0.08^{cde}	1.47 ± 0.05^{b}
3	0.5/1	0.99 ± 0.04^{fg}	1.29 ± 0.06^{f}	0.80 ± 0.05^{h}	1.49 ± 0.12^{e}	1.06 ± 0.06^{de}	1.67 ± 0.08^{a}
4	0.5/1.5	0.95 ± 0.05^{fg}	1.47 ± 0.08^{ef}	0.86 ± 0.04^{h}	1.70 ± 0.05^{de}	1.63 ± 0.04^{a}	1.26 ± 0.03^{cd}
5	0.5/2	1.07 ± 0.04^{efg}	1.59 ± 0.1^{de}	0.97 ± 0.06^{gh}	1.04 ± 0.04^{f}	1.36 ± 0.07^{b}	1.15 ± 0.09^{de}
6	1/0.5	2.29 ± 0.05^{a}	1.64 ± 0.1^{de}	1.03 ± 0.05^{gh}	1.41 ± 0.06^{e}	1.01 ± 0.06^{e}	1.47 ± 0.04^{cd}
7	1/1	1.10 ± 0.07^{ef}	2.17 ± 0.03^{c}	1.22 ± 0.04^{ef}	2.07 ± 0.08^{bc}	1.04 ± 0.08^{e}	1.25 ± 0.06^{ef}
8	1/1.5	1.61 ± 0.08^{d}	2.30 ± 0.07^{bc}	1.72 ± 0.04^{bc}	2.52 ± 0.07^{a}	1.14 ± 0.05^{cde}	1.07 ± 0.05^{gh}
9	1/2	1.80 ± 0.05^{cd}	2.57 ± 0.1^{a}	1.75 ± 0.09^{bc}	2.39 ± 0.08^{ab}	1.44 ± 0.06^{b}	0.88 ± 0.08^{fg}
10	1.5/0.5	2.22 ± 0.04^{a}	2.44 ± 0.1^{ab}	1.10 ± 0.05^{fg}	1.44 ± 0.12^{e}	1.28 ± 0.06^{bc}	0.95 ± 0.05^{bc}
11	1.5/1	1.77 ± 0.09^{cd}	2.59 ± 0.03^{a}	2.03 ± 0.1^{a}	1.68 ± 0.13^{de}	1.26 ± 0.06^{bc}	1.42 ± 0.06^{bcd}
12	1.5/1.5	1.72 ± 0.09^{d}	2.68 ± 0.06^{a}	1.57 ± 0.05^{cd}	2.17 ± 0.09^{bc}	1.40 ± 0.05^{b}	1.31 ± 0.05^{gh}
13	1.5/2	1.74 ± 0.09^{cd}	2.70 ± 0.07^{a}	1.67 ± 0.04^{bc}	2.13 ± 0.09^{bc}	1.07 ± 0.05^{de}	0.88 ± 0.07^{gh}
14	2/0.5	2.22 ± 0.04^{a}	2.07 ± 0.03^{c}	1.38 ± 0.09^{de}	1.60 ± 0.11^{de}	1.01 ± 0.05^{e}	0.85 ± 0.05^{fg}
15	2/1	2.08 ± 0.06^{ab}	2.62 ± 0.1^{a}	2.06 ± 0.07^{a}	2.39 ± 0.15^{ab}	1.12 ± 0.06^{cde}	0.94 ± 0.03^{h}
16	2/1.5	1.96 ± 0.03^{bc}	2.28 ± 0.06^{bc}	1.84 ± 0.04^{ab}	2.32 ± 0.06^{ab}	0.99 ± 0.08^{e}	0.75 ± 0.04^{h}
17	2/2	1.27 ± 0.04^{e}	1.75 ± 0.04^{d}	1.55 ± 0.06^{cd}	1.87 ± 0.14^{cd}	0.71 ± 0.06^{f}	0.70 ± 0.06^{i}

Mean values in the same column followed by the same letter are not significantly different at the 0.05 level according to the Duncan test

Essential oil compounds were identified by comparison of their retention indices (RI) with those reported in the literatures (Adams, 2004) and their mass spectrum with the Wiley library (Wiley 7.0).

Statistical Analysis: The experiments were performed in a factorial experiment based on completely randomized design with three replications. Two way ANOVA was used for data analyzing of combinational hormnal treatments with hormon concentration (17 levels) and hormonal combination (6 levels) as factors. One way ANOVA was used for data analysis of single hormone tratments. The comparison of means was performed using Duncan tests at $P\leq0.05$.

Results

Callus Induction: The results of present study showed, that whenever any callus induction take place in control group, callus formation promoted in all of media contained phytohormones of different Auxin/Cytokinin based combinations (Table 1, 2). However, NAA/BA combinations (1:0.5 mgl^{-1}) were significantly more effective to callus initiation than other hormonal mixtures like NAA/Kin, IBA/Kin, IBA/BA, 2,4-D/BA and 2,4-D/Kin at $P\leq0.05$. The highest amount of callus was obtained in medium supplemented with 1.5:2 mgl^{-1} of NAA/BA that in this treatment callus fresh weight reached to 2.70 g. Treatments IBA/BA with concentrations of 1:1.5 mgl^{-1} and NAA/KIN at a concentration of 1:o.5mgl^{-1} showed the highest callus after the first treatment (Table 2) and there was not significant diference between them.

Also the results indicated that callus production induced in media contained separately of auxin based hormones i.e. NAA, IBA and 2,4-D at concentration of $1mgl^{-1}$. In medium supplemented with NAA (1 mgl^{-1}) highest amount of callus fresh eight was recorded. Any callus induction observed in media contained individually of cytokinin based phytohormones like Kin and BA (Figure 1). However, it was found that callus formed in media with single hormone structure had low

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

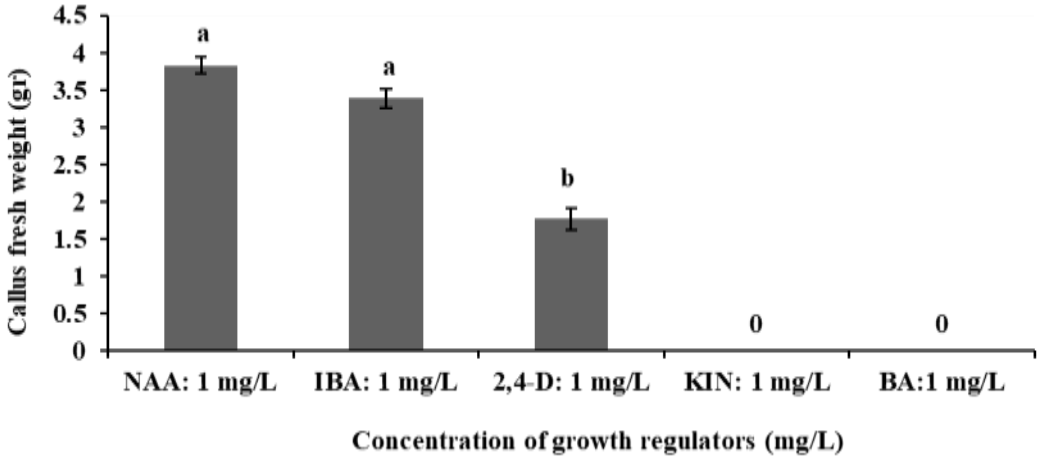

Fig. 1. Effect of non- combinational treatment with auxin and cytokinin based hormones on callus induction of *Stevia rebaudiana*

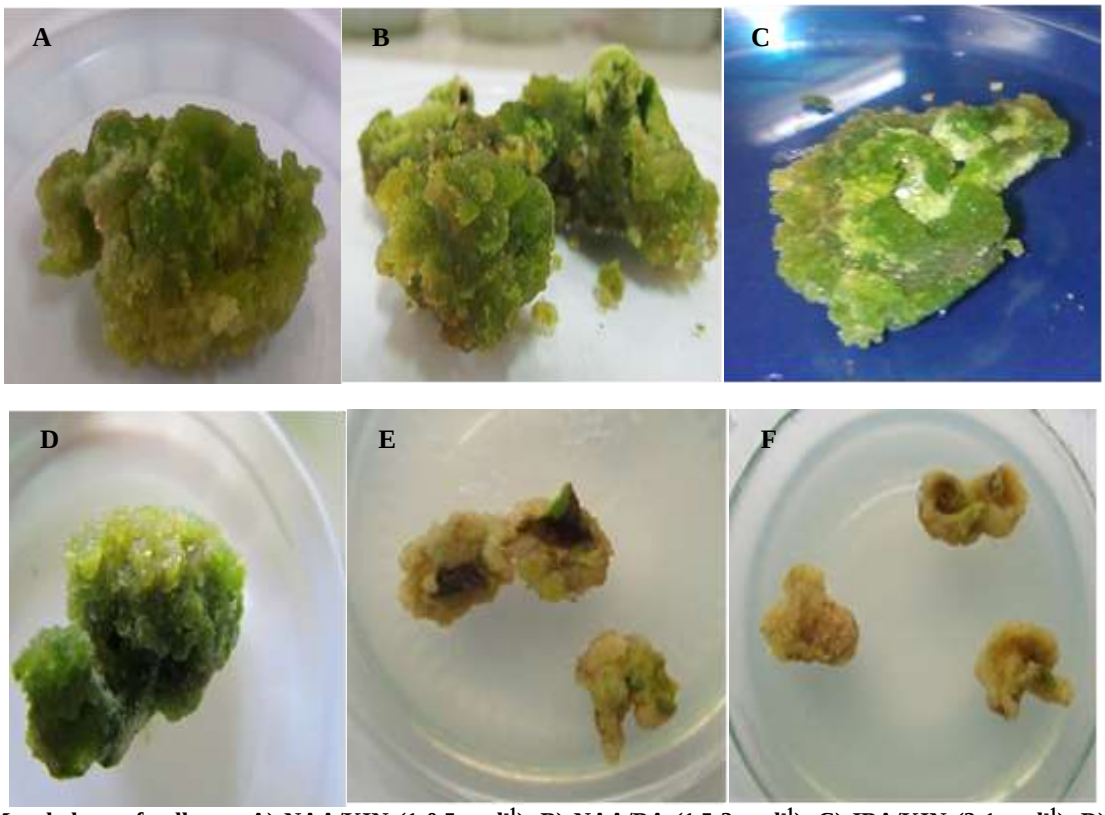

Fig. 2. Morphology of callus at A) NAA/KIN (1:0.5 mgl^{-1}), B) NAA/BA (1.5:2 mgl^{-1}), C) IBA/KIN (2:1 mgl^{-1}), D) IBA/BA (1:1.5 mgl^{-1}), E) 2,4-D/KIN (0.5:1.5 mgl^{-1}), F) 2,4-D/BA (0.5:1 mgl^{-1})
production in stevia based on callus cell shapand size (Figure 3.B).

viability and had been darken after 3 weeks of formation.

Callus Morphology: This study has revealed that the mostly calli obtained from media containing NAA/Kin and NAA/BA had a large size, the fragile structure and pale green color with extended growth and scattered position in MS medium. Calli formation obtained from media supplemented with IBA/KIN and IBA/BA had a large size, soft structure, beady with green colors. Callus produced from 2,4-D with BAP and KIN treatments had small size, firm and compact structure and yellowish cream color (Figure 2).

Microscopic examination of callus cells from different hormonal treatments showed that the cells morphology was significantly affected by the hormonal combinations. While cultures contained NAA/KIN combination forms callus cells with mostly spindle shape, sometimes horseshoe and rarely round shape with a cell dimension of 183±11 with 40.85±4.5 µm (Figure 3. A), the callus cells formed in medium with NAA/BA showed spindle to round shape and cell sizes of 156±15 with 40.70±2 µm (Figure 3. B). In the hormone treatments with IBA/KIN and IBA/BA callus cell shape were often narrow with a size of 192±12 with

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

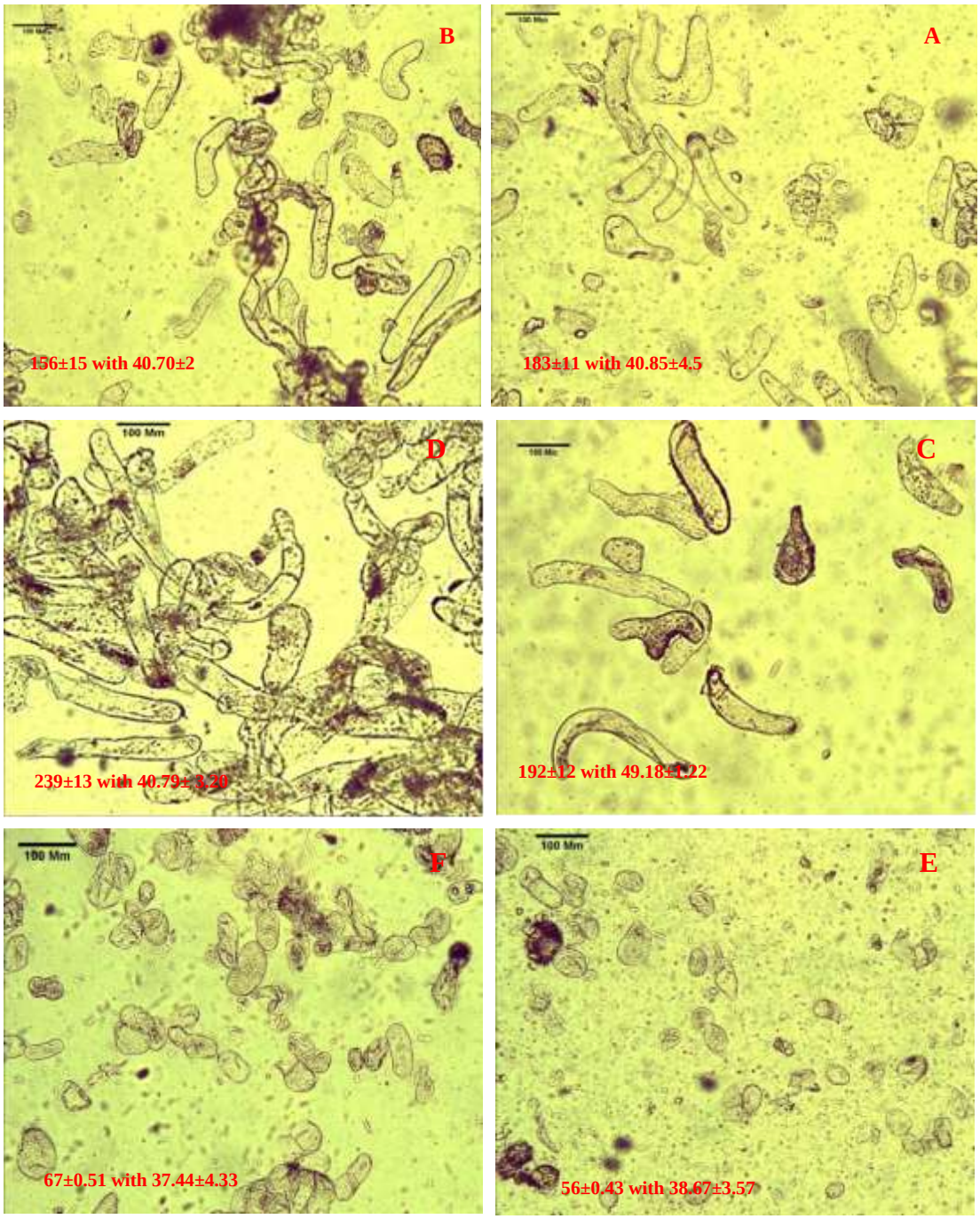

Fig. 3. Morphology of callus cells in the hormone treatments: A) NAA and KIN (1:0.5 mgl^{-1}), B) NAA and BA (1.5:2 mgl^{-1}), C) IBA and KIN (2:1 mgl^{-1}), D) IBA and BA (1:1.5 mgl^{-1}), E) 2,4-D and KIN (0.5:1.5 mgl^{-1}), F) 2,4-D and BA (0.5:1 mgl^{-1})

49.18±1.22 and 239±13 with 40.79± 3.20 µm, respectively (Figure 3. C,D). On the other hand, it was known that callus cells created with 2,4-D/KIN and 2,4-D/BA combination were generally small and round shaped with a average dimension of 56±0.43 with 38.67±3.57 and 67±0.51 with 37.44±4.33 µm, respectively (Figure 3. E,F). Therefore, IBA/BA combinations (1:1.5 mgl^{-1}) are more effectively in callus production in stevia based on callus cell shapand size (Figure 3.B).

Regeneration: This study has indicated that some hormonal combination might be effective for regeneration and can promote callus for organogenesis. It was revealed that shoot induction in callus happened at hormonal treatments as IBA/KIN (0.5:1.5 mgl^{-1}) and IBA/BA (0.5:1.5 mgl^{-1}) (Figure 4). Other hormonal combinations, were not capable to promote shoot organogenesis in callus. Root formation could induce using NAA/Kin, NAA/BAP, IBA/BA and IBA/Kin hormonal combination with concentration 1.5:0.5 mgl^{-1} and 2:0.5 mgl^{-1} (Figure 5). High rooting was obtained in media supplemented with NAA/Kin (1.5:0.5 mgl^{-1}) (Figure 6).

Rooted shoots exhibited 80% viability after 4 weeks

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

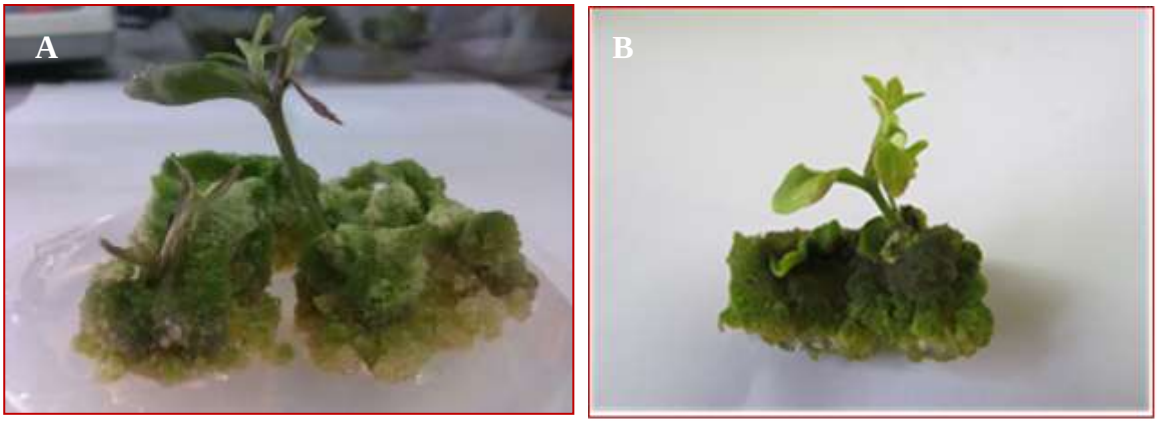

Fig. 4. A) Shoot induction at IBA/BA (0.5:1.5 mgl^{-1}), B) Shoot induction at IBA/KIN (0.5:1.5 mgl^{-1}).

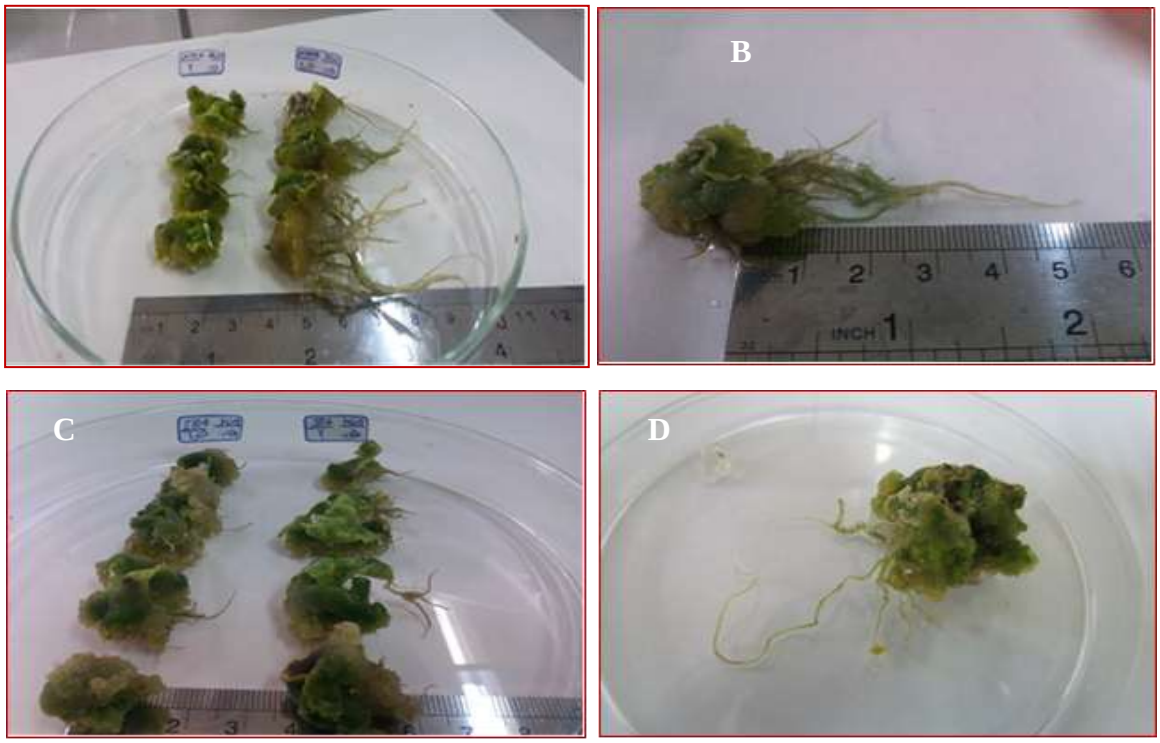

Fig. 5. A) Root induction at NAA/Kin (2:0.5; 1.5:0.5 mgl^{-1}); B) at NAA/BA (2:0.5 mgl^{-1}), C) at IBA/Kin (2:0.5; 1.5:0.5 mgl^{-1}), D) at IBA/BA (1.5:0.5 mgl^{-1}).

of transferring to soil/vermiculite contained pots (Tabe 3).

Callus oil Isolation: The results of the isolation and identification of the volatile constituents of callus obtained from treatment NAA/KIN (1:0.5mgl^{-1}), NAA/BA (1.5:2 mgl^{-1}), IBA/KIN (2:1 mgl^{-1}) and IBA/BA (1:1.5 mgl^{-1}) are presented in Table 5 in order to retention times. As it was seen, the callus oil obtained from NAA/Kin treatment (1:0.5mgl^{-1}, with maximum callus induction) is dominated with squalene (40.50%) as main compound. The callus formed in media contained NAA/BA (1.5:2 mgl^{-1}) is enriched with tricosane (38.57%). In addition, phytol (11.69%, 6.92%) is regarded as major constituent of the oil obtained from cultures supplemented with IBA/Kin and IBA/BA, respectively (Table 4).

Discussion

Our findings tend to depict that all tested auxin hormones like NAA, IBA and 2,4-D are capable to initialize callusing in *S.rebaudiana* leaf explants inoculated in MS medium. More callus production was obtained with application of combinations of auxins/cytokinins. Among auxins, NAA was more efficient for callus formation. This is in agreement with the report of Gupta *et al*. 2010, on establishment of callus culture of *S. rebaudiana* leaf and nodal explant. They were also observed only 10% callusing in leaf explant culture supplemented with 2,4-D (Gupta *et al.*, 2010). However, other reports implied on efficient role of 2, 4-D in callus induction of the stevia plant (Uddin *et al.*, 2006; Singh *et al.*, 2011). As it was previously pointed out callus attributes like color and shape differ

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

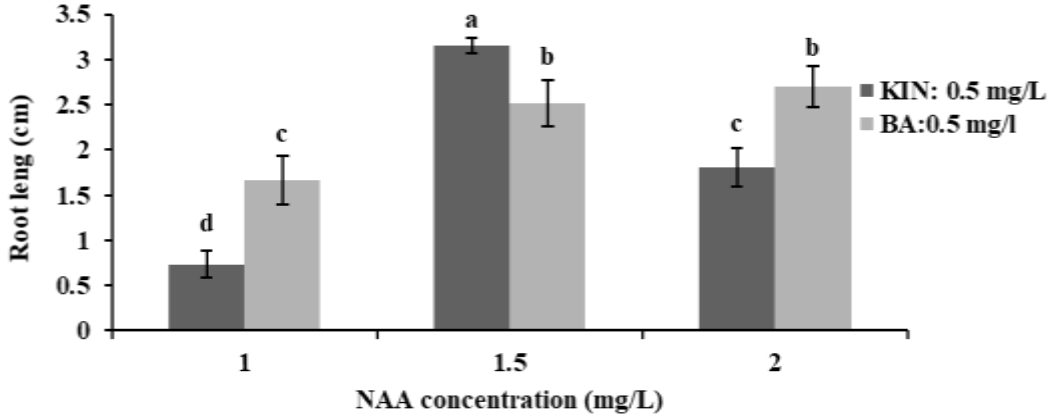

Fig. 6. Root length induced at different hormonal combination of NAA and KIN and NAA and BAP

Table 3. The viability percentage of regenerated stevia plants

Hormonal concentration / Viabity (%)	Treatment					
	NAA/KIN		NAA/BA	IBA/KIN		IBA/BA
	1.5:0.5 mgl^{-1}	2:0.5mgl^{-1}	2:0.5 mgl^{-1}	2:0.5 mgl^{-1}	1.5:0.5 mgl^{-1}	1.5:0.5 mgl^{-1}
	80%	20%	50%	25%	20%	30%

due to explant kind. Calli from leaf and root explants were shiny green while with nodal explants it was hard and brown (Pande and Gupta, 2013). Perrot's findings proved that kind of hormonal combination is discriminant callus attributes. In this study, it was found that the apparent response-maximum varies according to both the plant and the organ with shoots and roots showing differences in sensitivity of several orders of magnitude (Perrot-Rechenmann, 2010).

It is interesting to note that calli cells morphology and size were significantly different in cultures contained various hormonal combination. cell shapes were spindle to round in the hormonal treatments that may be attributed to different function of the hormones on mitosis. This may possibly be due to various effects of hormones on cytoskeleton and microtubules for determination of division plane orientation in calli cells (Blume *et al.*, 2012). A previous report described that auxin based hormones such as NAA promote cell elongation in stevia callus culture (Wada *et al.*, 1981). Our findings indicated that cell elongation took place at IBA/KIN and IBA/BA hormonal combination, as well as. These results are consistent with the researchers' report on the role of auxin in plant tissue culture. It reported that auxin causes elongation and prolongation of cells and cytokinin leads to cell division and tissue proliferation (Gupta *et al.*, 2010). The results of present work also indicated that there was the same morphology in calus cells in 2,4-D/KIN and 2,4-D/BA treatments. Alteration in cytokinin base hormone did not change the cell size and shape. As it was previously pointed out, 2,4-D as auxin based hormone, did not promote rooting and shooting. However it may be effective in somatic embryogenesis (Matsuta and Hirabayashi, 1989).

It was well known from the results that hormonal combination of auxins such as NAA or IBA with BAP with a ratio of 0.33 in MS medium efficiently differentiated shoots from callus in cultures. In contrast, high ratio of the combinations of 3-4 was essential for root induction. Previous studies indicated the role of NAA/BA combinations (0.1:0.5; 0.3:0.5 mgl^{-1}) in shoot and root production, respectively (Singh *et al.*, 2011). also we noted as in the early report full strength MS medium supplemented with NAA (1.5 mg/L) was the best medium for rooting (Hossain *et al.*, 2008). On the other hand, some authors emphasized on BA effects on shoot, root or embryo formation from leaf or stem explants of *S. rebaudiana* in MS media (Filho *et al.*, 1993; Rafiq *et al.*, 2007). So, cytokinins not only have promotive role in the shoot apical meristem, but also regulate the root negatively. In other words, some phenotypic changes can be explained by an altered ratio of the auxin–cytokinin. This indicates that these two hormones balance specifies only a subset of morphogenetic parameters taken together, influence of cytokinins on morpho-genesis is primarily achieved through cell cycle regulation cytokinins (Werner *et al.*, 2001). Studies have indicated that the role of cytokinins is stimulation of the cell elongation in coleoptile sections. In contrast, cytokinin inhibits auxin-induced cell elongation in stem and root sections (Evans, 1984). The high viability percentage in NAA/KIN (1.5:0.5 mg/l) may be attributed to high rooting process in this treatment then other ones (Guruchandran and Sasikumar, 2013).

A survey of literature showed that chemical

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

Table 4. Compounds identified in volatile oil of *Stevia rebaudiana* callus

Raw No	Compounds	Treatment							
		NAA/KIN (1:0.5mgl^{-1})		NAA/BA (1.5:2 mgl^{-1})		IBA/KIN (2:1 mgl^{-1})		IBA/BA (1:1.5 mgl^{-1})	
		Abundance[1] (%)	RI[2]	Abundance (%)	RI	Abundance (%)	RI	Abundance (%)	RI
1	Heptane, 2,3-dimethyl			0.39	nd[3]				
2	Heptane							1.23	700
3	n- Nonane	1.29	nd	0.39	nd			2.89	nd
4	Phenol	1.01	989	3.84	989	1.86	989	3.52	989
5	Decane	2.81	1000	3.29	1000			6.92	1000
6	Octanal					0.15	1004		
7	1,8-Cineole			2.39	1033				
8	Hexanal					0.38	1079		
9	Undecane	1.48	1100	1.39	1100			3.81	1100
10	Linalool					1.25	1101		
11	Nonanal					0.57	1104	1.85	1104
12	Dodecane	2.87	1200	2.27	1200			3.86	1200
13	Decanal							0.76	1206
14	Naphthalene			1.38	1214			2.98	1214
15	Octane, 2,6-dimethyl	0.81	1230	1.12	1230			2.32	1230
16	Tridecane					1.50	1300		
17	α-Longipinene					0.67	1351		
18	α-Copaene			1.85	1378				
19	β –elemene			0.20	1393				
20	Heptane,3,4, 5-trimethyl								
21	Tetradecane					1.81	1400	4.12	1400
22	Nonadecene	0.32	1900						
23	Obtusane	0.09	nd						
24	Hippuric acid					0.47	nd		
25	Acetaldehyde							0.45	nd
26	Octane, 1,1'-oxybis					0.25	nd		
27	2-Octyldodecan- 1-ol					0.51	nd		
28	Decane,1-iodo							1.96	nd
29	Caryophyllene					1.60	1419		
30	Trans-α-Bergamotene					0.86	1436		
31	α-Farnesene	3.01	1458						
32	t-β-Farnesene					3.01	1458		
33	β –Ionone							4.92	1484
34	Pentadecane					1.96	1500		
35	Propanoic acid	0.21	1550						
36	Caryophyllene oxide			0.68	1581	1. 07	1581		
37	gamma.-cis-sesquicyclogeraniol			1.02	1594	1.57	1594		
38	selinen-11-en-4-ol			0.89	1652				
39	Heptadecane							0/74	1698
40	Octadecane					1.56	1799		
41	Propane	0.26	nd	0.43	nd				
42	Eicosane					1.34	2000		
43	Heneicosane					1.32	2100		
44	Phytol			3.12	2114	1.69	2114	6.92	2114
45	Docosane			0.28	2200			2.12	2200
46	Pentacosane					0/55	2498		
47	Hexacosane							0.66	2600
48	Methylnonacosane					1.83	nd		
49	4-Octanone								
50	Heptacosane	0.39	2700						
51	Squalene	40.50	2829						
52	Decanedioic acid					2.36	nd		
53	Tetratriacontane					0.44	nd		
54	Triacontane					0.35	3000		

1. Content (%): Relative percentage calculated by GC/MS on an capillary column HP-5.
2. Retention index (RI) determined relatively to the retention time of a series of n-alkanes
3. not detected

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

Continue of table 4

Raw No	Compounds	Treatment							
		NAA/KIN (1:0.5mgl^{-1})		NAA/BA (1.5:2 mgl^{-1})		IBA/KIN (2:1 mgl^{-1})		IBA/BA (1:1.5 mgl^{-1})	
		Abundance[1] (%)	RI[2]	Abundance (%)	RI	Abundance (%)	RI	Abundance (%)	RI
55	Dotriacontane					1.57	3200		
56	Octadecane, 1-chloro								
57	Capnellane- 5.α.-ol					1.14	nd		
58	2-Methoxy myristic acid	2.16	nd						
59	4-hydroxy-3-nitrocoumar							0.31	nd
60	Tricosane			38.57	2300				
61	Octadecanoic acid					0.39	2163		
62	Ditridecyl ester							0.85	nd
63	Pyrrole-3,4-diacetic acid					1.54	nd		

1. Content (%): Relative percentage calculated by GC/MS on an capillary column HP-5.
2. Retention index (RI) determined relatively to the retention time of a series of n-alkanes
3. not detected

composition of the volatile oil of the leaves of *S. rebaudiana* was previously described (Hossein *et al.*, 2010). The comparison of the results of present work with those of Hossein *et al.* (2010) indicated that there is a considerable difference among volatile constituents profile of calli with the plant leaves. Whereas, the essential oil obtained from the plant leaves was dominated with monoterpenes and sesquiterpenes such as alpha-cadinol, caryophyllen oxide, spathulenol and beta-guinene, the oil from calli tissue induced from leaf segments composed of diterpenes, triterpenes and hydrocarbons like phytol, squalene and tricosan. It is consistent with the fact that plants metabolites produced in callus culture may be different from the plant organs. It is also worthy to mention that there is a considerable differences in the essential oil profile of calli induced from different hormonal combinations. It is thought that various hormonal treatments may tend to change in metabolic pathways. Differentiation stage influence as other factor on metabolic process and phyto- chemicals such as phenols in callus culture (Singh *et al.*, 2011).

References

Adams, R. P. (2004). Identification of essential oil component by gas chromategraphy/ quadrupole mass spectroscopy. Illinois: Allured publishing orporation.

Abdullateef, R. A. and Osman, M. (2011) Studies on effects of pruning on vegetative traits in *Stevia rebaudiana* Bertoni (Compositae). International Journal of Biology 4:146-152.

Bespalhok-Filho, J. C. and Hattori, K. (1997) Embryogenic callus formation and histological studies from *Stevia rebaudiana* (Bert.) Bertoni floret explants. Revista Brasileira de Fisiologia Vegetal 9: 185-188.

Blume, Y. B., Krasylenko, Y. A., and Yemets, A. I. (2012) Effects of phytohormones on the cytoskeleton of the plant cell. Russian Journal of Plant Physiology 59: 515-529.

Das, K., Dang, R., and Rajasekharan, P. E. (2006) Establishment and maintenance of callus of *Stevia rebaudiana Bertoni* under aseptic environment. Natural Product Radiance 5: 373-376.

Das, A., Gantait, S. and Mandal, N. (2011) Micropropagation of an elite medicinal plant: *Stevia rebaudiana Bert*. International Journal of Agricultural Research 6:40-48.

Evans, M. L. (1984) Functions of hormones at the cellular level of organization. In Hormonal Regulation of Development II. Pp. 23-79. Springer Heidelberg, Berlin.

Ferreira, T., and Rasband, W. (2012) ImageJ user guide. ImageJ/Fiji, 1.

Frederico, A. P., Ruas, M. A. Marinmorlaes, M. A., Ruas, C. F. and Nakajima, J. N. (1996) Chromosome studies in some Stevia (Compositae) species from southern Brazil. Brazilian Journal of Genetics 19: 605-609.

Goyal, S. K. and Goyal, R. K. (2010) Stevia (*Stevia rebaudiana*) a bio-sweetener: a review. International Journal of Food Sciences and Nutrition 61: 1–10.

Gupta, P., Sharma, S. and Saxena, S. (2010) Callusing in *Stevia rebaudiana* (natural sweetener) for steviol glycoside production. International Journal of Agricultural and Biological Sciences 1: 30-34.

Guruchandran, V. and Sasikumar, C. (2013) Organogenic plant regeneration via callus induction in *Stevia rebaudiana* Bert. International Journal of Current Microbiology and Applied Sciences 2: 56-61.

Hossain, M. A., Shamim Kabir, A. H. M., Jahan, T. A., and Hasan, M. N. (2008). Micropropagation of stevia. Int. J. Sustain. Crop Prod 3: 1-9.

Hossain, M. A., Siddique, A. B. Rahman, S. M. and Hossain, M. A. (2010) Chemical composition of the essential oils of *Stevia rebaudiana* Bertoni leaves. Asian Journal of Traditional Medicines 5:56–61.

[DOR: 20.1001.1.23222727.1397.7.28.2.5]

Janarthanam, B., Gopalakrishnan, M. and Sekar, T. (2010) Secondary metabolite production in callus cultures of *Stevia rebaudiana* Bertoni. Bangladesh Journal of Scientific and Industrial Research 45: 243-248.

Karuppusamy, S. (2009) A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. Journal of Medicinal Plants Research 3: 1222-1239.

Matsuta, N. and Hirabayashi, T. (1989) Embryogenic cell lines from somatic embryos of grape (*Vitis vinifera* L.). Plant cell reports 7: 684-687.

Pande, S. S. and Gupta, P. (2013) Plant tissue culture of *stevia rebeudiana* (Bertoli): A review. Journal of Pharmacognosy and Phytotherapy 5:26-33.

Patel, R. M. and Shah, R. R. (2009) Regeneration of Stevia plant through callus culture. Indian Journal of Pharmaceutical Sciences 71:46-50.

Perrot-Rechenmann, C. (2010) Cellular responses to auxin: division versus expansion. Cold Spring Harbor perspectives in biology 2: a001446.

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* Berton. Pakistan Journal of Botany 39:2467-2474.

Rathore, S., Yadav, K., Singh, N. and Singh, S. K. (2014) Comparative study on callus induction, proliferation and plantlets regeneration in two cultivars of *Stevia rebaudiana* Bertoni-the only non-caloric natural sweetener. Pert. Pertanika Journal of Tropical Agricultural Science 37: 499-508.

Singh, N., Yadav, K. and Kumari, S. (2011) Metabolic changes during differentiation in callus cultures of *Stevia rebaudiana* (Bertoni). Journal of Phytology 3:63-67.

Uddin, M. S., Chowdhury, M. S. H., Khan, M. M. M. H., Uddin, M. B., Ahmed, R., *et al*. (2006) In vitro propagation of *Stevia rebaudiana* Bert in Bangladesh. African Journal of Bio- technology 5:1238-1240.

Vanisree, M., Lee, C. Y., Lo, S. F., Nalawade, S. M., Lin, C. Y. and Tsay, H. S. (2004) Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. Botanical bulletin of Academia Sinica 45: 1-22.

Wada, Y., Tamura, T., Kodama, T., Yamaki, T. and Uchida, Y. (1981) Callus cultures and morphogenesis of Stevia rebaudiana Bertoni. Journal of Japan Oil Chemists Society 30: 215-219.

Werner, T., Motyka, V., Strnad, M. and Schmulling, T. (2001) Regulation of plant growth by cytokinin. Proceedings of the National Academy of Sciences 98: 10487-10492

[DOR: 20.1001.1.23222727.1397.7.28.2.5]