Assessment of Stevioside Content, Shoot Proliferation, and Root Induction of *Stevia rebaudiana* Bertoni under *In vitro* Conditions

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Abstract

Background: ***Stevia rebaudiana*** **Bertoni, an important anti-diabetic medicinal plant, becomes an inevitable alternative to sugar. Due to the propagation difficulties, tissue culture is the best alternative for rapid mass propagation of stevia plants.**

Objective: The present study was conducted to optimize a protocol for rapid micropropagation of *Stevia rebaudiana* by shoot tip explants and to investigate the effect of growth regulators concentration on steviol-glycosides content under *in vitro* culture conditions.

Methods: Young nodal stem explants were collected from a young growing plant and the effects of media and growth regulators on shoot proliferation were studied. Shoots produced on optimal medium for shoot proliferation were used for rooting experiments and the effects of IBA, NAA, and active carbon on root induction in both full MS and half MS media were investigated.

Results: Based on the results obtained from the proliferation stage, there was no significant difference between growth regulator levels. Analysis of data obtained from rooting experiment, revealed that there are significant differences between growth regulators in leaf length, shoot dry weight, and root fresh weight. Based on the results of mean comparison, plantlets grown on free active carbon media had the higher biomass than those grown on media supplemented with 2 $mg.L^{-1}$ active carbon. The highest content of stevioside (8.18%) was observed at half MS medium supplemented with 0.2 $mg.\ L^{-1}$ IBA, and 2 $mg.L^{-1}$ active carbon.

Conclusion: Plant growth regulators can be included among the factors affecting shoot proliferation and root induction of *Stevia rebaudiana.* Micropropagation of stevia can be improved by altering the macro elements concentration and adding activated carbon. In conclusion, half MS medium supplemented with 0.2 $mg.L^{-1}$IBA, and 2 $mg.L^{-1}$active carbon was superior for stevioside content.

Keywords: ***Stevia rebaudiana*****, Growth regulator, Micropropagation, Proliferation, Stevioside**

Introduction

Stevia rebaudiana Bertoniis one of the important anti-diabetic medicinal herbs, belongs to the *Asteraceae* family. *S. rebaudiana* was botanically classified in 1899 and initially called Eupatorium rebaudianum [1]. Studies revealed that Stevia has been used since ancient times for various purposes throughout the world [2]. Stevia has a remarkable water need but low nutrient requirements so, the stevia plants can be grown in relatively poor soil with frequent, shallow irrigation [3]. This plant is widely grown for its sweet leaves. The quantity of dry leaves that can be harvested varies from 15 to 35 g per plant [4]. One planted hectare can produce between 1000 and 1200 kg of dried leaves that contain 60–70 kg stevioside [5]. The stevia glycosides are the compounds responsible for the sweet taste. The leaves of stevia are the source of steviol glycosides such as stevioside and rebaudioside, which have 250–300 times the sweetness of sugar [6]. Stevioside was the first compound which was identified and applied as a sweetener in food products. In pure form, stevioside (4-α-13-[2-O-β-D-glucopyranosyl-β-D-glucopyranosyl] kaur- 16-en-18-oic acid *β-D*-glucopyranosyl ester) is a white crystalline powder with a melting point of 198 C, an optical rotation of -39.3^{D} in water, with a molecular formula of $C_{38}H_{60}O_{18}$, (MW: 804.88). Stevioside is sparingly soluble in water (1 g dissolves in 800 ml of water), but it is soluble in dioxane and slightly soluble in ethanol [7]. During the 1970s, other compounds were isolated, including rebaudioside A, with a sweetening potency even higher than stevioside [8]. Stevioside and rebaudioside A are the most widely studied steviol glycosides, for purposes of extraction, use as sweeteners and food additives, and for medicinal purposes [7]. There are a number of studies have reported that, stevioside, along with related other compounds such as rebaudioside A, steviol and isosteviol, may also offer therapeutic benefits [9]. In medical research, stevioside has shown promise in treating conditions such as diabetes, hypertension, tumors, and obesity. Stevia may also possess antimicrobial and antiviral properties. Although, data on these applications is still not supported by strong scientific literature, convincing evidence on stevia's antihypertensive properties was demonstrated in a clinical study [7]. Today, *Stevia rebaudiana* Bertoni as a natural sweetener plant with zero calorie content, becomes an inevitable alternative to sugar. Stevioside can be particularly beneficial to those suffering from obesity, diabetes mellitus, heart disease and dental caries [10]. *S. rebaudiana* Bertoni has attracted economic and scientific interests due to the sweetness and the supposed therapeutic properties of its leaf [1].

Stevia is a self-incompatible and insect-pollinated plant. A number of studies have reported poor percentages of viable seeds and Poor seed germination in stevia [1, 11]. Consequently, Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition [12, 13]. This plant is therefore usually propagated by stem cuttings which root easily, but require high labor inputs. The vegetative propagation is further limited by the

lower number of individuals that can be obtained simultaneously from a single plant. Due to the above-mentioned difficulties, tissue culture is the best alternative for rapid mass propagation of Stevia plants [14]. A number of studies have been conducted to evaluate the effects of tissue culture conditions and explant characteristics on micropropagation of stevia and also compare the stevioside content in the in vivo and in vitro leaves, supporting the effectiveness of micropropagation protocol generated [15]. In a study to assess the amounts of water-soluble vitamins in the Stevia leaf and callus extracts, reported that the contents of folic acid, vitamin C and vitamin B2 in the leaf extracts were significantly higher than those of the callus extracts. In the leaf extract, folic acid was found to be the major compound, followed by vitamin C. In the callus extract, vitamin C was the major compound, followed by vitamin B [16].

Even though in the recent past *S. rebaudiana* has received little attention in *in vitro* multiplication, but improvement in the yield of stevioside needs further attention. In the search for alternative to production of desirable medicinal compounds from Stevia, biotechnological approaches, particularly plant tissue culture is found to have potential in the industrial production of bio-active plant metabolites. The present study was conducted to optimize a protocol for rapid micropropagation of *Stevia rebaudiana* by shoot tip explants and to investigate the effect of growth regulators concentration on stevioside content under in vitro culture conditions.

Materials and Methods

Plant materials and sterilization procedure

Young nodal stem explant ranging in size from 1.5 to 2 cm were collected from a young growing plant maintained in the greenhouse. The explants were pre-sterilized in 70% (v/v) ethanol for 30 seconds and washed thoroughly with sterile distilled water. Further, sterilization was carried out using 2.5% (w/v) sodium hypochlorite. The explants were then rinsed three times with sterile distilled water.

Proliferation

Two MS medium [17] types (full and half MS) supplemented with three different concentration (0, 0.1 and 0.2 $mg.L^{-1}$) of GA were used for propagation (Table 1). The pH of culture medium was adjusted to 5.8, and 0.7% agar was added prior to autoclaving. The sterilized explants were cultured on six different media and maintained at a temperature of 25 ± 1 °C and a 16 h photoperiod. Shoot length (SL), shoot number (SN), Number of nodes (NN), leaf length (LL), and alive plant percentage (AP) were recorded after 4 weeks.

In vitro rooting

Shoots produced on optimal medium for shoot proliferation were used for rooting experiments. The effects of IBA, NAA and Active carbon on root Induction in both full MS and half MS media were investigated. Various concentrations of growth regulators (0.1 and 0.2 $mg.L^{-1}$ of IBA and NAA, separately) were tried for root induction and MS medium without growth regulators served

Table1- Studied factor and their levels in rooting experiment.

Factors	Levels																			
Growth regulator	Control (free hormone)				IBA ($0.1\ mg.l^{-1}$)				IBA ($0.2\ mg.l^{-1}$)				NAA ($0.1\ mg.l^{-1}$)				NAA ($0.2\ mg.l^{-1}$)			
Media	MS		$^{1/2}$MS		MS		$^{1/2}$MS		MS		$^{1/2}$MS		MS		$^{1/2}$MS		MS		$^{1/2}$MS	
Active carbon ($mg.l^{-1}$)	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2

as control. Both full and half MS media supplemented with 2 $g.L^{-1}$active carbon (AC) or without AC were used for in vitro rooting. The studied factors has been shown in Table 1.

Shoot length (SL), shoot number (SN), number of nodes (NN), leaf length (LL) and alive plant percentage (AP), root dry weight (RDW), shoot dry weight (SDW), root fresh weight (RFW), and shoot fresh weight (SFW), stevioside content (Stev.) were recorded after 5 weeks.

HPLC quantification of stevioside

In this research, stevioside content was determined in the callus and shoot from which the callus was developed by HPLC (High performance liquid chromatography) method.

HPLC system and conditions: HPLC analysis was performed by using a Knauer K2600A liquid chromatography (Germany) set with KNAUER-K1001 pump, solvent vacuum degasser, YMC-PackNH_2 column (ODS; 250 × 4.6 mm, 5 µm particle size), a variable wavelength detector, and an auto sampler with a 10 µL injection loop. YMC-Pack NH_2 is a normal-phase separation column utilizing the polarity of primary amino groups. It is also applicable to separations utilizing weak anion exchange. YMC-Pack NH_2 is often used for separation of sugars. Peak detection was made at 210 nm at the room temp. of 25°C by UV detector [KNAUER-UV (K2501)] from the above mentioned company. Separation was performed with a water and acetonitrile solvent system. Mobile phase was prepared from HPLC grade water (80%; A) and acetonitrile (ACN) (20%; B), respectively. The flow rate was 1.0 $ml.min^{-1}$ and the injection volume was 10 µL. Stevioside standard was purchased from Sigma Aldrich Ltd., USA (95 % purity). All the other chemicals used were of analytical grade. The standard was run on HPLC system and retention time for stev. was determined. Identification of stev. Indifferent *in- vitro* grown tissues was calculated by comparing the retention time of samples with standard. The stock and standard solution were diluted with water-acetonitrile (8:2) accordingly and were used for the confirmation of retention times.

Extract preparation: Callus tissues were sampled, and the contents of stevioside in calli were analyzed. Large friable light brown calli was formed in nearly 95% of the cultures. Extraction of stevioside (Stev.) from callus and shoot-derived callus was determined according to the recent method of Kailasam (2011) with slight modification [18]. In one set of our experiments, stevioside content of for 45 days were examined [19].The shoot-derived callus cultured of *Stevia rebaudiana* Bertoni were crushed and approximately 0.1 g of the powder was weighed into a 20 mL glass vial. Ten milliliters of 20% water and 80% ACN

mix was added to the vial which was then vortexed. The extraction was carried out by sonication for 60 minutes. The contents of the vial were centrifuged (5000 rpm) and the supernatant diluted 10 times with 20% water and 80% ACN mix. To an aliquot of the10 times diluted sample, we added 2,200 $\mu g.mL^{-1}$ stock solutions of stevioside to test analyte recoveries. The final concentration of each analyte in the spiked sample was 100 $\mu g.mL^{-1}$ [18]. The results obtained for each sample was expressed in percentage of dry weight. Observations were recorded and stevioside content were examined using four replicates. Data were subjected to Duncan's multiple range test ($P \leq 0.05$).

Data Analysis

The treatments were arranged in a factorial experiment based on completely randomized design (CRD) with three replications for experiment one (Shoot proliferation) and two (Root induction), and four replications for experiment three (Quantification of stevioside**)**. In the first experiment, two factors including, media, and growth regulators were studied. The second and third experiment was conducted to study the effects of three factors including media, active carbon, and growth regulators on the root induction and stevioside content. The collected data of each experiment were separately analyzed using factorial experiment based on completely randomized design. Statistical analysis of the data was carried out using SPSS 21 software and means comparison were done using Duncan's Multiple Range Test ($P \leq 0.05$).

Results

Based on the results obtained from the proliferation stage, there was no significant difference between growth regulator levels. The interaction effect of culture media and growth regulator also was not significant. However, the effect of media was significant for shoot length and number of nude. The plantlets grown on MS medium were found to have more shoot length and node number than those plantlets grown on half strength of MS medium (Table 2). Based on the results of mean comparison, free GA full MS was found as the best medium for shoot proliferation of stevia (Table 3). Previous studies have shown that cytokinins alone or along with auxins are essential for explant proliferation and proliferation rate raised considerably by increasing subculture times which is attributed to plant rejuvenation status [20]. As reported in some studies, integration of auxin and cytokinin is highly effective in proliferation of woody plants; however, auxin concentration should not be more than a significant level [20].

Analysis of data obtained from rooting experiment revealed that there are significant differences between growth regulators in leaf length, shoot dry weight and root fresh weight. Also the results revealed that there are significant differences between plantlets grown on full and half strength of MS medium. On the other hand, the results of analysis of variance showed that the subculture media supplemented with active carbon were significantly different than those that prepare without active carbon. As shown in Table 4, Active-carbon × Media interaction was

Table 2- Analysis of variance for the effect of growth regulator and medium and their interaction on shoot length (SL), Number of shoots (SN), nude number (NN), leaf length (LL) and alive plants (AP) in proliferation stage

S.O.V	df	Mean of squares				
		SL	SN	NN	LL	AP
Medium	1	38.7*	1.92^{ns}	9.3**	0.005^{ns}	0.22^{ns}
Growth regulator	2	22.1^{ns}	0.54^{ns}	0.95^{ns}	0.027^{ns}	0.096^{ns}
Medium× Growth regulator	2	2.6^{ns}	0.99^{ns}	0.3^{ns}	0.16^{ns}	0.016^{ns}
Error	12	6.3	0.7	0.7	0.2	0.07
C.V%		32	40	14	18	30

* indicates a significant difference at $P < 0.05$; ** indicates a significant difference $P \leq 0.01$; ^{ns} indicates non- significant difference

Table 3 - Number of nodes and shoot length of stevia in two different media (MS and half MS) in proliferation stage

	Number of node	Shoot length (cm)
MS	6.8^{a}	12.1^{a}
Half MS	5^{b}	9.4^{b}

There were no significant differences between the mean values shown with the same superscript letters at 5% probability level using Duncan's multiple range test.

Table 4 - Analysis of variance for the effect of active carbon, growth regulator and medium and their interactions on number of shoots (SN), nude number (NN), shoot length (SL) , leaf length (LL), alive plants (AP), root dry weight(RDW), shoot dry weight (SDW), root fresh weight (RFW) and shoot fresh weight (RFW) in rooting stage

S.O.V	df	Mean of squares								
		SN	NN	SL	LL	AP	RDW	SDW	RFW	SFW
Active carbon	1	2.97**	1.78^{ns}	24.7**	1.13**	0.15**	0.99^{ns}	0.13*	0.2*	81.1^{ns}
Media	1	3.15**	4.7^{ns}	0.01^{ns}	14.8**	0.01^{ns}	0.12^{ns}	0.64**	0.09^{ns}	94.7^{ns}
Growth regulator	4	0.13^{ns}	2.7^{ns}	1.2^{ns}	0.46*	0.01^{ns}	0.02^{ns}	0.09*	0.11*	85.7^{ns}
Active carbon × Media	1	0.59^{ns}	0.08^{ns}	0.04^{ns}	1.15**	0.01^{ns}	0.11^{ns}	0.21*	0.15*	74.8^{ns}
Activecarbon × Growthregulator	4	0.06^{ns}	0.33^{ns}	0.29^{ns}	0.07^{ns}	0.01^{ns}	0.04^{ns}	0.01^{ns}	0.02^{ns}	83.2^{ns}
Media × Growth regulator	4	0.35^{ns}	5.9**	1.5^{ns}	0.41*	0.01^{ns}	0.01^{ns}	0.08*	0.02^{ns}	82.7^{ns}
Activecarbon × Media × Growth regulator	4	0.02^{ns}	0.37^{ns}	0.63^{ns}	0.04^{ns}	0.01^{ns}	0.05^{ns}	0.01^{ns}	0.02^{ns}	79.8^{ns}
Error	40	0.16	1.2	1.1	0.13	0.01	0.05	0.03	0.036	82.1
C.V%		22	15	9	22	11	21	14	13	32

* indicates a significant difference at $P < 0.05$; ** indicates a significant difference $P \leq 0.01$; ^{ns}indicates non- significant difference

significant on leaf length (LL), shoot dry weight (SDW) and root fresh weight (RFW). The interaction effect of media and growth regulator on node number (NN), leaf length (LL) and shoot dry weight (SDW) was significant. However, Active carbon × Growth regulator interaction was not significant. The three-way interaction also was not significant. The highest node number was obtained in Half MS media without growth regulator (Figure 1). MS medium supplemented with 0.1 $mg.L^{-1}$ NAA and treatment of half MS supplemented with 0.2 $mg.L^{-1}$ IBA produced the highest leaf length and shoot dry weight, respectively

(Figure 2 and 3). As shown in table 5, the media supplemented with different plant growth regulators were significantly different in number of nodes, leaf length, shoot dry weight, and root fresh weight. Mean comparison revealed that The plantlets grown on MS medium supplemented with active carbon were found to have better characteristics compared to those grown on media without active carbon. In addition, full MS medium was significantly better than half strength of MS for shoot proliferation and micropropagation of stevia. Based on the

Table 5- Means comparison of growth regulator levels for significant traits in *Stevia rebaudiana* Bertoni, under *in vitro* culture conditions

	Number of nodes	Leaf length (cm)	Shoot dry weight (gr)	Root fresh weight (gr)
Control	7.6 ^{a}	1.74 ^{ab}	1.07 ^{b}	1.39 ^{b}
IBA(0.1mgL-1)	6.5 ^{b}	1.86 ^{a}	1.10 ^{b}	1.32 ^{b}
IBA(0.2mgL-1)	6.6 ^{b}	1.52 ^{bc}	1.22 ^{ab}	1.45 ^{ab}
NAA(0.1mgL-1)	6.5 ^{b}	1.67 ^{abc}	1.18 ^{ab}	1.39 ^{b}
NAA(0.2mgL-1)	7.1 ^{ab}	1.35 ^{c}	1.28 ^{a}	1.58 ^{a}

There were no significant differences between the mean values shown with the same superscript letters at 5% probability level using Duncan's multiple range test.

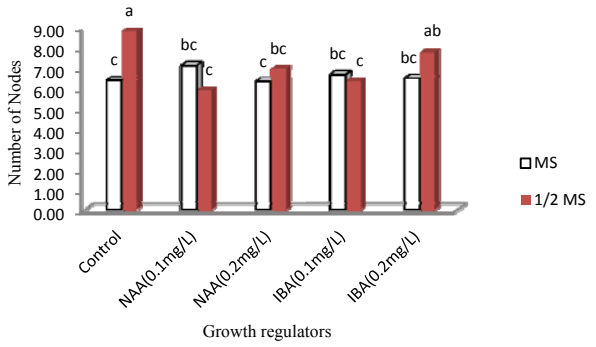


Figure 1- Effect of different levels of growth regulators on node number of stevia in MS and half MS media

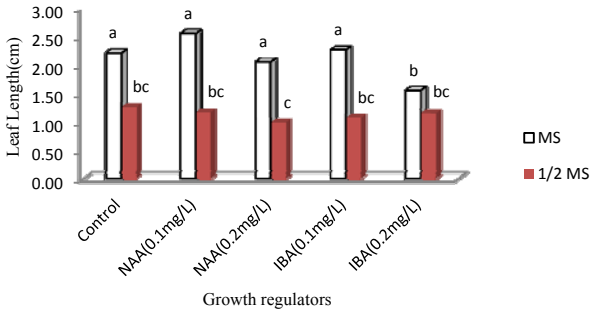


Figure 2- Effect of different levels of growth regulators on leaf length of stevia in MS and half MS media

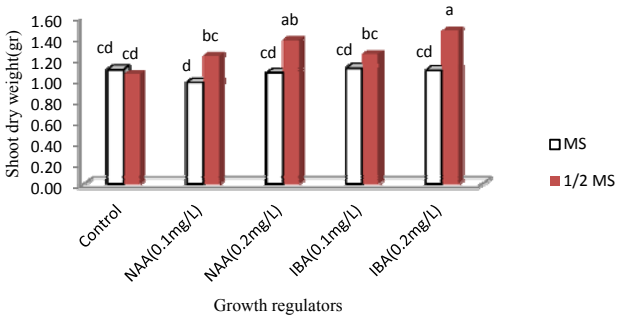


Figure 3- Effect of different levels of growth regulators on shoot dry weight of stevia in MS and half MS media

Table 6 - Results of the factorial analysis of variance (ANOVA) for effects of growth regulator, media and active carbon and their interactions on stevioside in *Stevia rebaudiana* Bertoni

S.O.V	df	Stev.
		Mean of squares (MS)
Growth regulator (G)	4	8.955**
Media (M)	1	13.178**
Active carbon (AC)	1	53.705**
Growth regulator (G) × Media (M)	4	.697^{ns}
Growth regulator (G) × Active carbon (AC)	4	.931^{ns}
Media (M) × Active carbon (AC)	1	19.337**
Growth regulator (G) × Media (M) × Active carbon (AC)	4	.429^{ns}
Error	60	.674
CV. (%)	-	17.51

* indicates a significant difference at $P < 0.05$; ** indicates a significant difference $P \leq 0.01$; ^{ns} indicates non- significant difference.

Table 7- Results of means comparisons effects of growth regulator, media, and active carbon, and their interactions on stevioside in *Stevia rebaudiana* Bertoni

Factors	Treatments	Stevioside (%)	
		Means	Std. Deviation
Growth regulator (G)	G_1 mean (Control)	3.908^{d}	0.983
	G_2 mean (0.1 mg.l^{-1} NAA)	4.228^{cd}	1.431
	G_3 mean (0.2 mg.l^{-1} NAA)	4.864^{b}	1.283
	G_4 mean (0.1 mg.l^{-1} IBA)	4.574^{bc}	1.202
	G_5 mean (0.2 mg.l^{-1} IBA)	5.861^{a}	1.698
Media (M)	M_1 mean (Full MS)	4.281^{b}	1.082
	M_2 mean (Half MS)	5.092^{a}	1.693
Active carbon (AC)	AC_1 mean (Free active carbon)	3.868^{b}	0.985
	AC_2 mean (2 mg.l^{-1} active carbon)	5.506^{a}	1.424
Media (M_1: Full MS)	AC_1 mean (Free active carbon)	3.953^{bc}	1.103
	AC_2 mean (2 mg.l^{-1} active carbon)	4.609^{b}	0.978
Media (M_2: half MS)	AC_1 mean (Free active carbon)	3.782^{c}	0.870
	AC_2 mean (2 mg.l^{-1} active carbon)	6.404^{a}	1.229

There were no significant differences between the mean values shown with the same superscript letters at 5% probability level using Duncan's multiple range test.

results of mean comparison, plantlets grown on free active carbon media, had the higher biomass than those grown on media supplemented with 2 $g.L^{-1}$ active carbon.

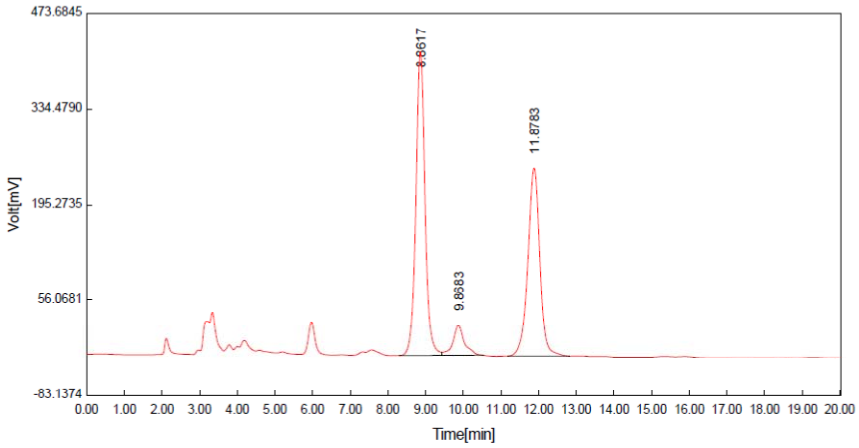
Forty-five days after inoculation, callus tissues were sampled at different growth regulators, media, and active carbon treatments, and the content of stevioside in calli were analyzed. In this part of study, analysis of stevioside was carried out from the callus in HPLC. In the present experiment, identification and quantification of stevioside content in the samples was done by comparing the retention time, UV spectra and peak area of sample with that of the standard. Figure 4 shows some selected peaks on HPLC chromatograms and fingerprinting of stevioside in *in vitro* with similar retention time, such as standard stevioside, control treatment, and treatment of 0.2 $mg.L^{-1}$ IBA + half MS medium + 2 $mg.L^{-1}$AC.The retention time (RT) of stevioside for standard were found to be 8.86 min and for callus 8.72 to 8.91 min (Figure 4). Generally, results showed that the growth regulators, media, active carbon, and interaction of media and active carbon had a significant effect on the content of stevioside ($P\leq 0.01$). There was no significant interaction between growth regulators and media with active carbon, growth regulators and media, and also, active carbon. But, between the media and application of active carbon was observed a significant interaction (Table 6).

Based on the results of mean comparison, the highest content of stevioside was observed at 0.2 $mg.L^{-1}$ IBA (5.86%), 1/2 MS medium (5.09%), and 2 $mg.L^{-1}$ active carbon (5.51%)by main effect of experimental factors, and half MS medium supplemented with 2 $mg.L^{-1}$ active carbon (6.40%) by interaction treatment. Also, the lowest of stevioside accumulation was obtained in the control treatment (3.91%), full MS (4.28%), and free active carbon (3.86%) by main effect of experimental factors, and full MS medium without active carbon (3.78%) by interaction treatment (Table 7).

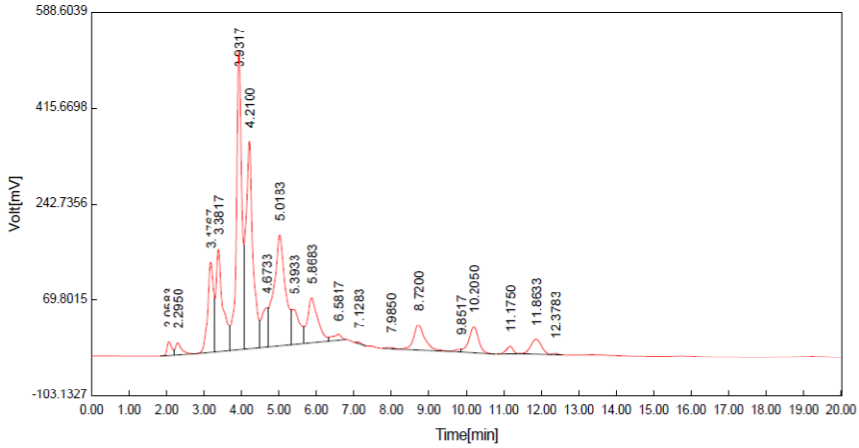
Discussion

Use of auxins for in vitro rooting stage has also been reported by many other researches [20]. Auxins play an important role in root induction because they promote adventitious root formation in the vast majority of species [21]. Indole acetic acid (IAA), indole butyric acid (IBA) and naphthalene acetic acid (NAA) are mainly three types of auxins used for root induction. However, plants respond quite differently to these auxins in regard to adventitious root formation [22]. A previous study demonstrated that plant growth regulators can be included among the factors affecting shoot proliferation and root induction of *Casuarina cunninghamiana* [23].

The quality (content of glycosides) of *S. rebaudiana* can be regulated by changes in the composition of the nutrient medium. HPLC analysis of callus cultured on MS medium revealed presence of stevioside 5.09% in half strength, and 4.28% in full strength. Therefore, it may be concluded that half MS medium is suitable for callus maintenance in growth regulator combinations of NAA and IBA. Further, the use of half MS medium was also



(*a*)



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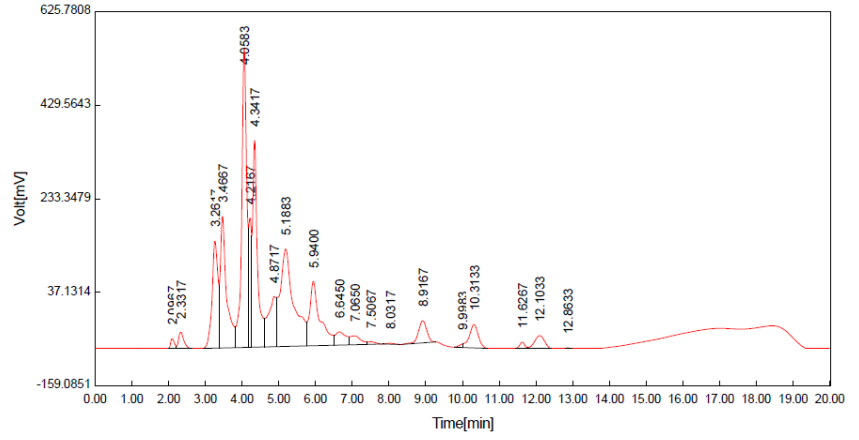

(*c*)

Figure 4 - Some selected peaks on HPLC chromatograms and fingerprinting of stevioside in *in vitro* with similar retention time (*a-c*), *a*; Standard stevioside (peak 8.86), *b*; control treatment (peak 8.72), and *c*; 0.2 mg.l^{-1}IBA+halfMS medium+2 mg.l^{-1}AC samples (peak 8.91)

found better with respect to percentage of stevioside content compared to full MS medium. For stevioside accumulation, MS to half strength was superior full strength. The inverse relationship between medium strength and secondary metabolite accumulation in stevia tissue cultures could be possibly explained by the fact that full nutrient media predominantly promote primary metabolism and cellular growth, in some cases hampering morphological and biochemical tissue differentiation. This hypothesis has been previously verified for the inhibition of differentiation of melon callus cultures by increased concentrations of soluble carbohydrates and selected macronutrient ions (K^+, Ca^{2+}) [24]. High frequency stevioside content from the callus occurred when the latter was transferred to half MS medium supplemented with different concentrations of IBA, and NAA. The higher stevioside content in certain hormonal treatments may be due to the addition of different plant growth regulators (PGRs) to the medium. The highest stevioside content was obtained on half MS medium containing 0.2 $mg.L^{-1}$ IBA, with active carbon. The effect of IBA is in concurrence with other studies where IBA is the most commonly used auxin for root formation and improve the production of stevioside. Recently, Dey *et al*. (2013) reported that addition of IBA in combination with chlorocholine chloride increased the stevioside content than other PGRs [25]. The current data are in agreement with the results of Khalil1 et al. (2014) [26]. Previous reports suggest that some of plant growth regulators help in the biosynthesis of metabolites such as ribotides, ribisides, and riboside-glucoside, but the exact mechanism as to how these plant growth regulators take part in the metabolic pathway is not elucidated yet. The results are confirmed with the findings of Sivaram and Mukundan (2003), and Babu et al. (2011) [27, 28]. Attempts on promoting accumulation of stevioside in callus by adding 2 $mg.L^{-1}$ active carbon to the medium were conducted. Active carbon is the used optimization method to get the minimum colour reduction with a maximum stevioside content. It is found that active carbon is the best absorbance to use for producing optimal stevioside content with minimum colour reduction [29].

Applications of different chemicals elicitors and bio-stimulants for alteration of plant secondary metabolism offer a novel approach to induce some beneficial changes in the production of photochemical, which can be exploited commercially [29]. Present investigation discusses the use and effect of IBA, active carbon on *in vitro S. rebaudiana*. Half MS in combinations with IBA, and active carbon found to be most effective for inducing certain beneficial changes for higher stevioside production (8.18%) in comparison to full MS without plant growth regulators, and active carbon (3.571%). In conclusion, half MS medium supplemented with 0.2 $mg.L^{-1}$ IBA, and 2 $mg.L^{-1}$ active carbon was superior for stevioside content. The major observations of this research indicate that application of IBA and active carbon in micropropagation of *S. rebaudiana* Bertoni is a promising approach and has commercial prospects, especially in the pharmaceutical industry.

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