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Biochemical Studies on the Production of Active Constituents in *Stevia rebaudiana* L. Callus

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Abstract: The role of some inducers; glucose (10, 30, 50 and 70 g/l), gibberellic acid (1, 2, 4 and 8 ppm), proline acid (10, 50, 100 and 200 ppm), glutamic acid (10, 50, 100 and 200 ppm) and 2-acetoxybenzoic acid (10, 50, 100 and 200 ppm) was examined for their effect on the production of active constituents in *Stevia rebaudiana* L. callus and their relation to callus production. Results showed that treatments with glucose, gibberellic and proline appeared to be effective on callus growth (fresh and dry weights) and the other treatments came in the second order. In this regard, the application of gibberellic acid (8 ppm) and proline acid (200 ppm) gave the best results of fresh weights. Also, the maximum value of dry weight was recorded when stevia callus was treated with proline at rate of 200 ppm. In the same direction, data showed the positive effect of glucose, gibberellic and proline to reduce the level of lipid peroxidation (malondialdehyde content), which used as a biomarker to measure oxidative stress in stevia callus. The lowest values were obtained when callus treated with proline at 200 ppm and glucose at 50 g/l. The accumulation of sweet component (stevioside) was evaluated in callus cultures through HPLC analysis. Application of glucose, proline and gibberellic acid had a promotive role in enhancing stevioside content in stevia callus. The maximum values were obtained when glucose, proline and gibberellic acids were applied at rates of 50 g/l, 200 ppm and 8 ppm, respectively. The percentages of increments were reached 90.12, 84.46 and 7.32% when compared with the control. These increases were associated with the accumulation of some free amino acids such as glycine, alanine, valine and phenylalanine. The results showed that glutamic acid and 2-acetoxybenzoic acid had a little effect on the active constituent (stevioside) in stevia callus and this was evident at the low concentration. On the other hand, the high concentrations negatively affected the production of active constituent in stevia callus. Twenty two amino acids were detected in stevia callus and the most abundant amino acids noticed in callus were serine, UFAA3 (unknown free amino acid), histidine, proline, alanine and valine. On the other hand, methionine, cysteine, UFAA1, UFAA2 and UFAA4 were presented in minute quantities. Other identified free amino acids in stevia callus had concentrations in between those extremes. This study highlights on the importance of use some chemical inducers; glucose, gibberellic and proline through tissue culture technique in order to produce stevioside in stevia callus on a large scale and at a low cost level. Also, the research recommended using this study to develop a protocol based on this technique in order to provide a good and permanent source of stevioside material, which has medical and industrial importance.

Key words: *Stevia rebaudiana* L. callus · Stevioside · Malondialdehyde · Free amino acids · growth

INTRODUCTION

Stevia rebaudiana L. is a natural non calorie sweetener plant, which belongs to family Asteraceae. Stevia leaves contain sweet components, which are called steviol glycosides and they are responsible for this sweetness. Sweet components vary between 4% to 20% of the dry weight of the leaves depending on stevia genotypes, treatments and growing conditions. In this regard, stevioside is the main sweet component in the

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leaves and tastes about 300 times sweeter than sucrose and it is safe when used as a sweetener. Other sweet compounds present in stevia leaves, but in lower concentration are: steviolbioside, rebaudioside A, B, C, D, E, F and dulcoside A [1, 2]. Stevioside is a diterpenic carboxylic alcohol with three glucose molecules [3, 4] and mainly used commercially as sugar substitute. In addition to its interesting sweetening property, stevia extract shows many pharmacological properties. Stevioside can be used as an antihyper glycaemic [5], antihypertensive [6], anti-tumor [7] as well as effective in blood pressure reduction [8, 9]. In addition, diet conscious and diabetic persons with hyperglycemia can use steviosides as an alternative sweetener [10]. Stevia can regenerate by seeds, but they are very small in size and infertile. Also, the seeds show a very low germination percentage, so large scale mechanized production of stevia through seeds is not fruitful [11]. In this concern, 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]. In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically plant tissue culture are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites [14]. Due to the above-mentioned difficulties, plant tissue culture or micropropagation can be used for rapid propagation and conservation of such valuable and endangered plant species [15, 16], which are difficult to propagate by conventional methods. In addition, this technology eliminates potential political and geographical boundaries against crop production and protection from weather flections, diseases, pests or soil problem. Recently, this technique used in modern laboratories in large scale to ensures the production of sufficient amounts of active biochemical constituents (high yielding) from endangered plants in very short span of time, which are difficult to propagate by conventional methods.

The present investigation aims to study the influence of some chemical materials on the production of active constituents in *Stevia rebaudiana* L. callus.

MATERIALS AND METHODS

Source of Explants: Stevia plants were obtained from the Sugar Crop Institute, Agricultural Research Center, Giza, Egypt. The plants were maintained under greenhouse conditions of the Desert Research Center, Cairo, Egypt, for at least 30 days prior to removal of material for culture. Leaves were removed from the branches and transferred immediately to the laboratory for sterilization. The leaves were washed for 15 minutes in running tap water then rinsed in sterile distilled water and sterilized under aseptic conditions by immersion for 20 minutes in 20% (v/v) commercial bleach (Clorox) followed by 3 minutes in 0.1% (w/v) mercuric chloride solution then washed 6 times with sterile distilled water to remove the traces of mercuric chloride.

Induction of Callus Cultures: Sterilized leaves were cultured on basal MS medium [17] supplemented with myoinositol (100 ppm), 30 g/l sucrose as a carbon source and 3g/l phytagel was used as gelling agent. To evaluate the plant growth regulators (PGRs) type and concentration on callus induction, explants were cultured on MS medium supplement with benzyl adenine (BA) at 0.5 ppm and either 2,4 dichlorophenoxy acetic acid (2,4-D) or naphthalene acetic acid (NAA) at different concentrations as follows:

1	0.0 ppm 2,4-D + 0.0 ppm BA	7	0.5 ppm NAA + 0.5 ppm BA
2	0.5 ppm 2,4-D + 0.5 ppm BA	8	1.0 ppm NAA + 0.5 ppm BA
3	1.0 ppm 2,4-D + 0.5 ppm BA	9	1.5 ppm NAA + 0.5 ppm BA
4	1.5 ppm 2,4-D + 0.5 ppm BA	10	2.0 ppm NAA + 0.5 ppm BA
5	2.0 ppm 2,4-D + 0.5 ppm BA	11	2.0 ppm NAA + 0.5 ppm 2,4-D + 0.5 ppm BA
6	2.0 ppm 2,4-D + 0.5 ppm NAA + 0.5 ppm BA		

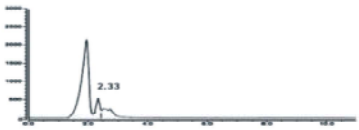
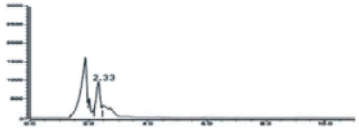
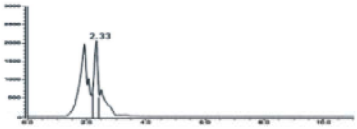
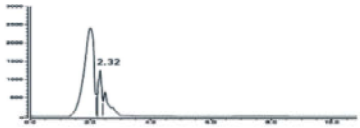
Each concentration was added to MS medium before the pH of the medium was adjusted to 5.7 with 0.1 N NaOH or HCl. Media were autoclaved at 121°C for 20 minutes. The cultures were incubated at 24± 2°C under light provided by white fluorescent tubes for 16 hrs/day.

Application of Some Chemical Inducers: To promote stevioside compounds production in stevia callus, equal amount of callus (150 mg) was cultured on MS basal medium (the best medium according to growth parameters of stevia callus (Fig. 2) with 2 ppm 2,4-D + 0.5 ppm NAA + 0.5 ppm BA with the following chemical inducers:

- Control (without inducers)
- Glucose (10, 30, 50 and 70 g/l)
- Gibberellic acid (1, 2, 4 and 8 ppm)
- Proline acid (10, 50, 100 and 200 ppm)
- Glutamic acid (10, 50, 100 and 200 ppm)
- 2-acetoxybenzoic acid (10, 50, 100 and 200 ppm)

The samples of fresh stevia callus were collected after 8 weeks from culture to determine growth parameters and some biochemical constituents. Also, fresh samples were lyophilized and stored at -20°C until analysis.

Table 1: Effect of glucose on stevioside content in *Stevia rebaudiana* L. callus

Solvent, authentic sample of stevioside and mother plant					
Types		**R T (min)**	**Peaks of stevioside**	**g/100 g (FW)**	
Solvent		00		**Solvent**	
Authentic sample of stevioside		2.32		**Authentic sample of stevioside**	
Mother plant		2.34		3.54	
Effect of glucose on stevioside content					
Treatments		**R T (min)**	**Peaks of stevioside**	**g/100 g (FW)**	**INC %**
Glucose	**Dose (g/l)**				
Control (without glucose)		2.26		3.36	00
Glucose (g/l)	**10**	2.33		2.76	Red
	30	2.33		3.91	16.36
	50	2.33		6.38	90.12
	70	2.32		4.47	33.03

RT=Retention Time, **FW** = Fresh weight , **INC%** = Increase of stevioside content and **Red**= Reduction of stevioside content compared with the control

Growth Measurements: Growth of callus was measured in terms of percentage of callus induction (%), fresh and dry weight (mg). Fresh weights of callus were taken after removing the excess of moisture on the surface using blotting paper. Dry weight of callus was determined by drying in a hot air oven at 40°C for 48 hrs.

Chemical Analysis

Determination of Lipid Peroxidation (Malondialdehyde Content): The level of lipid peroxidation in stevia callus was quantified by determination of malondialdehyde content (MDA), breakdown product of lipid peroxidation according to Health and Packer [18] and modified by Zaho *et al.* [19]. One gram of callus was homogenized in 1 ml of 0.1% (w/v) trichloroacetic acid with a prechilled mortar and pestle. The homogenate was spun at 10,000 G for 5 minutes. Two ml of TBA (Thiobarbituric acid) reagent was added to 0.5 ml aliquot of the supernatant. The mixture was heated at 95ºC for 15 minutes and cooled immediately. The absorbance was read at 532 nm and the value was corrected for non specific absorption at 600 nm in spectrophotometer (Spectronic Genesys 5). The concentration of MDA-TBA complex in callus was converted from ppm (calculated from MDA standard curve) to ç mol/g fresh weight.

Determination of Sweet Component (Stevioside): Stevia callus were extracted by mortaring in methanol according to the method of Brandle [20] and Nikolai *et al*. [21]. The stevioside obtained by methanol extract analyzed by High Performance Liquid Chromatography (HPLC) as described by Nishiyama *et al.* [22]. The HPLC system was a Dionex Ultimate 3000 equipped with an auto-sampler, quaternary pump and a diode array detector. The analytical column was BDS Hypersil C8 column. Separation was performed with acetonitrile and water (85: 15 v/v) as the elusion solvent at flow rate of 0.7 ml/min and the detection wavelength was 205/210 nm. Under these analytical conditions, the typical retention time of stevioside was 2.32 min (Table 1). It is worth mentioning that the standard addition was done by using stevioside pure material to confirm the results, before the analysis of stevioside in samples of stevia callus. Standard addition is a technique that helps qualify dubious test results. The reason for using the standard addition of stevioside is that the samples of stevia contain other components that interfere with the stevioside causing inaccuracy in the determined concentration. In addition, the separation of stevioside carried out by using column C8, which differed from column C18 in their separation. The idea is to add known volume of stevioside (known concentration) to the sample and the change in peak area was noticed. The change in peak area between the sample and the sample with standard is assumed.

Determination of Free Amino Acids: From each samples (fresh stevia callus), 2 gm were extracted with 70% ethyl alcohol. The ethanolic solution was filtered, concentrated and passed through a column of purified cation exchange resin (Dowex 50). Elution was carried out with 70% ethyl alcohol to take all carbohydrates, pigments and lipids present, then with ammonia solution for elution of free amino acids. The same steps were repeated again using HCl instead of ammonia solution to complete elution of free amino acids [23-25]. Each eluent was concentrated to a small volume by evaporation under vacuum at 45°C and kept deepfreezed until being determined by amino acid analyzer (Sykam).

Statistical Analysis: The experiments were subjected to completely randomized design. Analysis of variance (**ANOVA**) and Duncan's multiple range test [26], as modified by Snedecor and Cochran [27], were performed to analyze the obtained data.

RESULTS AND DISCUSSION

Induction of Callus Cultures: Callus cultures were initiated from leaves of *Stevia rebaudiana* L. Growth regulators; 2, 4-D, NAA and BA are frequently used to induce callus tissues in many plant species [28]. Therefore, MS medium supplemented with different concentrations of 2, 4-D and NAA with BA were tested to obtain the best callus induction and fresh weight (Fig. 1). Visible callus formation was obtained within two weeks and observations were taken after six weeks of the culture. Compact greenish yellow callus was induced from wound sites in the leaf explants. The highest callus induction percentages and callus fresh weights are presented in Fig. 2. Also, the percentage of callus induction and callus fresh weight varied depending on PGRs and their concentrations. Comparing the effect of different tested concentrations of PGRs on callus growth, it could be noticed that callus induction percentage and fresh weights were gradually increased with increasing the concentration of 2,4-D and NAA from 0.5 to 2 ppm.

Moreover, the maximum callus induction and fresh weight of callus were obtained with PGRs combination of 2 ppm 2,4-D with 0.5 ppm NAA and 0.5 ppm BA, which gave 100% callus induction and 0.33 g callus from leaves,

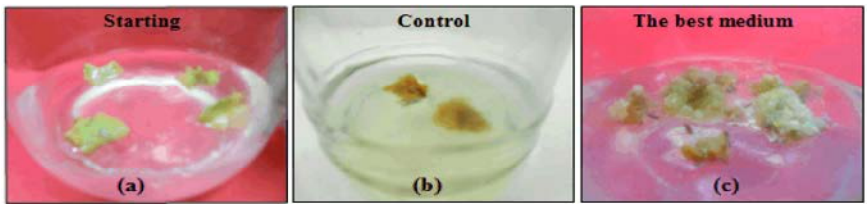


Fig. 1: Callus growth from leaves of *Stevia rebaudiana* L. (a) after 2 weeks and on (b) control medium and (c) MS medium containing 2 ppm 2,4-D + 0.5 ppm NAA + 0.5 ppm BA (best medium).

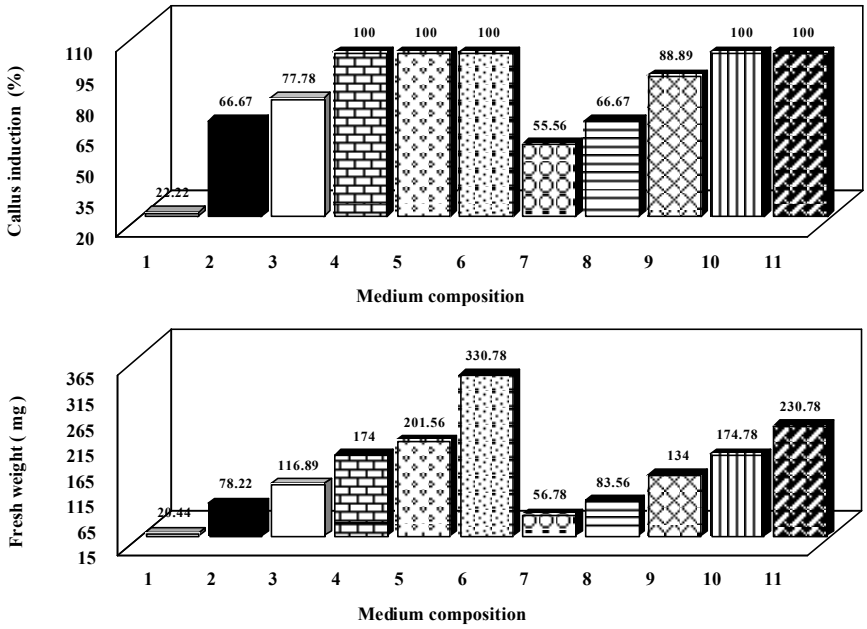


Fig. 2: Effect of 2,4-D, NAA and BA on *Stevia rebaudiana* L. callus induction percentage and fresh weight.(1-11= medium composition as mentioned in the materials and methods).

followed by 2 ppm NAA with 0.5 ppm 2, 4-D and 0.5 ppm BA, which gave 100% callus induction and 0.23 g callus. This study is in broad agreement with Gopi and Vatsala [29], who reported that the maximum callus growth was found with auxins such as 2,4-D and NAA and also with BA among the cytokinins. Also, Agarwal and Kamal [30] reported that the presence of 2,4-D has been shown to be essential for callus formation in *Momordica charantia*. In addition, Hou and Jia [31] reported that 2 ppm 2,4-D and 1 ppm kin could induce high frequency of calli from *A. melilotodies* hypocotyl and stem explants. The lowest callus induction (22%) and fresh weight (0.02 g) were observed in explants cultures in the absence of PGRs. Data in the same figure suggest that 2 ppm 2,4-D with 0.5 ppm BA and 2 ppm NAA with 0.5 ppm BA included callus fresh weight, but was lower that callus fresh weight in explants cultured in 2,4-D with NAA or NAA with 2,4-D without BA. Decreasing the concentration of 2,4-D or NAA to 0.5 ppm remarkably lowered callus induction and callus fresh weight. Likewise, among the two evaluated auxins, it was found that 2,4-D is most effective than NAA for biomass production from stevia leaf explants.

Effect of Inducers on Callus Growth: The results indicated that, moisture content, fresh and dry weights of callus were depending on the concentration of inducers. For this study MS medium supplemented with 2 ppm 2,4-D + 0.5 ppm NAA + 0.5 ppm BA was selected as the standard medium from the previous experiment based on callus induction (%) and fresh weight. Calli were cultured on different concentrations of glucose, gibberellic acid, proline acid, glutamic acid and 2-acetoxybenzoic acid as inducers (Figs 3 and 4). Data showed that application of gibberellic acid (except 2 ppm) had a positive effect on fresh weight compared with the control (without gibberellic acid). Also, the same growth parameter was increased when callus was treated with proline at rates 50 and 200 ppm. In the same direction, fresh weight was increased

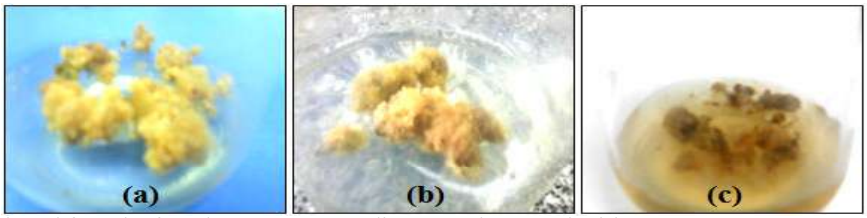


Fig. 3: Callus developed from leaf explants on MS medium supplemented with 2 ppm 2,4-D + 0.5 ppm NAA + 0.5 ppm BA with (a) 8 ppm gibberellic acid (b) 200 ppm proline acid (c) 100 ppm 2-acetoxybenzoic acid.

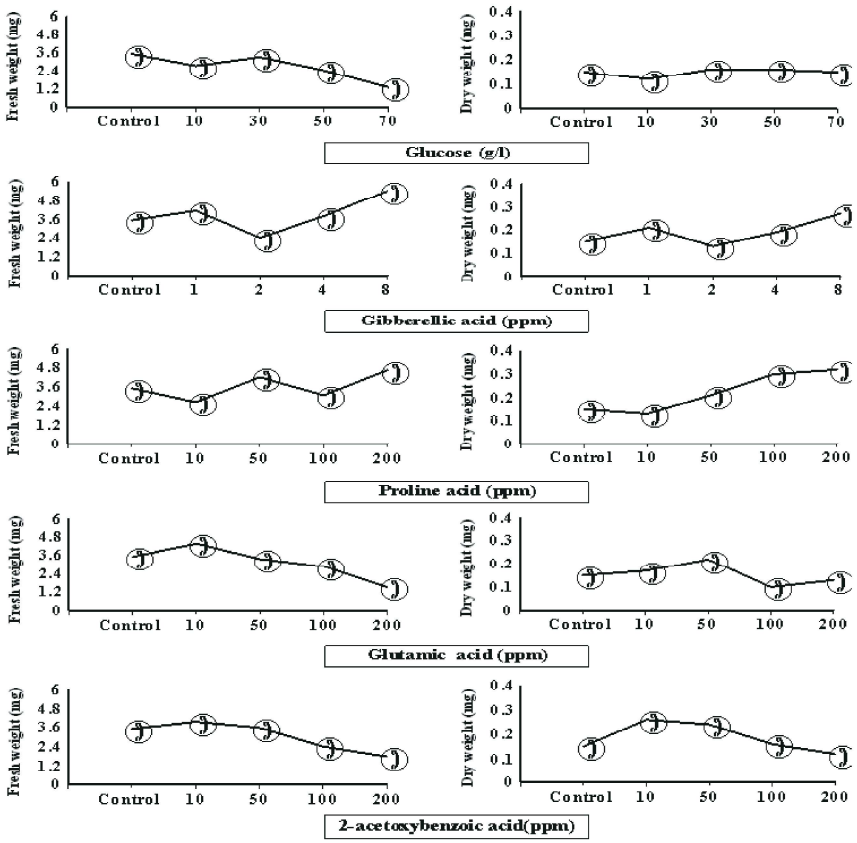


Fig. 4: Effect of glucose, gibberellic acid, proline acid, glutamic acid and 2-acetoxybenzoic acid on fresh and dry weights in *Stevia rebaudiana* L. callus.

after application of glutamic acid and 2-acetoxybenzoic acid at rates 10 and 50 ppm compared with the control.

Concerning dry weight, it was increased after treatment with glucose at rates of 30, 50 and 70 g/l. Also, callus applied with gibberellic acid (except 2 ppm) and proline (except 10 ppm) showed promotive effects on dry weights compared with the control. On the other hand, the results showed that the high levels of glutamic acid (100 and 200 ppm) affected negatively on dry weights of stevia callus. Also, the highest level (200 ppm) of 2-acetoxybenzoic acid showed the same trend compared with the control. Generally, application of gibberellic acid (8 ppm) and proline acid (200 ppm) gave the best results of fresh weights, which recorded the highest values. In this concern, the maximum value of dry weight was recorded when stevia callus was treated with proline at rate of 200 ppm. There are many researches that explain the positive role of proline on growth such as Kishore and Dange [32], who showed that the increase in dry weight of callus tissue of cotton was due to the more accumulation of proline. It is one of the possible means of overcoming osmotic stress [33]. It acts as a compatible solute that adjusts the osmotic potential in the cytoplasm and plays an important role in defense mechanisms of stressed cells [34]. Also, Mohamed *et al*. [35] found that

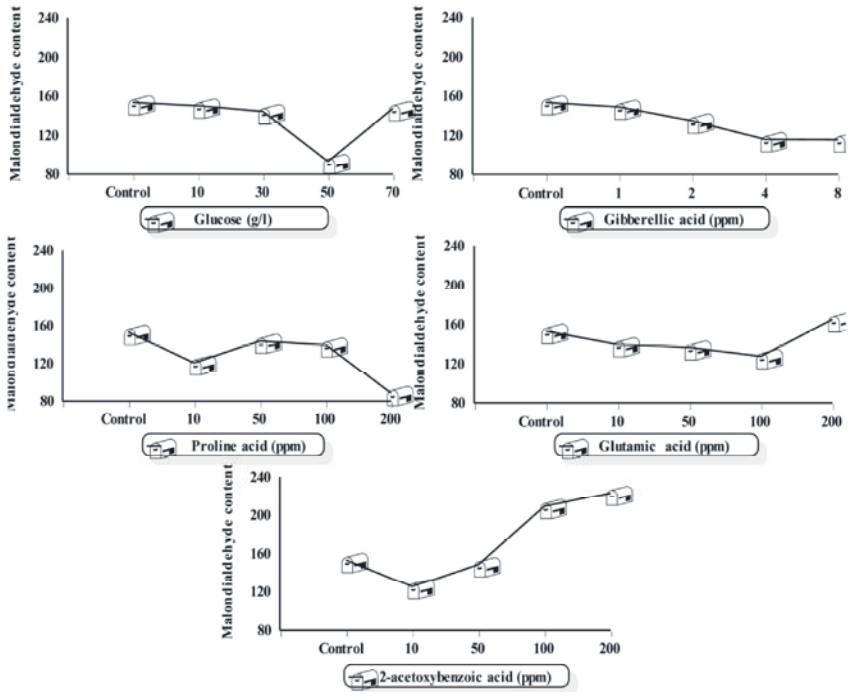


Fig. 5: Effect of some inducers on malondialdehyde content (η mole/g fresh weight) in *Stevia rebaudiana* L. callus.

fresh and dry weights of strawberry increased in callus grown on media supplemented with proline. In this regard, gibberellic acid promotes growth via an increase in the turgor pressure through the hydrolysis of sucrose [36]. Also, Yasuhiro *et al*. [37] showed that production of cell cultures of *Coptis japonica* was enhanced by the addition of gibberellic acid to the medium. Gibberellic acid also knows as an effective elicitor for production of secondary metabolites [38].

Effect of Inducers on Biochemical Constituents

Lipid Peroxidation Product (Malondialdehyde Content): The level of lipid peroxidation in stevia callus was quantified by determination of malondialdehyde (MDA) content (Fig. 5) and it was used as a biomarker to measure oxidative stress in callus. The data clearly demonstrated that the application of glucose at all levels decreased MDA content compared with the control (without glucose). The minimum value was recorded when glucose applied at rate of 50 g/l. Data in the same figure showed that gibberellic acid alleviated the MDA toxic product in callus. It was decreased with the increase of gibberellic acid concentration compared with the control (without gibberellic acid). Also, treatment with proline had a negative role on the accumulation of MDA. In this regard, the lowest value of MDA (88.35 ηmole/g fresh weight) was obtained when stevia callus was treated with proline at 200 ppm. Concerning the treatment with glutamic acid, it was tended to accumulate MDA content in stevia callus, especially at the highest concentration (200 ppm). Also, MDA content responded positively after treated with 2-acetoxybenzoic acid at the high concentrations. In this concern, the maximum values of MDA content were obtained after the treatment with 2-acetoxybenzoic acid at 100 and 200 ppm compared with the control. The positive effect of some inducers (glucose, gibberellic and proline) to reduce the content of MDA in stevia callus probably is due to their ability to activate antioxidant enzymes and thus reduce reactive oxygen species and also reduce the decomposition of unsaturated fatty acids (reactive oxygen species degrade polyunsaturated lipids, forming MDA). In addition, this compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells [39]. Also, the production of MDA is used as a biomarker to measure the level of oxidative stress in an organism [40, 41]. In this regard, Ahmad *et al.* [42] studied the free radical scavenging activity of regenerated tissue of stevia to find new potential sources of natural antioxidants.

The Enhancement of Stevioside Content in *Stevia rebaudiana* L. Callus: The present study describes the enhancement of stevioside content in stevia callus.

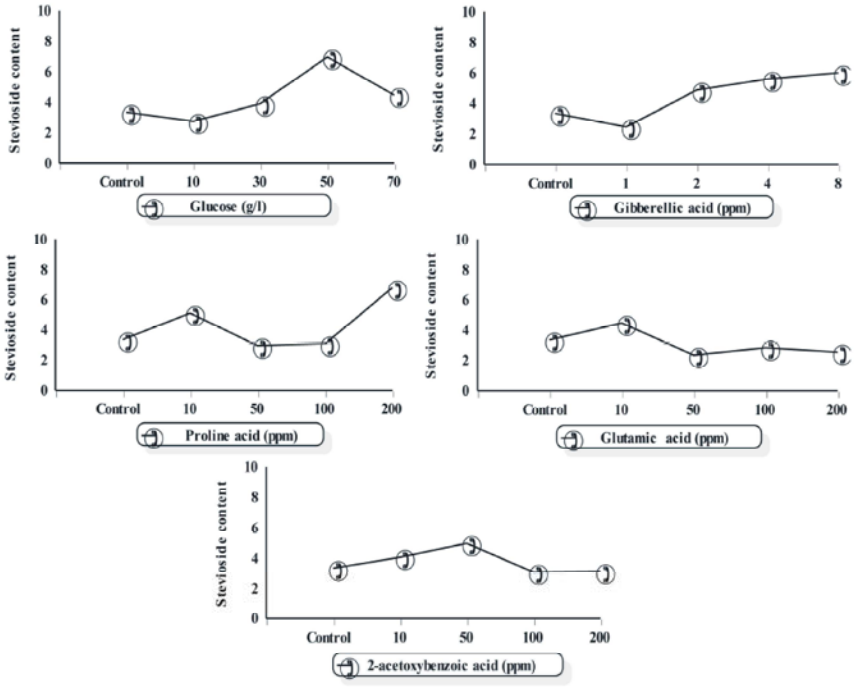
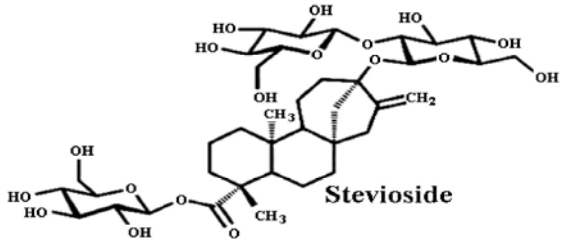


Fig. 6: Effect of some inducers on stevioside content (g / 100g fresh weight) in *Stevia rebaudiana* L. callus.

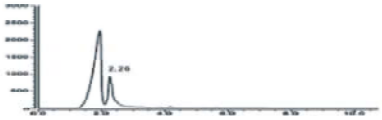
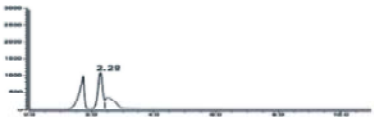
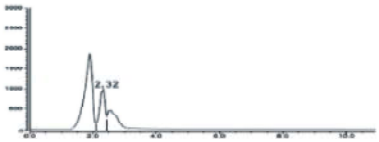
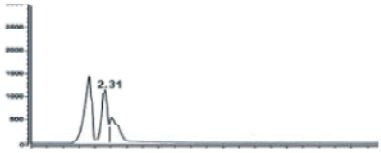
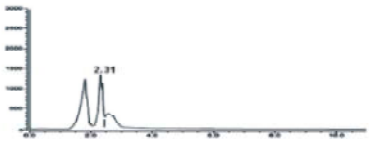
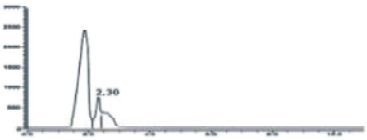
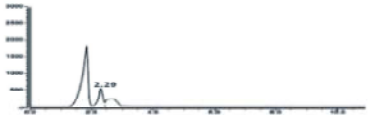
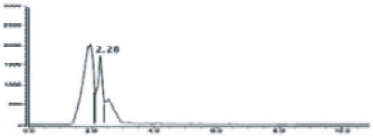
The different chemical materials like glucose, gibberellic acid proline acid, glutamic acid and 2-acetoxybenzoic acid were used in media culture.



Effect of Glucose on Stevioside Content in *Stevia rebaudiana* L. Callus: It is evident from the results in Table 1 and Fig. 6 that application of glucose had a positive effect on stevioside content (except 10 g/l) compared with the control (without glucose). The highest value of stevioside was obtained when glucose applied at rate of 50 g/l which reached 6.38 g/100g (1.89 fold increase than control). While, the lowest value was recorded after the treatment with 10 g/l. The percentages of increment of stevioside content were reached 16.36, 90.12 and 33.03% after treatment with glucose at rates of 30, 50 and 70 g/l, respectively. In this regard, the use of glucose in culture medium was noticed by many authors [43-47]. There are also researches that study the production of stevioside in stevia callus [48, 49]. The important role of glucose molecule in the plant cell is probably due to its source of energy in the plant cell, which burns in the cytoplasm and mitochondria to release carbon dioxide, water and energy. This energy is trapped in the ATP molecule and used for everything in the plant cell. Also, glucose is very hydrophilic (water loving) and attracts water. So, cells containing large numbers of glucose molecules would have a high osmosis and would be constantly fighting the incessant movement of water from the outside of the cell to the inside. In addition, glucose promoted secondary embryogenesis in *Prunus incisa* [50].

Effect of Gibberellic Acid on Stevioside Content in *Stevia rebaudiana* L. Callus: It is apparent from data in Table 2 and Fig. 6 that application of gibberellic acid had a promotive role in enhancing stevioside content in callus. In this concern, treatment with gibberellic acid at rate of 8 ppm gave the highest value of stevioside content compared with the control. On the contrary, the minimum value was produced by adding gibberellic acid at rate of 1 ppm. The increments reached 45.53, 61.01 and 73.21% when gibberellic acid was applied at rate of 2, 4 and 8, respectively. In this regard, Chen and Li [51] showed that the content of stevioside was the highest in callus cultured on medium supplemented with gibberellic acid at rate of 1ppm. Also, they founded that stevioside content in differentiated callus was higher than that in undifferentiated callus. The content of stevioside in

Table 2: Effect of gibberellic and proline acids on stevioside content in *Stevia rebaudiana* L. callus

Treatments		R T (min)	Peaks of stevioside	g/100 g (FW)	INC%
Chemical inducers	Dose (ppm)				
Control (without inducers)		2.26		3.36	00
Gibberellic acid (ppm)	1	2.30		2.47	Red
	2	2.28		4.89	45.53
	4	2.32		5.41	61.01
	8	2.31		5.82	73.21
Proline acid (ppm)	10	2.31		5.12	52.38
	50	2.30		2.95	Red
	100	2.29		3.08	Red
	200	2.28		6.19	84.46

RT=Retention Time, **FW** = Fresh weight , **INC% =** Increase of stevioside content and **Red=** Reduction of stevioside content compared with the control

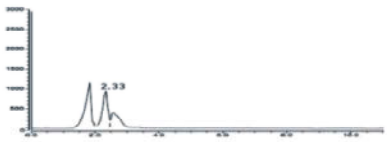
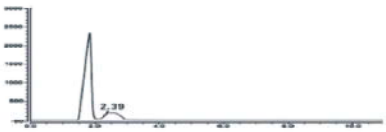
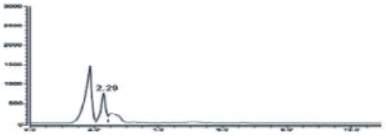
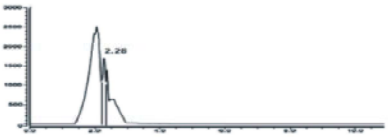
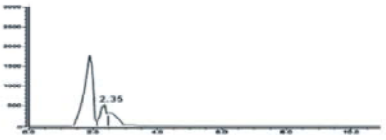
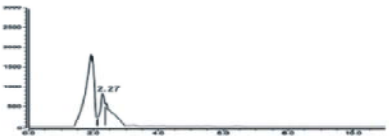
leaves of plants derived from the differentiation of callus was twice that of plants cultured in the field. In the same direction, Modi *et al*. [52] showed that stevioside content was increased in stevia leaves after gibberellic acid was applied on plants. The acidic nature of gibberellic acid could be an important factor in making the culture medium more acidic. In addition to its role in activating the expression of genes [53], gibberellic acid may be involved in lowering the pH of the cell wall. This drop in the pH of cell wall may result in the activation of certain cell wall hydrolysis. The hydrolysis of the bonds in certain cell wall components is an important factor for plant cell. In this regard, Arbabian *et al*. [54] showed that certain concentrations of gibberellic acid resulted in a higher rate of differentiation of vascular tissue in young sunflower leaves under both *in vitro* and *in vivo* conditions. In addition, gibberellic acid, at certain concentrations, has been shown to be beneficial for the physiology and metabolism of many plants [55, 56], which may provide a mechanism to regulate physiology, biochemistry, growth and development as a function of water availability [57].

Effect of Proline Acid on Stevioside Content in *Stevia rebaudiana* L. Callus: Data presented in Table 2 and Fig. 6 indicated that application of proline enhanced the increment of stevioside in stevia callus. The maximum value was recorded by applying proline at rate of 200 ppm; the percentage of increment was reached 84.46% compared with the control (1.84 fold increase than control). On the other hand, stevioside content was decreased when proline applied at rates of 50 and 100 ppm. Proline is responsible for scavenging the reactive oxygen species (ROS) and other free radicals. Excessive levels of ROS result in oxidative damage to plants, e.g., nucleic acid damage, oxidation of proteins and lipids [58]. In this regard, the activities of antioxidative enzymes (catalase, peroxidase and superoxide dismutase) were significantly enhanced when proline was applied exogenously in tobacco suspension cultures [59]. They showed that the activities of APX (ascorbate peroxidase), MDHAR (monohydro ascorbate reductase) and DHAR (dihydro ascorbate reductase) enzymes, which are the components of ascorbate-glutathione (ASC-GSH) cycle, were significantly enhanced by exogenous proline application. In another study, Hong *et al.* [60] concluded that the role of proline as a free radical scavenger is more important in alleviating stress than its role as a simple osmolyte. Also, proline had little effect on ammonium concentration even though it enhanced callus weight enormously [61]. In addition, Rao *et al*. [62] showed that proline enhanced the frequency of embryogenesis.

Effect of Glutamic Acid on Stevioside Content in *Stevia rebaudiana* L. Callus: The results showed that glutamic acid had a little effect on the active constituent (stevioside) in stevia callus and this was evident at the low concentration (10 ppm) as shown in Table 3 and Fig. 6. Nevertheless, the high concentrations of glutamic acid negatively affected the production of active constituent in stevia callus. In this regard, it was decreased after the treatment with 50, 100 and 200 ppm compared with the control (without glutamic acid). There are many interpretations which describe the role of glutamic acid in promoting callus: i) Glutamine did not affect growth but in most cases improved slightly callus induction of olive [63]. ii) Glutamic acid induced somatic embryos of *Cucumis melo* L. [64]**.** iii) Glutamic acid significantly reduced the ammonium concentration in maize callus [61]. iv) Addition of free amino acids (glutamic acid) to the culture media improved callus formation and development of maize [65], increased regenerative ability of rice calli [66], strongly stimulated somatic embryo formation of carrot [67] and increased the frequency of callus formation, plant regeneration and number of rice calluses [68].

Effect of 2-Acetoxybenzoic Acid on Stevioside Content in *Stevia rebaudiana* L. Callus: From data presented in Table 3 and Fig. 6, treatment with 2-acetoxybenzoic acid at rates of 10 and 50 ppm led to an increase in stevioside content, which reached 21.42 and 48.51% respectively, compared with the control (without 2-acetoxybenzoic acid). On the other hand, the highest concentrations of 2-acetoxybenzoic acid (100 and 200 ppm) clearly decreased stevioside content. In addition, 2-acetoxybenzoic acid known as acetylsalicylic acid (ASA) and it is a commercially available form of salicylic acid. It is known that in aqueous solutions, ASA is hydrolyzed almost entirely to salicylic acid, which is an active ingredient. Salicylic acid is an endogenous growth regulator with phenolic nature, which participates in regulation of several physiological processes [69, 70]. The positive effect of salicylic acid may be attributed to: i) Salicylic acid may switch on pathways that result in preventing of oxidative damage or repair that damage [71]. ii) Salicylic acid molecule acts as a potential non enzymatic antioxidant as well as plant growth regulator, which plays number of plant physiological processes [72, 73]. iii) Regulating some chemical contents such as total soluble proteins, total phenols, proline, total soluble carbohydrates and sugars [74].

Table 3: Effect of glutamic and 2-acetoxybenzoic acids on stevioside content in *Stevia rebaudiana* L. callus

Treatments		R T (min)	Peaks of stevioside	g/100 g (FW)	INC%
Chemical inducers	Dose (ppm)				
Control (without inducers)		2.26		3.36	00
Glutamic acid (ppm)	10	2.33		4.48	33.33
	50	2.39		2.35	Red
	100	2.28		2.84	Red
	200	2.30		2.55	Red
2-acetoxybenzoic acid (ppm)	10	2.29		4.08	21.42
	50	2.28		4.99	48.51
	100	2.35		3.10	Red
	200	2.27		3.14	Red

RT=Retention Time, **FW** = Fresh weight , **INC%** = Increase of stevioside content and **Red=** Reduction of stevioside content compared with the control

Table 4: Effect of glucose, gibberellic acid and proline acid on free amino acids in *Stevia rebaudiana* L. callus.

	Treatments												
	%												
		Glucose (g/l)				Gibberellic acid (ppm)				Proline acid (ppm)			
Free amino acids	Control	10	30	50	70	1	2	4	8	10	50	100	200
Aspartic	2.72	3.17	3.21	4.04	2.56	2.54	2.03	2.59	2.17	2.31	3.25	2.69	1.40
Serine	23.61	30.27	18.99	5.25	31.97	22.40	14.71	47.18	41.40	30.09	30.66	28.68	9.36
Glutamic	0.42	4.09	0.55	1.82	0.22	2.20	0.57	2.12	1.956	0.11	3.04	0.11	0.40
Proline	1.90	0.79	3.56	4.93	6.87	1.60	4.51	0.57	1.61	3.32	1.49	4.70	12.59
UFAA1	0.15	0.13	n.d	0.31	0.13	0.50	0.55	n.d	n.d	0.11	n.d	0.27	n.d
Glycine	0.64	1.91	1.01	1.42	0.51	1.58	0.56	0.79	1.39	0.43	1.01	0.59	0.85
Alanine	2.34	5.04	5.44	12.34	4.83	1.79	5.05	2.85	2.84	3.36	4.63	3.94	4.86
Cysteine	n.d	n.d	0.08	n.d	n.d	0.27	0.37	n.d	0.09	0.23	n.d	0.20	0.13
Valine	1.72	1.92	2.12	3.24	2.03	1.92	2.04	1.72	2.20	2.09	2.38	2.53	2.65
Methionine	0.06	0.03	0.05	n.d	0.05	0.05	n.d	n.d	n.d	0.08	0.07	0.16	0.06
Isoleucine	0.24	0.54	1.02	1.41	0.45	0.61	0.64	0.49	0.01	0.34	0.52	0.51	0.62
Leucine	1.11	0.77	0.81	0.88	1.02	0.91	1.69	0.52	0.59	1.11	1.160	1.25	1.38
Tyrosine	1.45	0.32	3.21	2.46	5.30	0.13	14.10	0.43	0.67	7.47	2.45	2.26	0.86
Phenylalanine	0.09	1.04	0.44	0.25	0.08	0.81	0.66	0.53	1.01	0.08	0.38	0.15	0.27
UFAA2	n.d	0.14	0.11	0.19	n.d	0.41	n.d	n.d	n.d	n.d	n.d	0.09	n.d
UFAA3	15.22	8.27	15.01	14.89	13.97	17.23	32.58	9.37	11.83	31.65	28.24	23.45	12.36
Histidine	4.24	5.87	5.21	4.39	6.32	4.13	5.58	2.80	3.19	4.95	4.15	5.70	12.38
UFAA4	0.11	0.21	0.05	n.d	0.27	0.16	0.19	0.10	0.05	0.37	n.d	0.52	0.28
UFAA5	0.59	2.64	0.41	0.56	0.48	0.49	0.23	0.54	0.90	0.81	0.35	0.66	0.34
Lysine	0.84	1.12	1.01	1.51	1.27	0.76	0.53	0.56	0.48	0.68	0.50	1.07	0.46
AM	38.97	26.42	33.72	34.32	19.36	35.54	10.90	22.95	21.72	7.92	15.23	17.06	34.68
UFAA6	1.43	0.85	0.87	0.63	0.92	1.23	1.02	1.45	1.77	1.17	n.d	1.57	1.98
Arginine	2.15	4.47	3.12	5.15	1.39	2.72	1.50	2.45	4.11	1.32	0.49	1.83	2.10

- UFAA = Unknown free amino acids from 1 to 6, AM = Ammonia (not amino acid), n.d = not detectable.
- Amino acids found in the table were arranged according to the retention time of amino acids which separated from column of amino acid analyzer apparatus.% = Area% of free amino acids.

Free Amino acids in *Stevia rebaudiana* L. Callus: Data listed in Tables 4 and 5 shows the effect of some inducers on the pattern of free amino acids in stevia callus. Amino acids found in the tables were arranged according to the retention time of amino acids, which were separated from column of amino acid analyzer apparatus. Twenty two free amino acids were detected in stevia callus and the most abundant amino acids noticed in all samples were serine, UFAA3, histidine, proline, alanine and valine (Fig. 7). In this regard, stevia callus treated with glucose at rates of 10 and 70 g/l showed an increase of serine compared with the control. In the same direction, serine was increased in callus after treatment with gibberellic and proline at rates (4 and 8 ppm) and (10, 50 and 100 ppm), respectively. Serine has an important role in the plant cell, where it enters in the biosynthesis of some important compounds such as glycinebetaine. In this regard, glycinebetaine derive from the oxidation of choline that in turn, derives from serine through ethanolamine [75, 76]. In this concern, Modi *et al*. [52] showed that free amino acids were increased in stevia after application of gibberellic acid on plants. Such content in callus was increased when applied glutamic acid at rate 10 ppm and 2-acetoxybenzoic acid at rates 10 and 50 ppm compared with the control. Regarding histidine content, it was increased by adding glucose, glutamic acid and 2-acetoxybenzoic acid at all doses compared with the control. In this connection, data indicated accumulation of such content in stevia callus when gibberellic acid applied at rate 2 ppm. Also, the same trend was true after treatment with proline at rates 10, 100 and 200 ppm.

Data cleared that application of glucose, gibberellic acid, proline acid and glutamic acid resulted in an increment in proline content when adding to medium at rates (30, 50 and 70 g/l), (2 ppm), (10, 100 and 200 ppm) and (50, 100 and 200 ppm), respectively compared with the control. Also, application of 2-acetoxybenzoic acid appeared to be effective on proline accumulation in stevia callus at all rates. In this regard, Koc [77] indicated that

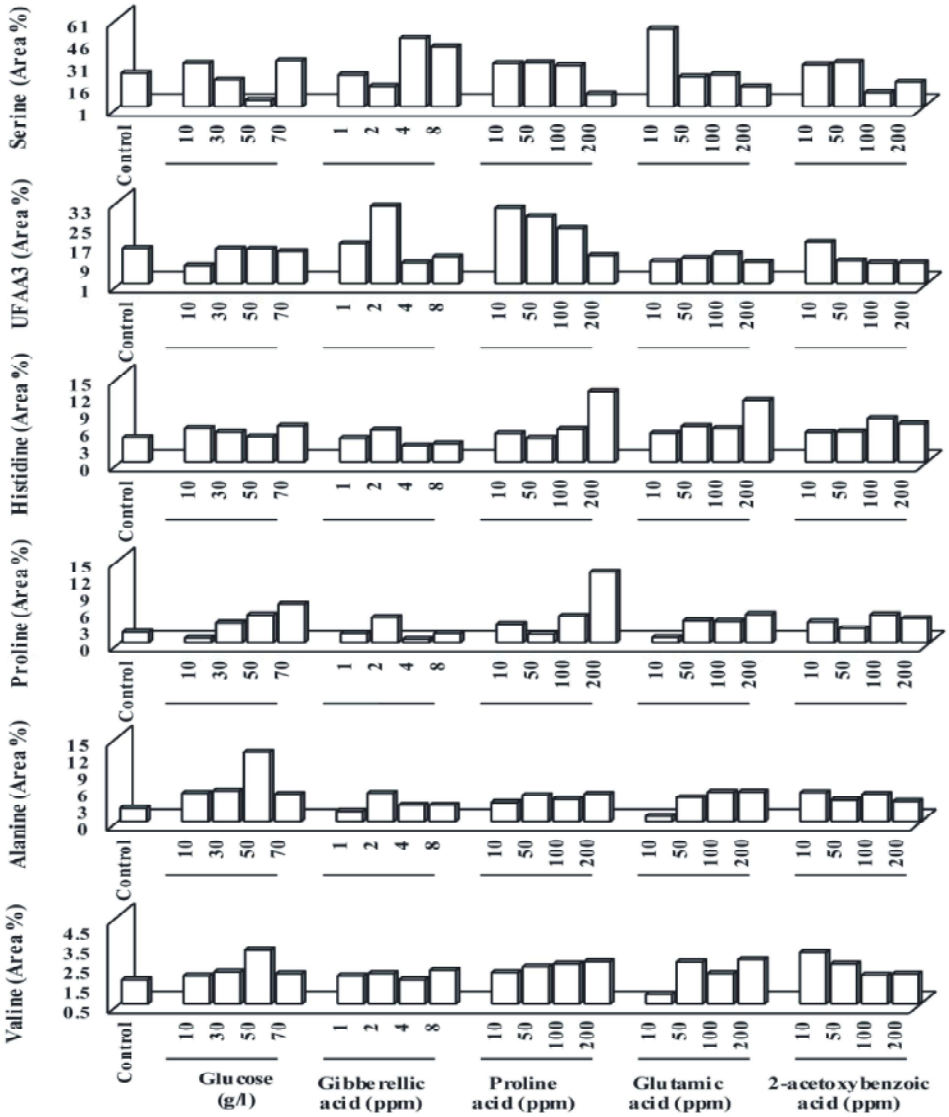


Fig. 7: Effect of some inducers on serine, UFAA3, histidine, proline, alanine and valine acids accumulation in *Stevia rebaudiana* L. callus

proline and salicylic treatments led to the accumulation of proline in pepper callus. Moreover, Hayat *et al*. [78] found that the important role of proline in plants attributed to: 1) It protects the plants from various stresses and also helps plants to recover from stress more rapidly. 2) When applied exogenously to plants exposed to stress, proline results in enhanced growth and other physiological characteristics of plants. 3) Exogenous proline scavenges the reactive oxygen species (ROS) generated in plants under various biotic and abiotic stresses. 4) Exogenous proline application affects plant-water relations by maintaining turgidity of cells under stress. In a study by Hare *et al*. [79] it was shown that proline applied exogenously at a low concentration enhanced *in vitro* shoot organogenesis in *Arabidopsis*, whereas growth was inhibited at higher concentrations. In another study, addition of exogenous proline to the culture medium increased the dry weight and free proline content of *Medicago sativa* callus [80]. On the other hand, methionine, cysteine, UFAA1, UFAA2 and UFAA4 are presented in minute quantities in stevia samples. Other identified free amino acids in stevia callus have concentrations in between those extremes and decreased or increased depending on the concerned amino acid and different chemical materials used (type and dose).

Table 5: Effect of glutamic and 2-acetoxybenzoic acids on free amino acids in *Stevia rebaudiana* L. callus

	Treatments								
	%								
		Glutamic acid (ppm)				2-acetoxybenzoic acid (ppm)			
Free amino acids	Control	10	50	100	200	10	50	100	200
Aspartic	2.72	1.99	1.71	1.69	3.50	2.84	2.06	2.01	1.51
Serine	23.61	53.50	21.24	22.53	14.50	29.28	30.91	10.38	17.63
Glutamic	0.42	1.80	1.45	1.53	2.27	1.06	1.08	1.16	0.96
Proline	1.90	0.89	3.92	3.81	5.02	3.71	2.53	4.97	4.31
UFAA1	0.15	0.02	0.30	0.21	0.42	0.58	0.05	0.14	0.08
Glycine	0.64	0.73	0.81	0.67	0.90	0.86	0.94	0.90	0.90
Alanine	2.34	0.92	4.22	5.22	5.24	5.14	3.79	4.85	3.52
Cysteine	n.d	0.07	n.d	n.d	0.21	0.22	0.16	n.d	0.15
Valine	1.72	0.99	2.63	2.04	2.75	3.11	2.53	1.95	2.02
Methionine	0.06	0.07	0.11	0.04	0.38	0.17	0.10	0.21	0.06
Isoleucine	0.24	0.21	0.44	0.40	0.92	0.86	0.61	0.64	0.50
Leucine	1.11	0.43	1.24	1.01	1.48	1.59	1.35	1.25	1.11
Tyrosine	1.45	0.04	0.98	1.41	1.07	1.71	0.34	0.35	0.22
Phenylalanine	0.09	0.13	0.21	0.09	0.26	0.26	0.44	0.51	0.37
UFAA2	n.d	n.d	n.d	0.09	n.d	n.d	0.10	0.11	0.11
UFAA3	15.22	9.99	11.21	12.95	9.58	17.83	10.25	9.33	9.35
Histidine	4.24	5.00	6.24	5.83	10.83	5.16	5.26	7.57	6.64
UFAA4	0.11	0.60	0.25	0.27	0.28	0.34	0.28	0.39	0.32
UFAA5	0.59	0.92	0.27	0.36	0.32	0.58	0.56	0.49	0.52
Lysine	0.84	0.53	0.91	0.80	1.05	1.05	1.14	1.27	1.41
AM	38.97	16.96	38.2	36.15	36.00	20.85	32.92	47.81	45.69
UFAA6	1.43	2.32	1.78	1.91	1.02	1.48	1.62	2.19	1.64
Arginine	2.15	1.89	1.88	1.01	2.00	1.31	0.99	1.54	0.95

- UFAA = Unknown free amino acids from 1 to 6, AM = Ammonia (not amino acid), n.d = not detectable.
- Amino acids found in the table were arranged according to the retention time of amino acids which separated from column of amino acid analyzer apparatus. % = Area % of free amino acids.

CONCLUSION

The present study concludes that treatments with glucose, gibberellic acid and proline acid appeared to be effective on callus growth and this was associated with clear decrease in malondialdehyde content, which used as a biomarker to measure oxidative stress in stevia callus. Also, these treatments had a promotive role in enhancing stevioside content in stevia callus, which was associated with the accumulation of some free amino acids. Glutamic acid and 2-acetoxybenzoic acid had a little effect on stevioside content and this was evident at the low concentration, but in the high concentration had a negative effect on the production of active constituent (stevioside) in stevia callus.

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