IHJPAS. 36 (3) 2023

Ibn Al-Haitham Journal for Pure and Applied Sciences
Journal homepage: jih.uobaghdad.edu.iq

In Vitro Effect of Mannitol Stress and γ-ray on Secondary Products From Golden Sunrise Cherry Tomato Callus

Shaimaa N. Mizil*
Department of Biology Science College
AL-Mustansiriyah University, Baghdad, Iraq.

Ekhlas. A.J. ElKaaby
Department of Genetic Engineering Ministry of Sciences and Technology, Food and Biotechnology Center.

Maher Z. F. Al-Shammary
Department of Biology, College of Education for Pure Science Ibn Al-Haitham, University of Baghdad, Baghdad, Iraq.

***Corresponding Author:** Shaymaa.m1979@uomustansiriyah.edu.iq

Article history: Received 28 November 2022, Accepted 2 January 2023, Published in July 2023.

doi.org/10.30526/36.3.3117

Abstract

Effect of drought stress induced by mannitol on secondary products in callus of Golden Sunrise cherry tomatoes were studied in vitro. Seeds were irradiated with gamma at the doses (0, 20, or 40 Gy), and germinated on Murashige and Skoog, (MS) basal medium. Callus were initiated from cotyledon leaf explants using a combination of (2.0 kin + 2.0 IAA mg. l^{-1}) then transferred into MS medium supplemented with mannitol at concentrations of (0, 40, and 60 g.l^{-1}). Vitamin C(vit C), enzyme activity, Ascorbate peroxidase (Apx), and Amylase enzymes, Proline amino acid and β- carotene were quantified by high-performance Performance Liquid Chromatography (HPLC). Results revealed that, highest contents of (Apx) 128.16 µg. ml^{-1} and 132.6 µg. ml^{-1} for amylase at 60 g.l^{-1} of mannitol and 0 Gy of gamma, respectively. Furthermore, the content of VIT C (265.57 µg. ml^{-1}) at 20 Gy and 60 g.l^{-1} of mannitol. The results also showed that a combination of 40 Gy and 60 g.l^{-1} of mannitol was optimum for the production of high proline amino acids (761.0 µg. ml^{-1}) and (113.54 µg. ml^{-1}) for β- carotene content in callus culture.

Keywords: Secondary Products, Cherry Tomato Callus, Mannitol Stress, Plant Tissue Culture.

1.Introduction

In the sixteenth century, tomatoes (*Solanum lycopersicum L.*) were brought from the Andes to Europe. This plant has gained popularity in modern times and has developed into a significant crop for the economy [1]. It is a perennial crop belonging to the Solanaceae family [2].

Plants have been utilized for therapeutic purposes all across the world since prehistoric times. Plants' pharmacological capabilities are based on their phytochemical components, particularly secondary metabolites, which are excellent sources of value-added bioactive chemicals. Secondary Metabolites (SMs) are compounds with a complicated chemical makeup that are formed in response to various types of stress to carry out several physiological processes in plants [3]. Furthermore, (SMs) are substances that are not required for a cell (organism) to survive but play a function in the cell's (organism's) interaction with its environment [4]. These chemicals are frequently engaged in plant defense against biotic or abiotic stressors. A few SMs are utilized as chemicals, particularly pharmaceuticals, flavors, perfumes, insecticides, and dyes, and so have a high economic value [5]. Secondary molecules accumulate heavily in response to a number of environmental stresses, including light, temperature, soil water, soil fertility, and salinity. For most plants, a change in one element can affect SMs content [4, 6]. Many researchers investigate the accumulation of secondary products by exposing callus to different stresses [7, 8].

Plant cell and tissue cultures (PTC) have a high regular capacity to produce a variety of beneficial secondary metabolites . It's a continuous, stable source of plant medicines [9] and independent of meteorological and geographical variables, allow continuous, sustainable, affordable, and viable secondary metabolite production [3].

The advancement of plant biotechnology has created several potentials for tomato plant engineering [10]. In vitro mutagenesis in plant organ, tissue, and cell cultures allows for the rapid and prevalent spread of mutant plants [11]. Mutation induction by gamma radiation (GR) has been reported in field-collected buds and seeds, as well as tissue cultures, protoplasts, and calluses [12]. Plants can be managed under controlled conditions, in small spaces, and at any time of year using this technique [13]. In vitro biotechnological techniques ensure quality and safety, which, when combined with mutagenesis techniques, allows the selection of outstanding agronomic characteristics in economically and productively important plants [14]. Plants developed several mechanisms to cope with stress, including morphological and structural changes and increased secondary production [15]. Plants can cope with these stresses via a variety of antioxidant enzymes [16] and non-enzymatic antioxidants [17].

The main objective of this study is to investigate the effect of drought stress and gamma rays on secondary products of cherry tomato callus.

2.Materials and methods

Seeds of Cherry tomato CV (Golden Sunrise) were irradiated with gamma cobalt -60 source (CO^{60}) at the doses (0, 20 or 40 Gy), and germinated on Murashige and Skoog, (MS) basal medium[18] supplemented with organic components and non organic compounds and combination of (2.0 kin + 2.0 IAA mg. l^{-1}) for callus induction. One month later, 100 mg of callus fresh weight (FW) were cultured on MS medium supplemented with mannitol at concentration of (0, 40 and 60 $g.l^{-1}$). The cultures were incubated in culture room condition.The cultures were kept in a controlled growth chamber at 25±2°C with a light photoperiod of 18/6 darkness for 30 days.

2.1. Determination of active compounds in stressed callus by high-performance liquid chromatography (H. P.L.C)

2.1.1. Analysis of soluble Vitamin C (vit C) in stressed callus of cherry tomato

A. Separated conditions:

Vitamin C was separated on the Fast Liquid Chromatography (FLC) column under the optimum conditions as follows: Column:STR ODS-II 1,3µm particle size (50 x 4.6 mm I.D.) column, Mobile phase: (A) 0.1 M potassium phosphate, PH 2.6:5 M octane sulphonate (ion-pair); (B) acetonitrail A; B (9:1, V:V) Detection UV set at 270 nm; flow rate 1.5 ml/min (40 C temperature). Standard concentration: 25 µg. ml^{-1} Retention time 1.052.

B. Preparation of sample:

Extraction and estimation of vitamin C according to the following method by [19] 1.0 g of sample was weighed and then dissolved in 100 ml of HPLC methanol. water 60:40 V/V, the sample was shaken and agitated in Ultrasonic bath for 10 minutes, then concentrated by evaporating the solvent with a stream of liquid N2 until it reached nearly 0.5 ml, then added some of the mobile phase to reach 1 ml. Dried samples were re-suspended in 1.0 ml of HPLC-grade methanol by vortexing. The mixture was passed through 2.5 µm disposable filters, and then 20 µl were injected on an HPLC column. The retention time and peak of area for the standard were determined (**Figure 1**), and then the concentration for each sample was quantitatively determined by comparison of the standard peak of area with the sample peak of area.

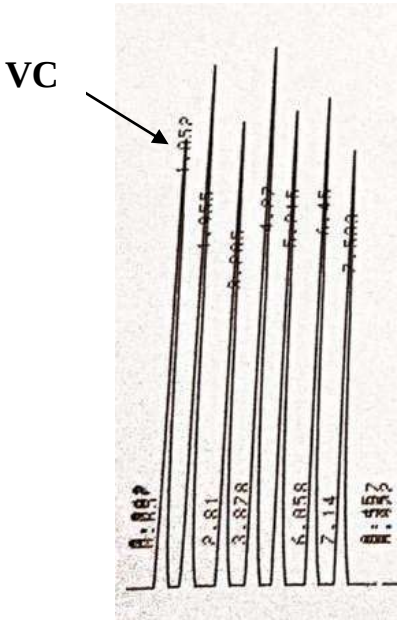

Figure 1 .Retention time of standard VC (1.052)

2.1.2.Analysis of Ascorbate peroxidase (Apx) and Amylase enzymes in stressed callus of cherry tomato

A. Separated conditions:

Apx and Amylase enzymes separated on Fast Liquid Chromatography (FLC) under the optimum conditions as follows:

Column: 7 µm particle size, Nuclosil 4000-7PEI, anion exchange for enzyme and protein (125X 4.0 mm I.D) column,

Mobile phase: solvent (**A**) 2 $mM.l^{-1}$ tris-acetate, PH 8.0. Solvent (**B**) 20 $mM.l^{-1}$tris- acetate PH 8.0. 0.1 M Phosphate buffer PH + 1.5 mM KCl isocratic separation using solvent (A: B, 40:60 V/V). Detection UV set at 280 nm Flow rate: 1.5 ml/min. Standard concentration: **125** µg. ml^{-1} Retention times are 2.05 and 3.70 for the Apx and Amylase enzymes, respectively.

B. Preparation of sample:

A method described by [20] with slight modifications as follows: 1.0 g of each sample was weighed and homogenized with phosphate buffer, then the suspension was subjected to Ultra-sonication (Bransonifier, USA) at 60% duty cycles for 10 min at 4ºC followed by centrifugation at 7,500 rpm for 10 min. at the clear supernatant of each sample containing the enzyme was extracted with phosphate buffer, then centrifuged. 5 mg of $(NH_4)_2SO_4$ precipitation was performed on the extracted enzyme, dialyzed, loaded, and eluted from a Sephadex G-200 column with phosphate buffer. The overall purification was collected. A fast separation at 4ºC was maintained during the purification process and extraction. H.P.L.C separation conditions were optimal PH and temperature, which were determined as 8.0 and 25ºC. According to the optimal conditions given above. The mixture was passed through 2.5 µm disposable filter, and stored at 4ºC for further analysis. 25 µl of each sample is injected into H.P.L.C system according to the optimal conditions. The retention time and peak of area for the standard were determined (**Figure** 2), and then the concentration for each sample was quantitatively determined by comparison of the standard peak of area with the sample peak of area.

Figure 2 . Retention time of standard enzymes Apx 2.05 and Amylase 3.70

2.1.3. Analysis of proline amino acid in stressed callus of cherry tomato

A. Separated conditions:

The pre-column Derivatization Method is used, where the amino acids are derivatized before injection, and then the reaction products are separated and detected. This concept is illustrated on the right (Figure 3). The column is Shimpack XR-ODS (50X4.mm I.D), 3 µm particle size).

Detector UV set 254 nm Gradients were formed between two degassed solvents as follows: Solvent **(A):** 5% methanol in 0.1 N Sodium acetate buffer pH (7.0). Solvent **(B):** methanol, linear gradients in form 0-25 min. 25µl injection, and 35°C .

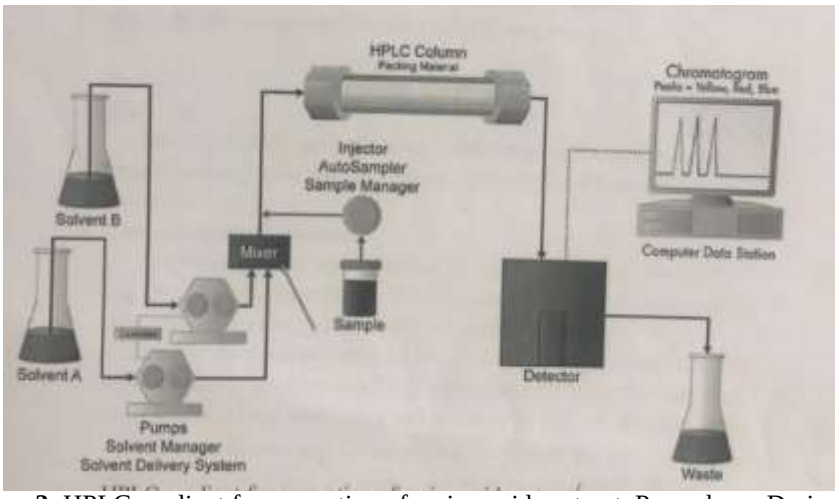

Figure 3: HPLC gradient for separation of amino acids extract Pre-column Derivatization

B. Sample Preparation:

The method of Gokulakumar and Narayanaswamy [21] was followed to extract the amino acid Proline from stressed callus of cherry tomato:

A 100ml of boiled D. D water was added to (1) g dry weight of the callus from each treatment. After grinding, filtered using filter paper, the solution was kept over night at room temperature. Then centrifuged at 12,000 rpm, for 1 minute and Keep in bottles at 20°C until estimated.Next day the mixture centrifuge at 12,000 rpm for 1 min, filtered and concentrated using a rotary evaporator at a temperature of 30 ° C until the final volume reached (5) ml. the clear medium was transferred to small bottles .Amino acids were determined by Jones and Gilligan [22] derivatives and prepared as followed.

A 50 mg of Orthopthaldehyde (OPA) and 50 µg of 2-mercaptoethanol dissolve in 1.25 ml of ethanol, then fill the volume to 50 ml by adding 50 mol of sodium borate, pH adjusted to 5.9, and the solution kept in the dark for 24 hours. The retention time and peak of area for the standard was determined (**Figure 4**)

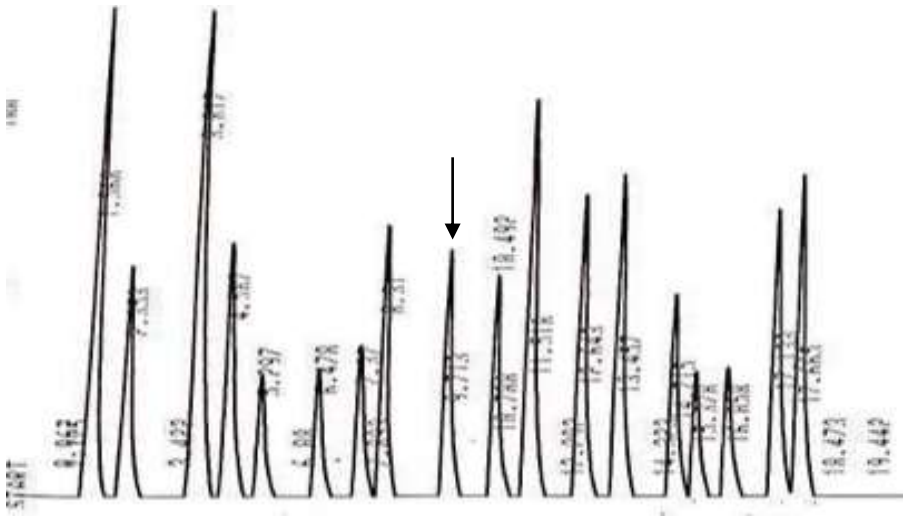

Figure 4.Standard proline retention time (9.7)

2.1.4. Analysis of β- carotene in stressed callus of cherry tomato

The standard of active compounds (pigments) was separated on Fast Liquid Chromatographic (FLC). The column Zobax Clipse XDB- C-18, 3 µm particle size(50X4.6 mm I.D). Mobile phase Solvent **(A):** 0.1% acetic acid. Solvent **(B):** methanol, using linear program from 0%B to 100% B for 9 min. Detector UV set 370 nm.Temperature 30°C, Flow rate 1.5ml/min. Injection volume 25µl. The retention time and peak of area for the standard was determined (**Figure 5**).

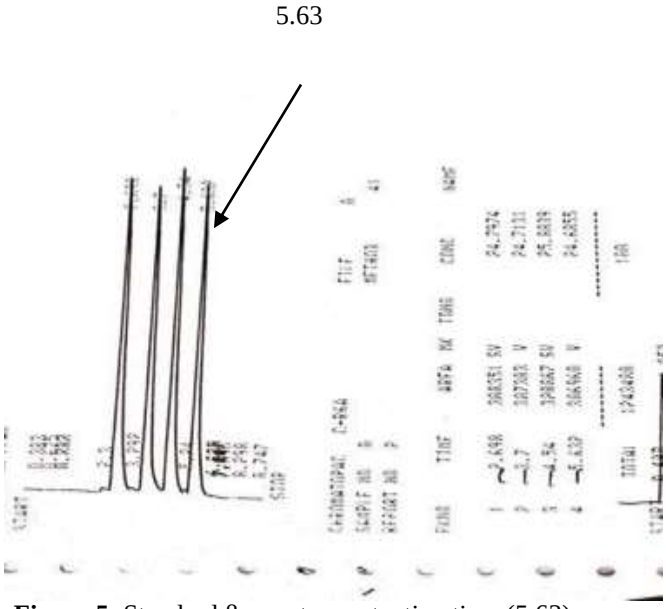

Figure 5: Standard β- carotene retention time (5.63)

Calculation: The concentration of (enzymes, Vit C, β- carotene and amino acids) were quantified using the following equation [23]

$$\text{Concentration of sample } (\mu g.\ ml^{-1}) = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{conc. of standard} \times \text{dilution factor}$$

***** For statistical analysis each treatment replicated trice time

Statistical Analysis

The experiments were setup in completely randomized design (C. R. D). Each treatment replicated three times. Data collected and analyzed using GenStat softwere (12^{ed}) and means were separated using Duncan's multiple range test at 5% level.

4.Results and discussion:

4.1.Effect of Gamma and mannitol stress and their interaction on Apx and Amylase concentration in stressed callus:

For enzymes concentration (**Table 1&2**) HPLC analysis showed that the highest accumulation of Apx and Amylase were found when callus enhanced with 60 $g.l^{-1}$ of mannitol with values (109.45 µg. ml^{-1} and 128.32 µg. ml^{-1}) respectively compare with less amount in the control and

40 g.l^{-1} treatment were the value respectively for Apx (94.04 µg. ml^{-1} , 92.89 µg. ml^{-1}) and (107.14 µg. ml^{-1},112.32 µg. ml^{-1})for amylase enzyme .When the callus was exposed to gamma doses of 20 and 40 Gy ,the concentration of Apx were (92.72 µg. ml^{-1}, 96.53 µg. ml^{-1}) respectively and the highest value was (107.13 µg. ml^{-1})in control and the value was (114.96, 114.24 µg. ml^{-1}) while the superior was for control that recorded (118.58 µg. ml^{-1}) for Amylase concentration and it is clear that there are no significant differences among them. For the Interaction effect on accumulation of enzymes according to table (1&2) the highest values for APX and Amylase were (128.16µg. ml^{-1}, 132.6 µg. ml^{-1}) in 60gm.l^{-1} mannitol with (0) dose of gamma respectively, while it recorded the lowest value of APX that reached (88.2 µg. ml^{-1}) in (20 Gy, 40 g. l^{-1}) and for amylase it was 132.6 µg. ml^{-1} .
(**Table 1&2**).

Table 1. Effect of Gamma ray and mannitol stress on APX µg.ml^{-1} in tomato callus

Gamma	Mannitol			Mean of gamma radiation
	0	40	60	
0	96.76 c	96.47 c	128.16 a	107.13 a
20	93.03 d	88.2 e	96.94 c	92.72 c
40	92.34 d	94.01 cd	103.24 b	96.53 b
Mean of mannitol	94.04 b	92.89 b	109.45 a	

***Each value represent mean of three replicates**
***Means followed by the different letters are significantly differed from each other within the single variant or their interactions at 5% level according to Duncan's multiple range test.**

Table 2. Effect of Gamma ray and mannitol stress on Amylase µg.ml^{-1} in tomato callus

Gamma	Mannitol			Mean of gamma radiation
	0	40	60	
0	98.66 g	124.49 c	132.6 a	118.58 a
20	110.22 e	110.68 e	123.97 c	114.96 b
40	112.53 d	101.8 f	128.39 b	114.24 b
Mean of mannitol	107.14 c	112.32 b	128.32 a	

***Each value represent mean of three replicates**
***Means followed by the different letters are significantly differed from each other within the single variant or their interactions at 5% level according to Duncan's multiple range test**

Plants tend to amass complex chemical composition groups of organic compounds in response to various types of stress in order to enhance their resistance to environmental conditions and the stimulation of defensive mechanisms [24]. The addition of mannitol to the culture media inhibited growth and increased the sub-culturing duration in various crops [25]. A higher mannitol content lowered the nutritional medium's water potential and made water absorbtion more difficult, resulting in osmotic stress [26]. The osmotic potential of plants treated with mannitol steadily reduced, indicating that osmolyte buildup increased with time. Mannitol is extensively utilised in several studies to explore the in vitro osmotic stress response of many plant species [27]. .

Our study agrees with the results of Anca [28], which found that APX, CAT were enhanced at the transcriptional level and in terms of enzyme activity, indicating the activation of antioxidant defense. APX activity is reduced when exposed to gamma doses of Co^{60} [29]. Beyaz's findings [30] clearly reveal that exposure to gamma irradiation pretreatment, alone or in conjunction with

drought stress, resulted in substantial increases in APX activities. It is noted that any increase in amylase enzyme callus content may be attributed to the amylase enzyme's role in converting starch into simple sugars to regulate osmosis and be absorbed by plant cells in order to release the necessary energy that is important in the vital processes of survival and continuity under stress [31]. The peroxidase enzyme developed more under stress situations, since it is regarded as one of the antioxidant enzymes that operate as a defense system for plants, trapping hydrogen peroxide and oxide radicals O-, where O- transforms to O_2 with H_2O_2 molecules to eventually turn into H_2O water molecules. The enhanced enzyme activity reflects the plant's capacity to endure stress [32], and these findings are in line with previous findings [33].

4.2. Effect of Gamma ray and mannitol stress on Ascorbic acid (vit C) in cherry tomato callus:

As for Ascorbic acid in callus content, results in (Table 3) showed superiority for mannitol concentrations of 60 $g.l^{-1}$ with values (247.94µg.ml^{-1}).While the highest concentration of vitamin C was found at the control treatment (0Gy) compared with (20 and 40) Gy. For the interactions between gamma radiation and mannitol concentrations, results in (Table 3) clarified that 20 Gy in combination with 60 $g.l^{-1}$ mannitol accumulated the highest mean of vitamin C reached (265.57mg.g^{-1}). L-ascorbic acid (vitamin C) is a primary antioxidant in plants, and it plays an important role in reducing excessive cellular reactive oxygen species activity produced by a variety of abiotic stressors. Plant ascorbate levels fluctuate according to the degree of stress and species sensitivity [34]. Plants with higher ascorbate content can effectively scavenge the excessive ROS generated during stress conditions, and confer increased tolerance to abiotic stresses.

Table 3. Effect of Gamma ray and mannitol stress on VIT C. µg. ml^{-1} in tomato callus

Gamma	Mannitol			Mean of gamma radiation
	0	40	60	
0	231.2 d	236.73 c	253.48 b	240.5 a
20	108.08 g	237.92 c	265.57 a	203.9 b
40	113.62 f	238.7 c	224.77 e	192.4 c
Mean of mannitol	150.96 c	237.8 b	247.94 a	

***Each value represent mean of three replicates**
***Means followed by the different letters are significantly differed from each other within the single variant or their interactions at 5% level according to Duncan's multiple range test**

2.5. Effect of Gamma ray and mannitol stress on proline amino acid in cherry tomato:.

As for Ascorbic acid in callus content, results in (**Table 3**) showed superiority for mannitol concentrations of 60 $g.l^{-1}$ with values (247.94µg.ml^{-1}).While the highest concentration of vitamin C was found at the control treatment (0Gy) compared with (20 and 40) Gy. For the interactions between gamma radiation and mannitol concentrations, results in (**Table 3**) clarified that 20 Gy in combination with 60 $g.l^{-1}$ mannitol accumulated the highest mean of vitamin C reached (265.57mg.g^{-1}). L-ascorbic acid (vitamin C) is a primary antioxidant in plants, and it plays an important role in reducing excessive cellular reactive oxygen species activity produced by a variety of abiotic stressors. Plant ascorbate levels fluctuate according to the degree of stress and species sensitivity [34]. Plants with higher ascorbate content can effectively scavenge the excessive ROS generated during stress conditions, and confer increased tolerance to abiotic stresses.

Hadi [35] investigated that increasing mannitol concentration led to an increase in the accumulation of proline, and another study using PEG to induce stress found an increase in proline content [36].

According to Xiong and Zhu [37], under stress circumstances, plants not only generate antioxidants but also accumulate suitable solutes such as proline, which were previously assumed to operate as osmotic buffers. They do, however, appear to have a role in maintaining the functional state of macromolecules as part of osmotic adjustment, most likely by scavenging reactive oxygen species. Proline protects cells by scavenging oxidative free radicals such as the hydroxyl radical [38, 39]. It helps to lower cell acidity, which leads to an increase in the production of NADP+, which is required for biological processes. When plant cells are stressed, the production of NADP + increases by increasing the gene expression of the enzyme (P5CS) pyrroline-5-carboxylate synthetase, which is responsible for converting glutamate to proline [40, 31].

Table 4. Effect of Gamma ray and mannitol stress on proline amino acid .µg.ml^{-1} in tomato callus.

Gamma	Mannitol			Mean of gamma radiation
	0	40	60	
0	482.1 e	533 d	604.7 c	539.9 b
20	402.1 f	655.8 b	666.6 b	574.9 a
40	402.1 f	508.2de	761.0 a	557.1 ab
Mean of mannitol	428.8 c	565.7 b	677.4 a	

***Each value represent mean of three replicates**

***Means followed by the different letters are significantly differed from each other within the single variant or their interactions at 5% level according to Duncan's multiple range test**

2.6.Effect of Gamma ray and mannitol stress and their interaction on β- carotene accumulation in cherry tomato callus:

Table (5) show that the concentration of β- carotene rises as the concentration of mannitol increases, with significant differences appearing where a concentration of 60 of mannitol was recorded at a rate of 105.9 µg.ml^{-1}, while the lowest concentration was recorded in the control treatment, at a rate of 75.96 µg.ml^{-1}.While gamma doses recorded highest mean (98.32 µg.ml^{-1})at 40GY and the lowest concentration was (87.26 µg.ml^{-1}). Furthermore, the interaction between gamma dosages and mannitol concentration revealed that combining 60 g.l^{-1} mannitol with 40 Gy of gamma therapy resulted in a larger rise in β- carotene accumulation (113.54 mg. g^{-1}) that outperformed all other interaction parameters. It is clear from the results that there is a significant increase in β- carotene accumulation with increasing mannitol and gamma doses together.

Table 5. Effect of Gamma ray and mannitol stress on β- carotene .µg.ml^{-1} in cherry tomato callus.

Gamma	Mannitol			Mean of gamma radiation
	0	40	60	
0	66.61 f	106.11b	99.73 c	90.82 b
20	70.67 e	86.71 d	104.42 b	87.26 c
40	90.61 d	90.81 d	113.54 a	98.32 a
Mean of mannitol	75.96 c	94.54 b	105.9 a	

***Each value represent mean of three replicates**

***Means followed by the different letters are significantly differed from each other within the single variant or their interactions at 5% level according to Duncan's multiple range test**

5.Conclusion

Our results clarified that Gamma rays at 20 or 40 Gy and in vitro biotechnology under stress conditions in the presence of 40 or 60 $g.L^{-1}$ mannitol have been considered reliable approaches for enhancing secondary metabolite production from cherry tomato callus.

References

1. Paduchuri, P.; Gohokar, S.; Thamke, B.; Subhas, M. Transgenic tomatoes. *Int J Adv Biotechnol Res* .**2010**, *2*, 69–72. http://www. bipublication.com.
2. Mamidala, P.; Nanna, R.S. Effect of genotype, explant source and medium on in vitro regeneration of tomato. *Int. J. Gen. Molec. Biol.*, **2011**, *3*(*3*), 45–50.
3. Chandran, H., Meena, M., Barupal, T.; Sharma, K. Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. *Biotechnology Reports*, **2020,***26*, p.e00450.
4. Yang, L.; Wen, K.S.; Ruan, X.; Zhao, Y.X.; Wei, F.; Wang, Q. Response of plant secondary metabolites to environmental factors. *Molecules*, **2018**,*23*(*4*), 762.
5. Pagare, S., Bhatia, M., Tripathi, N., Pagare, S. ; Bansal, Y.K. Secondary metabolites of plants and their role: Overview. *Current Trends in Biotechnology and Pharmacy*, **2015**,*9(3)*, 293-304.
6. Zykin, P.A.; Andreeva, E.A.; Lykholay, A.N.; Tsvetkova, N.V. ; Voylokov, A.V. Anthocyanin composition and content in rye plants with different grain color. *Molecules*, **2018**, *23*(*4*), 948.
7. Jassim, E.H.; Ameen, S.K. Effect of sucrose and mannitol on Ajmalicine production from leaves induced callus of *Catharanthus roseus* LG Don *in vitro*. *J. Biotechnol. Res. Center*. **2014**, *1,8*(*2*), 27-34.
8. Ibrahim, I.R.; Ameen, S.K. Influence of stress on secondary metabolites production from callus of Moringa oleifera in vitro. *The Iraqi J. Agric. Sci*. **2017**, *48*(*4*), 1099.
9. Vijayasree, N.; Udayasri, P.; Aswani, K.Y.; Ravi, B.B.; Phani, K.Y. ; Vijay, V.M. Advancements in the Production of Secondary Metabolites. *J. Nat. Prod*. **2010**, *3*, 112-123.
10. Gerszberg, A.; Hnatuszko-Konka, K.; Kowalczyk, T. ; Kononowicz, A.K. Tomato (*Solanum lycopersicum* L.) in the service of biotechnology. *Plant Cell, Tissue and Organ Culture (PCTOC)*, **2015**, *120*(*3*), 881-902.
11. Spencer-Lopes, M.M.; Forster, B.P.; Jankuloski, L. *Manual on mutation breeding* (No. Ed. 3). Food and Agriculture Organization of the United Nations (FAO), **2018**.
12. Suprasanna, P.; Vitthal, S.B.; Yadav, P.V. *In vitro* mutagenesis and selection in plant tissue cultures and their prospects for crop improvement. *Bioremediation, Biodiversity and Bioavailability*. **2012**, *6(Special Issue 1)*, 6-14.
13. Hernández-Muñoz, S.; Pedraza-Santos, M.E.; López, P.A.; Gómez-Sanabria, J.M.; Morales-García, J.L. Mutagenesis in the improvement of ornamental plants. *Revista Chapingo. Serie horticultura*. **2019**, *25*(*3*), 151-167.
14. Al-Safadi, B.; Elias, R. Improvement of caper (Capparis spinosa L.) propagation using in vitro culture and gamma irradiation. *Scientia Horticulturae*, **2011**, *127*(*3*), 290-297.
15. Cecoli, G.; Ramos, J.C.; Ortega, L.I.; Acosta, J.M.; Perreta, M.G. Salinity Induced Anatomical and Morphological Changes in Chloris gayana Kunth Roots. *Biocell*., **2011**, *35*(*1*), 9-17.
16. Rajput, V.D.; Singh, R.K.; Verma, K.K.; Sharma, L.; Quiroz-Figueroa, F.R.; Meena, M.; Gour, V.S.; Minkina, T.; Sushkova, S.; Mandzhieva S. Recent developments in enzymatic

antioxidant defence mechanism in plants with special reference to abiotic stress. *Biology*. **2021**, *26*, *10(4)*, 267.

17. Liang, X.; Zhang, L.; Natarajan, S.K.; Becker, D.F. Proline mechanisms of stress survival. *Antioxidants & Redox Signaling*, **2013**, *19*(*9*), 998-1011.

18. Murashige, T.; Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, **1962**, *15*(*3*),473-97.

19. Mitic, M.N. Rapid and Reliable HPLC Method for the Determination of Vitamin C in Pharmaceutical Sample. *Trop J Pharm Res.* **2011**, *10*(*1*), 105-111.

20. Yang, L.; Xi, Y.; Luo, Y.; Ni1, H.; Hai-Hang, L.i. Preparation of peroxidase and phenolics using discarded sweet potato old stems. *Scientific Reports*, **2019**, *9*, 1-10.

21. Gokulakumar, B.; Narayanaswamy, R. High Performance Liquid Chromatography for the Estimation and Detection of Amino Acids in Root Rot Disease in Sesame. *Advan. Biol. Res*, **2009**, *3*(*5-6*), 162-167.

22. Jones, B.N.; Gilligan, J.P. O-phthaldialdehyde precolumn derivatization and reversed-phase high - performance liquid chromatography of polypeptide hydrolysates and physiological fluids. *J. Chr. A.* **1983**, *266*, 471-482.

23. Budhiraja R.P. *Separation chemistry.* New Age International Ltd, Publishers, New Delhi, **2004**, 171-239

24. Murthy, N.H.; Lee E.J.; Peak K.Y. Production of secondary metabolites from cell and organ cultures: Strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell Tissue and Organ Culture*. **2014**, *118*(*1*), 1- 16.

25. Lopez-Puc, G.An effective in vitro slow growth protocol for conservation of the orchid *Epidendrum chlorocorymbos* SCHLTR. Trop. *Subtrop. Agroecosyst*. **2013**, *16*, 61–68.

26. El-Bahr, M.K.; Abd EL-Hamid, A.; Matter, M.A.; Shaltout, A.; Bekheet, S.A.; El-Ashry, A.A. *In vitro* conservation of embryogenic cultures of date palm using osmotic mediated growth agents. *J. Genet. Eng. Biotechnol*. **2016**, *14*, 363–370.

27. Huang, G.T.; Ma, S.L.; Bai, L.P.; Zhang, L.; Ma, H.; Jia, P.; Liu, J.; Zhong, M.; Guo, Z.-F. Signal transduction during cold, salt, and drought stresses in plants. *Mol. Biol. Rep*., **2012**, *2*, 969–987.

28. Macovei, A.; Garg, B.; Raikwar, S.; Balestrazzi, A.; Carbonera, D.; Buttafava, A.; Bremont, J.F.; Gill, S.S.; Tuteja, N. Synergistic exposure of rice seeds to different doses of-ray and salinity stress resulted in increased antioxidant enzyme activities and gene-specific modulation of tc-ner pathway. *BioMed research Int.*, **2014**, 2014.

29. Beyaz, R.; Sancak, C.; Yildiz, Ç.; Kuşvuran, Ş.; Yildiz M. Physiological responses of the M_1 sainfoin (*Onobrychis viciifolia* Scop) plants to gamma radiation. *Appl Radiat Isot*., **2016**, *118*, 73-79. doi: 10.1016/j.apradiso.2016.09.005.

30. Beyaz, R. Impact of gamma irradiation pretreatment on the growth of common vetch (Vicia sativa L.) seedlings grown under salt and drought stress. *International Journal of Radiation Biology*, **2020**, *96*(*2*), 257-66.

31. Azizi, Kh.; Moradii, J.; Heidari, S.; Khalili, A.; Feizian, M. Effect of different concentrations of gibberellic acid on seed yield and yield components of soybean genotypes in summer intercropping. *Inter. J. Agri.Sci*. **2012**, *2*(*4*), 291-301.

32. Quiles, M.J.; Lopez, N. I. Photoinhibition of photosystems I and II induced by exposure to high light intensity during oat plant grown effects on the chloroplastic NADH dehydrogenase complex. *Plant Sci*., **2004**, *166*, 815-823.

33. Kareem, T.K.; Abbas, T.K. Biochemical and physiological Changes of Callus growth and Lycopene Pigment Production from Tomato (*Lycopersicon esculentum* Mill.) under Drought Stress. International Journal of innovative. *Science and Technology*. **2018**, *3*(*2*), 7-21.

34. Venkatesh, J.; Park, S.W. Role of L-ascorbate in alleviating abiotic stresses in crop plants. *Botanical Studies*. **2014**, *55*(*1*), 1-9.

35. Hadi, S.M.; Ibrahim, K.M.; Yousif, S.I. Effect of shock and elevated levels of mannitol on callus growth, regeneration and proline accumulation in *Ruta graveolens* cultures. *Int. J. Curr. Microbiol. App. Sci*., **2014**, *3(11)*, 479-88.

36. Alaakel, S.; AL-Ouda, AS. ; Youssef, A.A. Effect of Salt and Osmotic Stresses on the Activity of Some Antioxidant Enzymes and Biochemical Traits in *Catharanthus roseus*. *Baghdad Science Journal*. **2021**, *18*(*3*).

37. Xiong, L.;Zhu, J.K. Molecular and genetic aspects of plant response to osmotic stress. *Plant Cell Environ*., **2002**, *25*, 131– 139.

38. Shulaev, V.; Cortes, D.; Miller, G. ; Mittler, R. Metabolomics for plant stress response. *Physiological Plant*, **2008**, *132*(2), 199-208.

39. Teixeira, J.; Fidalgo, F. Salt stress effects glutamine synthetase activity and mRNA accumulation on Potato plants in an organ dependent manner. *Plant physiological. Biochem*, **2009**, *47*(*9*), 807-813.

40. Szabados, L.; Savoure, A. Proline: a multifunctional amino acid. *Trends Plant Sci*., **2010**, *15*(*2*): 89-97.