



Biochemical and physiological changes of callus growth and Lycopene pigment production from Tomato (*Lycopersicon esculentum* Mill) under drought stress

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Abstract : This experiment was conducted in faculty of Science labs, Kufa University, carried out during 2014 to applied methods for extraction, purification and Quantitative of Lycopene red pigments, from callus tissue and tomato fruits mother plant (*Lycopersicon esculentum* Mill). This study include of three parts. Firstly, Tomato seeds (Supper queen) hybrid were germinated in free MS medium and callus induction from shoot tip (3cm pieces) by using MS medium supplemented with Dichlorophenoxyacetic acid (2,4-D) at different concentration (1, 2, 3) mg/l with (BA) benzyl adenine at concentration of (0.2) mg/l. Secondly, identically callus fresh weight recultured in the same Ms medium supplemented with high molecular weight (polyethylene glycol) PEG was used as selective agent at level of (5,15,20 and 30 %). Thirdly, comparisons study were made between *in vitro* and *in vivo* grown plant. Powder of control (lycopene) used as standard solution. The content of lycopene was done by using HPLC (high performance liquid chromatography), and compare of the quantitatively of lycopene with these content in fruits of mother plant, and callus tissue. Also, include alcohol extraction of Lycopene from tomato fruit by using acetone and hexane mixture. The result showed significant increased ($P < 0.05$) of Lycopene production and the superiority of lycopene content in Callus than the content in fruits of mother plant. Antioxidant enzymes activity like Catalase (CAT), guaiacol peroxidase (POX) and Superoxide dismutase (SOD) were high in callus under drought stress than in fruit of mother plant. However, Proline and total sugar content were at higher levels in callus under drought stress than in fruit of mother plant.

Key Words: Culture media, callus, CAT, POX, SOD, Lycopene pigment.

Introduction

The worldwide demand for natural dyes are derived from naturally sources such as plants, insects, animals and minerals is nowadays of great interest due to the increased awareness on therapeutic properties of natural dyes in public (26).

Medicinal plants produced a great amounts of phytochemical compound that widely used in diverse applications, including pharmaceuticals, foods color, dyes or attraction of pollinating insects and protection against pests and pathogens (99). "Carotenoids" plant Pigments groups of more than 600 different plant pigments, which are responsible for many colors. Tomatos belongs to family are a valuable source of several micronutrients and phytochemicals including a variety of antioxidants natural lycopene, α and β -carotene, lutein, zeaxanthin, polyphenolics such as kaempferol and b-cryptoxanthin. Lycopene is a carotenoid that is present in tomatoes is responsible red colour of the fruit. It constitutes about 80–90% of the total carotenoid content of redripe tomatoes.(86),(80) (26). Tomato fruits is considered two of the most important economic

vegetable crops in Iraq .Tomato plants is the most sensitive and critical stage due to the drought stress with 85% of all the dietary sources of lycopene depending on genotype and environmental conditions (77). Lycopene an aliphatic hydrocarbon with molecular formula $C_{40}H_{56}$ is a fat-soluble antioxidant as free radical scavenger (31). However,Lycopene is insoluble in water, but soluble in organic solvents(21) synthesized by many plants and microorganisms that responsible for the red to pink colors found in ripe tomato ,guava, pink, papaya, grape, and watermelon fruit (24), (25). Although 80% of the lycopene is found in the linear, all-trans conformation, human tissues (particularly liver, adrenal, adipose tissue, testes and prostate) contain mainly cis-isomers that may contribute to specific biological properties (25).Dietary lycopene protected lipids, proteins and DNA from oxidation, is inversely associated with the risk for a number of pathologies including most notably prostate, lung, stomach, cervical, breast, oral, colorectal cancer and esophageal, pancreatic, osteoporosis and cardiovascular disease. (25), (22). (8), had reported that tomato skins contained a high amount of lycopene act as protective ultraviolet light-induced skin damage. Plant tissue culture contribute in improvements evaluation and screening of plant genotypes for drought tolerance and conceder now an important techniques being used for rapid production of secondary metabolite from medicinal plants . *In vitro* plant callus culture has been found to depend on many factors, are composition of the basic medium, growth regulators, light intensity photoperiod, temperature, cultivation vessel (4). Drought stress continues to be a major global problem for the crop plants growth and development. Generally drought stress occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation (43). Improving growth rate and crop production in drought conditions may be achieved through breeding crops that are more tolerant to drought(79). *In vitro* technique useful a good methods for studying many aspects of plant growth and development or studying the physiological and biochemical processes, which contribute to drought tolerance or effects of drought at the cellular level under controlled conditions (87) ,(86).Secondary metabolite production in vitro culture technology has proven as an efficient alternative production system on many plants (37), (39). A wide range of controlled plant *in vitro* culture can generate of carotenoid production (55). There is a series of distinct advantage to producing a valuable secondary product in plant cell culture, rather than *in vivo* in whole crop plant. These include, production can be more reliable, simpler, and more predictable and isolation of the phytochemical can be rapid and efficient , as compared to extraction from complex whole plants ,also, compounds produced in vitro can directly parallel compounds in the whole plant and cell culture, can yield a source of defined standard phytochemical in large volumes. *In vitro* studies indicated phytonutrients such as carotenoid and any phenolic compounds may play a significant role in addition to vitamin in protecting biological systems from the effects of oxidative stress(45). Because of these received attention of lycopene pigments in tomato fruits with respect to its antioxidant activity and potential in preventing prostate cancer and cardiovascular diseases in humans. Researchers work to increasing of Lycopene contains in the diets by using new technique of biotechnology such as plant tissue culture in order to enhancement of production although, it has been used as a new source of production. Finally, the determining of carotenoid include lycopene, the AOAC method has been a standard method(11). This involves isolation of carotenes by column chromatography and quantification by visible spectroscopy. The purpose of my work is to examine the effect of different growth regulators on callus induction from tomato shoot tip explants and enhanced lycopene production in callus tissue.

Antioxidants are referred to as compounds which are able to inhibit the oxidation of different biomolecules and helps in repairing the damages caused to the body tissues due to oxidation processes(61). Although ,Investigation of study is to select callus lines tolerant to drought stress and characterize callus lines with respect to growth, lycopene content, osmolytes accumulation and activity of antioxidant enzymes and compare with its content in fruit mother plant.

Material and methods

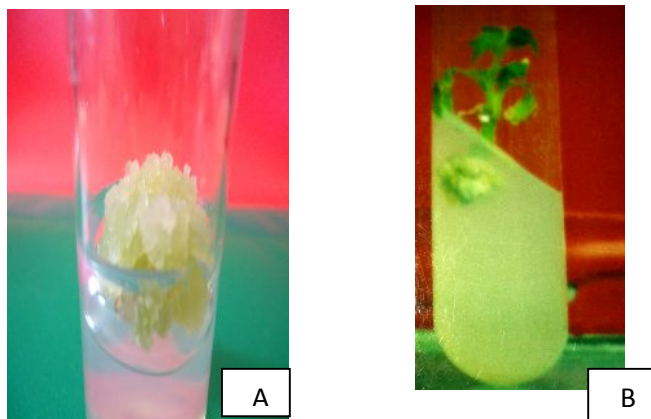
In vitro seeds sterilization and germination

All treatment in this work were done under aseptic conditions inside the Laminar airflow cabinet . Tomato seeds were collected from Babylon city , about 45 seeds were placed in flask and washed with distilled water three times to eliminate dust and other surface contaminants and soaked in flask containing sodium hypochlorite 6 % for 10 min flowed by soaked in ethanol 70% for 5 min and washed at three times with double distilled water. Tween 20 was used as a wetting agent at one drop for 20 min (2). Sterilizing seeds were cultured about two seeds in each tube containing 15 ml of free MS medium (Murashige and Skoog (1962) and

incubated in the dark conditions for four days; there after it was transferred to the chamber room and maintained 16/8 light/ dark condition for 2 weeks for seeds germination (67).

Callus induction

After seeds germination *in vitro*, the shoot tip explants were excised and cultured in MS media supplemented with 0.2 mg/l BA and 2,4-D at concentration 1, 2 and 3 mg/L respectively for callus induction with 12 replicate of each cultivar at each treatment of plant regulators (auxin and cytokinins) were employed Fig(1A) . The pH of the media was adjusted to 5.8 and autoclaved at 121°C for 15 min. The cultures were incubated at 25±1°C with 16/8 light/dark cycle. After 2 weeks callus initiated in the base of explants and completed fresh weight after 4 weeks Fig(1B).



**Fig(1) A: Shoot tip of *L. escolenetum* grown in (2mg/L)2,4-D and BA(0.2mg/L)
B: Callus induced from shoot tip of *L. escolenetum* grown in (2mg/L)2,4-D and BA(0.2mg/L)**

Callus fresh(g) and dry weight (mg) determination

After 40 days of shoot tip cultivation in MS medium, callus induced, fresh weight was calculated for all concentrations of 2,4-D and BA used in culture medium. Where callus harvested from glass tubes and measured fresh weight using an electric sensitive balance after removal of medium residue suspended on callus by washing with distilled water. However, fresh callus was placed in a glass petri dishes by two dishes each containing 6 pieces of callus and dried in oven at 45C for 24 hours and then get out and callus dry weights were determined then calculated.

Drought treatments

After 4 weeks of callus induced from the basis of explants fresh weight of callus induced in medium of 2 mg /L 2,4-D and 0.2 mg/L BA consider as the best treatments in relation with fresh callus weight Fig(1B) , callus remove from vessels under sterile condition and cut a suitable amount of callus (500 mg) fresh weight and subculture on the same medium for further proliferation. Added with a range of polyethylene glycol (PEG) at level of (5,15,20 and 30 %) to initiate selection stress treatments with 12 replicate of each cultivar at each drought level were employed. After five weeks treatment callus was harvested and washed with distilled water to remove agar remains and dried by tissue paper to remove the surface moisture(67).

Tomato sample preparation

Tomato fruits(Supper Queen) used for this study were from the 2014 harvest and were obtained from Babylon fields, according to method of (103), Fresh fruits were finely ground to a pure in an same weight of distilled water with an electric tissue grinder before sampling and assay. Tissue purees were kept on ice and out of light after preparation and until assayed. Purees were stirred on ice on a magnetic stirring plate during replicate sampling. Sampling and weighing of replicates were performed in reduced room light.

Lycopene pigment extraction

Lycopene pigments were extracted according to method of (78). Tomato fruits (10 grams) and callus (5 grams) dried samples were grinded and homogenized and were added with 5 ml solution of 0.05% (w/v) butylatedhydroxytoluen (BHT) in acetone, 5 ml ethanol and 10 ml hexane. Homogenate were incubated under low temperature with ice and shaken with stirrer for 15 minutes, then were added with 3 ml deionized water on every vials and were shaken for 15 minutes in cold. Samples were left at room temperature for 5 minutes in order to separate the two phases.

Thin Layer Chromatography (TLC)

TLC was conducted according to the method of (65) qualitative analysis of lycopene was done by using thin layer chromatography (TLC) to isolate of lycopene in fruits and callus crude extraction. Standard solutions used were Lycopene (Sigma). TLC using ethyl acetate: glacial acetic acid: formic acid: water with the proportion of [100:11:11:25, v/v/v/v] as solvent system for the separation of bioactive compounds in crude extracts of tomato fruit intact plant and callus at UV light 366 nm (65). Silica gel 60 F254 plates were used at thickness of 0.25 mm and dimensions of 20 × 20 cm. where 2 ml of crude extract of the fruits and callus were took and concentrated as spots by using of Capillary tubes at equal dimensions (2cm away from Edge) on silica gel plates. and then plates were placed in jar container contain the mobile phase and covered provisions, after the arrival of the solvent to approximately the end of plate, then removed the plates and left to dry in the laboratory atmosphere, then separated spots sites was identified with the naked eye and then under the ultraviolet rays (UV) at 366 wavelength.

Preparation of standard lycopene solution

According to methods in (65) 0.5 mg/ml solution of pure lycopene (Sigma Aldrich) were made in absolute ethanol for preparation of the standard Lycopene spots.

Estimation of proline

According to (15) method, proline was estimated in tomato fruit and induced callus tissue by placed 5 g of tomato fruit and 1g fresh weight callus tissue and crush well in 3 % aqueous sulfosalicylic acid and centrifuged at 2000 r/min for 10 minutes. Added 2 ml of Ninhydrin solution and 2 ml of glacial acetic acid to mixture homogenate and placed in glass test tubes and heated to 100 ° C in a water bath for 30 minutes and leave to cool and then the reaction mixture was extracted with Estimated the proline content by measuring red layer containing toluene by a spectrophotometer at a wavelength of 520 nm and compared with the standard curve of proline, while Planck solution composed of 5 ml of toluene 1 ml toluene and mixed vigorously shaking for 20 seconds. Proline content was measured as mg/g DW.

Estimation of carbohydrate Content (Soluble Sugars)

Spectrophotometric method (model UV/VIS) shows potential for the analysis of Carbohydrate at 400-800 nm (67). Soluble sugars were determined based on the method of phenol sulfuric acid. 0.1g dry weight macerated sample (fruits and callus) was homogenized with distilled water, extract was filtered and the extract treated with 1% phenol and 98% sulfuric acid, mixture remained for 1 hour and then absorbance at 485 nm was determined by spectrophotometer. Contents of soluble sugar were determined by using glucose as standard. The total soluble Sugars was then estimated using the standard curve of Glucose, cuvette containing pet-ether (blank) was used to calibrate the spectrophotometer to zero point. Carbohydrate as represented by the equation: (52).

$$\frac{\text{Absorbance of unknown}}{\text{Concentration of unknown}} = \frac{\text{Absorbance of standard}}{\text{Concentration of standard}}$$

Standard samples

Dissolve 100 mg of glucose in 100 ml distilled water. Working standard of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene (52). The total soluble carbohydrate was then estimated using the standard curve of Glucose (78).

Estimation of catalase enzyme activity

Catalase activity was measured according to method of (23). One gram of frozen callus was homogenized in a pre chilled pestle & mortar with 5 ml of ice cold 50 mM phosphate buffer. The extract was centrifuged at 4 °C for 20 Min at 12,500 X g. The supernatant was used for enzyme assay. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7.0) 0.4 ml 15 mM H₂O₂ and 0.1 ml of enzyme extract. The decomposition of H₂O₂ was followed by decline in absorbance at 240 nm. Catalase activity was expressed in unit mg/1 protein. One unit was defined as the amount of enzyme catalyzing the decomposition of 1 n mol H₂O₂ per m /1 per mg/1 protein.

Estimation of peroxidase enzyme activity

Peroxidase activity was assayed by the method of (53). Assay mixture contained 2 ml of 0.1 M Phosphate buffer (pH 6.8) 1 ml of 0.01 M Pyrogallol, 1 ml of 0.005 M H₂O₂ and 0.5 ml of enzyme extract. The solution was incubated for 5 min at 25 °C after which the reaction was terminated by adding 1 ml of 2.5N H₂SO₄. The Purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5N H₂SO₄ at zero times. The activity was expressed in unit mg/1 protein. One unit (U) is defined as that amount of enzyme, which forms 1 mol of purpurogallin per minute per mg protein under the assay conditions.

Estimation of Superoxide dismutase activity

Superoxide dismutase activity was estimated in terms of inhibition of the photochemical reduction of Nitroblue tetrazolium (NBT) as per the procedure of (16). The reaction mixture (3 ml) consisted of 50 mM phosphate buffer (pH 7.8) and 0.1 mM EDTA, 14.3 mM methionine, 82.5 mM NBT and 2.2 mM riboflavin. The reaction was initiated by adding 100 µl of enzyme extract. The tubes were kept 30 cm below a light source (2×40 W fluorescent tubes) for 30 min. The reaction was stopped by switching off the light. The reduction of NBT was measured by monitoring the change in absorbance at 560 nm. The activity was expressed in terms of units mg/1 protein. One unit of enzyme was defined as the amount of enzyme that brings about 50 % inhibition of NBT under the assay conditions.

Quantitative determination of Lycopene content by High performance liquid chromatography

HPLC, composes as potentially source of identification of Lycopene well as their quantity, Lycopene content in the extracting fruits and callus tissue was determined by using methods of (21) were analyzed by HPLC system (Shimadzu Corp., Japan) was used as a reference method. A C30 YMC reverse-phase column (4.6 " 150 mm, 3 µm, YMC, Wilmington, NC), was used as methods for carotenoid separation with mobile phases consisting of 86% acetonitril, 10% water, 4% formic acid (38). The flow-rate was 1.5 ml/min, and the retention time was 6 min with detection at 472 nm .

Statistical analysis

1-A complete randomized block design in a factorial experiment was used with nine replicates. Treatment means were compared according least Significant Difference (LSD) at 0.05 level. 2-The data between Callus and fruits of Lycopene content analysis by using the (T-test) analysis (90).

Result and Dissections

Callus Fresh and dry weight in MS medium

According to many results in the same study shoot tip cultures medium of tomato plant, BA and 2,4-D applications was able to induced callus tissue and development in culture medium. Nutrient media for tomato shoot tip cultures contained BA at concentration 0.2 mg/l, and 2,4-D in the concentration of (1. 2 and 3 mg/l) to initiation of callus tissue. Callus induced from tomato shoot tip were collected from culture media and weighed. Table (2) show the optimal concentrations of auxin (2,4-D) applied in culture medium affected callus initiation from shoot tip of sterile mother plant, however, BA affected callus fresh weight significantly and maximum fresh callus tissue was achieved on MS medium with BA at concentration of 0.2 mg/l and 2,4-D at concentration of 2 mg/l about (2.29 g/L) than other concentrations of 2,4-D(1 and 3 mg/l) recorded (1.13 and

0.86 mg/l fresh weight) respectively. Callus induced in this study at MS medium with BA(0.2 mg/l and 2,4-D 2mg/l) concenter as the pest medium for callus initiation which uses in drought treatment. Also, Result in the same table indicate that BA and 2,4-D concentration affect callus dry weight at all concentration in cultures medium. However, callus grew in MS medium with 0.2 BA mg/l and 2 mg/l 2,4-D significantly increased in dry weight about (0.61mg/l) comparison with (1 and 3) 2,4-D and 0.2 BA which about(0.26 and 0.20 mg/l respectively) .

Finally, The maximum callus fresh weight *Hyoscyamus aureus L.* archived after 40 days only on MS media containing 2 mg/l NAA (1.817 gm) and after 100 days from culture on MS media containing 1mg/l 2, 4-D (1.41gm) (49).

Callus Fresh and dry weight under drought Stress

Drought treatments strongly affect callus fresh and dry weight at average for all cultures treatments. Control treatment were callus grew in MS medium without PEG. Callus fresh and dry weights was determined after 40 days in drought treatment. It was observed that callus cultures of both the cultivars showed a gradual decrease in fresh weights with an increase in PEG levels as compared to the control treatment. The results showed in Table(2) callus grown in MS medium with (5%) drought level significantly decreased in fresh weight it reached about (2.56 g/l) in relation with control (0%) drought level it reached about (2.29 g/L) that greatest significantly with control cultures. Application of (15 and 20 %) of PEG added more reduction in these parameters about (1.77 and 1.02 g/L) respectively in comparison with control treatment. However , 30% drought treatment recorded lowest callus dry weight and significantly decrease in comparison with all PEG treatment. Also, data in the same table show significantly more increased in dry callus weight that treated with PEG in culture medium at levels of 5 and 15% it reached to (0.68 and 0.45) mg/L respectively in comparison with control treatment(0% PEG) about (0.89 mg/L) and more significantly increase in callus dry weight at level of 20% about (0.28 mg/L) compare with control treatment. Results of application of PEG at 20% level caused more significantly increased in dry weight of tomato plant callus in comparison with 5% and 15% PEG treatments and significantly effect in comparison with control treatment. However ,lower callus dry weight recorded in 30% drought treatment and significantly decrease in comparison with all PEG treatment. Drought stress, is an complex phenomenon which includes osmotic stress, specific ion effect, nutrient deficiency etc. thereby affecting various physiological and biochemical mechanisms associated with plant growth and development (94). The results in this study showed that the callus fresh weight was significantly influenced by increased water tension (Table 1). In this study observed a reduction in callus fresh weight and more increase of dry weight treated with PEG in all treatment except the 30% treatment that recorded low amount of fresh and dry call weight. Several study in the same experiment observed that decreased growth in presence of PEG in callus medium((34),(1). More specifically, the dry weight of the control callus was significantly lower than those of the callus treated with PEG.

Table(1) Effect of BA and 2,4-D drought treatments on callus fresh and dry weight

Average	Dry weight mg/l	Fresh weight g/l	2,4-D Concentration mg/L	BA Concentration mg/L
0.69	0.26*	1.13 *	1	0.2
1.59	0.89**	2.29**	2	
0.53	0.20*	0.86*	3	
	0.35	1.42	Average	
Drought treatments %				
Average	Dry weight mg/l	Fresh weight g/l	PEG %	
1.59	0.89	2.29	0	
1.62	0.68**	2.56**	5	
1.11	0.45*	1.77*	15	
0.65	0.28	1.02	20	
0.47	0.07	0.88	30	
0.86	0.57	1.91	Average	

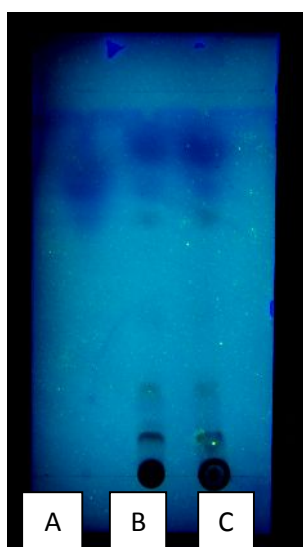
The harmful effect of drought stress on intact plants was increased with increasing levels of drought in callus conditions. In plant tissue culture technology callus fresh and dry weight induced from different part of tomato showed change in growth response to drought conditions as compared to mother plant. It might be due to high concentration of proline content and metabolic solute in callus grown in medium with drought treatments. Several physiological studies suggested that under stress conditions different form of carbohydrates are nontoxic molecules accumulate although to varying degree in different plant species suggested that sugars synthesize in response to osmotic stress either act as osmotica and/or protect specific macromolecules and contribute to the stabilization of membrane structures (47),(20). These molecules may have a primary role of turgor maintenance but they may also be involved in stabilizing proteins and cell structures and do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentrations under osmotic stress (27).The concentration of soluble sugars increased at a rate closely corresponding to the increase in fresh weight (59).

TLC determination Of Lycopene pigments

Lycopene pigments that isolated from callus grown under drought stress and fruits crude extracts of (*L. esculentum*) by (TLC) technique. The developing solvent was able to separate different pigments having different retention factor (Rf value) present in crude extracts.

Table(2): Characteristic of lycopene and carotenoids compounds of extract of.

Color in UV light and filter spot	Relative flow (Rf)	Color in UV light	No. of spot	Explants
Red	0.45	Pink red	*	Lycopene
Red	0.46	Orange	1	Callus
Orange	0.66	Dark yellow	2	
Dark yellow	0.72	Yellow	3	
Blue	0.81	Orange	4	
Red	0.46	red	1	Fruits
yellow	0.36	orange	2	
yellow	0.56	yellow	3	
Orange	0.75	(blue)	4	
Blue	0.80	Orange	5	



Fig(3): Figure TLC of Lycopene from tomato fruits and callus A) Puree Lycopene: B) Tomato fruits: (C) Callus.

Table (2) and Fig (2A) shows the characteristics of puree Lycopene recorded only one spot referred to Lycopene stander with R_f equal to (0.45), Fig(2B) with same table shows the characteristics of Lycopene pigment that found in crude extract of callus tissue grown in MS medium with 20% PEG in order to concenter as the pest treatments for Lycopene production under drought stress revealed that contain four spots with relative flow (R_f) equal to 0.46 (red color), 0.66 (orange), 0.72 (yellow) and 0.81 (Blue). While Fig (2C) indicate that , fruits extract gave five spots with relative flow (R_f) equal to 0.46 (red color), 0.36 (orange) 0.56 (yellow) 0.75 (Orange) and 0.81 (Blue) respectively .The results were documented and used for the comparison of the obtained profiles with the R_f value of Lycopene standard gave red ($R_f=0.46$). Spots had a R_f value of 0.46 refers to Lycopene pigment and spot with 0.81 refers to Catachin compounds and R_f value 0.72 represent of β -carotene (18). This results indicates that the tomato crude extracts contained Lycopene products were detected by UV 366 wavelength. In almost all the investigated samples the presence of band corresponding to identical compounds was indicated with an arrow tomato plant. This results obtained from this study was agrees to our study of several researchers use TLC chromatography technique to separation of deferent type of phytochemical from medicinal plant *in vitro* or *in vivo* (28); (18); (54), (79).

Proline and total sugar contents and its Correlation with Lycopene production under drought Stress .

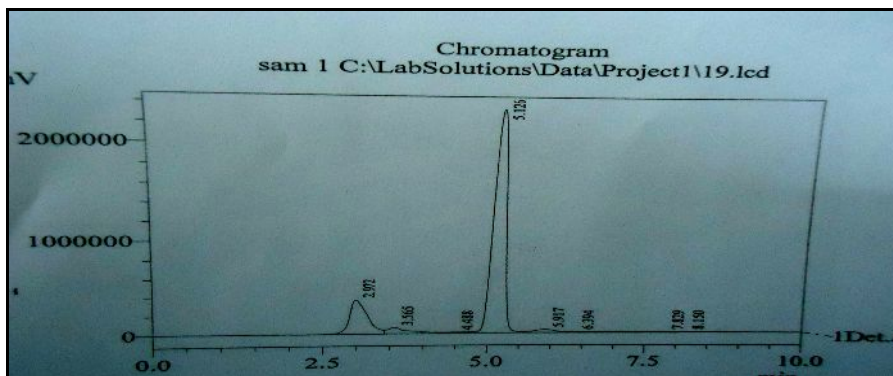
Drought stress had a significant effect on proline and total soluble sugar content causes enhancement of proline biosynthesis pathways and increased of starch conversion to sugar (34). Data present in table(3) indicate that ,the lowers proline content was found in callus grown in MS medium with drought treatment (5%) was about(3.43 mg/ g DW) in comparison with all PEG treatment and increased significantly in compare with callus grown in MS medium without PEG(1.20 mg/ g DW), while the higher value was observed in (20%) drought treatment(11.51 mg/ g DW) as a result in than those in (5,15 and 30 % PEG about (3.43,6.56 and 2.45 mg/ g DW) respectively at the stressful treatments .Also , result in the same table reveled that total soluble sugars content in callus grown in all PEG treatment significantly increased under drought condition except 30% was found as (4.34 mg/ g DW) which significantly decrease , however soluble sugar levels in callus culture medium with 20% PEG was found as (14.55 mg/ g DW) was more than control treatment and with fruit sugar content was found as (5.19 mg/ g DW). Also, the lowest content was found in callus grown in high PEG level 30% about (4.34 mg /g DW).However, Soluble sugar contents in callus grown in MS medium without PEG significantly higher compared to fruits contents . Callus showed higher amount of osmolytic content than tomato Fruit. Accumulation of free proline and total soluble sugar in response to different environmental stresses seems to be wide-spread among plants (14); (98). Proline can protect plants from stress through different mechanisms, including osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of proteins/enzymes (72). Proline and total soluble sugar content was higher in callus tissue than tomato fruits have showed positive correlation with Lycopene productions in callus tissues compare with fruits plants species as shown in (Table 3). Conclusively, the level of drought treatments in callus medium showed considerable effect on total carbohydrate and Lycopene concentrations accumulations. Absorbance peak of callus sample with retention time which closed to retention time to Lycopene standard sample Fig(4). The maximum Lycopene concentrations was recorded at 20% PEG was found as (8.86 mg/ g DW) in callus culture medium significantly increased under drought stress. In this study, Lycopene content significantly increased with increased of PEG level in callus medium except 30% was found as (4.12 mg/ g DW) which significantly decrease , however PEG levels in callus culture medium with 20% PEG was found increased of lycopene content more than control treatment and with fruit lycopene content in 5% and 15% PEG level was found as (8.86 and 14.32 mg/ g DW) respectively. Also, the lowest content was found in callus grown in MS medium without PEG level about (5.81mg /g DW) significantly higher in compare with lycopene content in tomato fruit was found as (6.88 mg/ g DW) . However, the higher content of Lycopene was found in callus grown in MS medium with 20% PEG level about (19.54 mg /g DW) and significantly higher in compare with lycopene content in tomato fruit was found as (6.88 mg/ g DW) .Sugars modulate many vital processes that are also controlled by hormones during plant growth and development.(57).Accumulation of proline and carbohydrate was investigated in callus tissue with all PEG treatment plants, synthesize and accumulated compatible solutes such sugar amino acids, glycine betaine in response to osmotic stress (20) They do not affected normal metabolism and accumulate predominantly in the cytoplasm at high concentrations under osmotic stress (reviewed in 27).Also, they have adaptive value of its the metabolic pathways(37) and involved in scavenging reactive oxygen species (27) These molecules may have a primary role of turgor maintenance but they may also be involved in stabilizing proteins and cell structures (73). The increasing of sugar content in drought condition could be contributed to decreasing of callus water content followed by maintain of plant

water potential (71) and increasing of osmolytic content and different elements accumulation(30) , as well as which plays an important role in maintain growth rate and secondary metabolite production such as Lycopene pigment. The suggestion that compatible solutes contribute to the detoxification of reactive oxygen species (29).

(17) observed that six genotypes of tomato differed for their responses to abiotic stress factors . Overall, the growth and most of biochemical parameters decreased with PEG concentrations, increasing, the content of chlorophyll *a* and *b*, content of carotenoid and proline in plants.

Accumulation of proline in response to excess PEG levels has been observed in several plants (73). Due to this study, the content of lycopene found in callus tissues at all levels of PEG used in callus medium was chosen based on our previous report (79) which indicate that callus line grown on different concentrations of PEG concenter as better lycopene content than callus when grown on medium without PEG. It might be due to high concentration of proline content in callus.

(5) Show that Lycopene content in callus tomato has been reported to vary with concentration and combination of hormone in MS medium , light, incubation, genotypes and explants used. Various studies of Lycopene pigments enhancement reported in plant tissues technique . Proline is synthesized from glutamate and proline has been found to serve as a substrate for respiration and as a source of nitrogen and other metabolites (95) ,(101). The role of proline in adaptation and survival of plants had been observed by (103) and (89) as a solute that protects macromolecules against denaturation and as a means of reducing acid it in the cell(50). A complex essential role of soluble sugars in plant metabolism is well known as products of hydrolytic processes, substrates in biosynthesis processes, energy production .Other studies show that the free proline content and soluble sugars can be used as drought tolerance indicators for selecting drought resistant genotypes.



Fig(4) Peak of standard of Lycopene pigment by HPLC

Antioxidant Enzymes activity and its Correlation with Lycopene production under drought Stress .

A significant difference ($p < 0.05$) was observed between all drought stress treatments and antioxidant enzymes in tomato callus tissues. Results revealed that an increase in drought stress on tomato callus culture , there was a increased in antioxidant enzymes activity and increased of Lycopene accumulation.

Superoxide dismutase activity

In this study ,data in table (4) indicate that the callus induced from tomato shoot tip grown in MS medium without PEG treatment of drought stress significantly increased in superoxide dismutase activity in comparison with control plant fruits. PEG at level of (5,15 and 20%) increased there activity of SOD enzyme as was (38.13, 42.45 and 44.55%) respectively except in treatment of 30% was recorded decreased of SOD enzyme activity was about 20.23% . Callus when treated with PEG as result of environmental stress factors, ROS levels can dramatically increase with increase of PEG level in culture medium and this increase in the later stage, leads to oxidative stress can cause damage to cellular macromolecules, including proteins, lipids, carbohydrates and DNA(35).

Table(3)Effect of PEG on Proline, Carbohydrate and Lycopene content in fruit and callus of tomato

Biochemical changes							PEG%
Lycopene content mg/g DW		Total soluble sugars mg/g DW		Proline content			
Average	callus	fruit	callus	fruit	callus	fruit	
6.08	5.81	6.36	5.19	6.22	1.20	0.89	0
-	8.86	-	7.65	-	3.43	-	5
-	14.32	-	9.45	-	6.56	-	15
-	19.54	-	14.55	-	11.51	-	20
-	4.12	-	4.34	-	2.45	-	30
Mean of level Biochemical changes =0.58	10.53	-	8.35	-	5.03	-	Average
	L.S.D. (0.05)						Mean of Drought levels =0.69

Catalase enzyme activity

Drought -induced reactive oxygen species have devastating global effects in plants are manipulates by a number of antioxidant enzymes. There were different results show in this study between callus culture under different drought stress levels and callus grown in culture medium without drought treatment in catalase enzyme activities, catalase enzyme activities decreased gradually with increased of PEG in callus medium and the minimums catalase enzyme activities occurred in callus with respect to 30 % drought stress.

Peroxidase activity

Results in this study it was observed that POX enzyme activity in callus culture medium significant increased in callus with all treatments of PEG. The maximum stimulation of its enzymes activity was observed when callus of tomato were exposed to 20% drought treatment and minimum activity was observed when callus of tomato were exposed to 30% PEG in culture medium (Table 4). However, peak inhibition of peroxidase activity was observed when callus tissue were exposed to high level of drought stress.

Antioxidant Enzymes activity and its Correlation with Lycopene production under drought Stress

Environmental stresses, including drought produce reactive oxygen species in callus tissue are extremely injury to cells at high concentrations and it subjected to "oxidative stress." Fig(4) reveled that drought stress significantly increases Lycopene contents in callus tissues in comparison with control tomato fruits plants. Level of PEG (20%) in callus culture medium caused significantly increased in lycopene content in comparison with(15 and 5%) PEG about(19.54, 14.32and 8.86 mg/g DW) respectively. Over all drought conditions, the content of lycopene pigment were nearly positive effect in response to increased of PEG levels in callus culture medium. Drought stress affected lycopene content-active pigment of the callus tomato plant with positive increased of antioxidant enzymes activity such as POX, SOD and CAT. The Drought stress at levels of (5%) in the callus growth medium enhanced the catalase enzyme activity and lycopene content significantly increased up to (1) fold with callus grown in culture medium without drought treatment and about (1.5) fold in comparison with lycopene content in tomato fruit extracts. Also, in callus grown in medium with

15% PEG (Fig4) it increases up to (2) fold increase in Lycopene content was observed in the callus grown with 5% and (4) fold increases in lycopene content were found in 20 % in comparison with 30% PEG .

The enhanced production of reactive oxygen species during environmental stresses can adversely affect the cellular activities by causing the oxidation of proteins, peroxidation of lipids, and preventing the activity of enzymes, which eventually results in cellular deactivation (85). Plants have both enzymatic and nonenzymatic mechanisms for scavenging reactive oxygen species. The enzymatic antioxidants include (SOD), (CAT), (GPX), phenolics, and carotenoid, which act as potent nonenzymic antioxidant inside the cell (96). Several researches has shown that the treatment of drought stress on tomato plants can enhance lycopene and potentially other antioxidant concentrations in fruits. The increase in lycopene in response to stress in the tomato fruits varied from 20% to 80%. evidence suggests that increasing antioxidant concentrations is a primary physiological response of the plant to drought stress. Additionally drought stress during cultivation increased the antioxidant capacity of tomato fruit while maintaining the lycopene concentration (10). Effect of PEG in MS medium with different levels on growth , Osmolytes and accumulation and antioxidant activity of callus tomato is reported by (79), (75), (69).

Increase the antioxidant enzymes capacity may enhance the callus tolerance to drought stresses thus increasing the survival rate and increase lycopene accumulation. lycopene are antioxidant substances accumulated in the chloroplast and protect the physiological function when a callus is subjected to stress, by scavenging the excessively free radicals (51). Also, it has been reported that the kinetics of lycopene accumulation in callus tissue reveals a stress-specific pattern of accumulation that is consistent with a physiological role under drought stress . Generally, drought stress increase free radicals level in callus tissues lycopene is strongly involved in improving nutrient assimilation and in stimulating the anti-oxidative defense system. To enhance lycopene production from callus induced from tomato shoot tip *in vitro* cultures were treated with PEG in culture medium during the culture incubation, or in combination of auxin (2,4-D) and cytokinin(BA) to produce varying ratios of Lycopene pigments in callus tissues. Plant tissue culture techniques are the more advantage methods to studying and observing morphological, physiological and biochemical changes in callus cultures and organized tissue (i.e. shoot tip, mature embryo, parent plant) levels (75).

Comparison of Lycopene production from callus Cultures in different media drought stress and fruit mother plant

Plant growth hormone (PGRs) in callus culture medium are also responsible for the increase of secondary metabolite production (48). In *L. escoletume* plant the combination of 2,4-D and BA had greater influence at initial stage of Callus growth and Lycopene production. A low concentration of 2,4-D in MS medium may help to reduce the accumulation of Lycopene and exhibited a negative effect on callus growth. In this study, MS medium supplemented with 2,4-D and BA was used for successful establishment of shoot tip culture showed responses in producing callus when cultured *in vitro* was found very effective. The ability to control cell expansion was noted to be accomplished by association with polysaccharides and corresponding hydrolytic enzymes of the cell wall. It was also observed that among auxins (2, 4-D, NAA and IAA) tested 2, 4-D was very effective for callus induction, this is due to the reason that 2, 4-D is much more stable and less inactivated during culture processes than the other auxins (86). Callus culture accumulated the greatest amount of Lycopene pigment with PEG than callus treated without PEG had greater overall lycopene accumulation than the tomato fruits . The results in this study support the finding that callus tissue under drought stress had the potential to achieved many physiological and biochemical changes such as increased of antioxidant enzyme activity and increased of osmolytic solutes including proline amino acid and total sugar content in responses to drought stress that normally lead to processes including Lycopene accumulation. Callus induced from shoot tip grown in drought stress conditions exhibits more tolerance to stress. However, the tomato mother plant did not show this same trend, suggesting that there may be to a genotype of tomato is the most sensitive and critical stage due to the drought stress depending on genotype and environmental conditions (77). (33) suggested that nine genetic loci known within tomatoes that control fruit pigmentation in *Solanum lycopersicum* previously reported to have enhanced lycopene production under stress conditions. (19) suggested that several enzymes modulated and can be used to induce or enhance secondary metabolite production and accumulation *in vitro* cultures . The antioxidant enzyme system activity was in significantly positive correlation with lycopene contents, suggesting a strong sensitive of the cell redox system and activation of defense responses under drought stress in *L. escoletume* shoot tip cultures. However, Soluble sugar content was high during callus grown in stress condition , this enhanced level was reported to be developed by the over-uptake of sugar from

the medium. The activities of POD, SOD, and CAT increased significantly in callus tissue after treatment with different levels of PEG are usually used to evaluate physiological and biochemical responses of plants to biotic and abiotic stresses. and in studies of plant systemic acquired resistance system and prevented oxidative injury of membrane and enhanced oxidative stress tolerance in plants. These compatible solutes can accumulate to high levels and mitigating oxidative damage caused by free radicals, and maintaining the enzyme activities under stress (106). (48) reports that induced synthesis of triterpenoids/other secondary metabolites in cell and tissue cultures of plants like *Daucus carota* L.

Table(4) Effect of PEG on Antioxidant enzyme activity and Lycopene content in fruit and callus of tomato

Biochemical changes									PEG%
Lycopene content			Antioxidant enzyme activity						
			CA		SOD		POX		
Average	Callus	fruit	Callus	fruit	Callus	fruit	Callus	fruit	
5.08	5.81	4.36	3.47	3.21	35.41	28.55	26.63	25.99	0
-	8.86	-	3.93	-	38.13	-	28.32	-	5
-	14.32	-	4.67	-	42.45	-	33.16	-	15
-	19.54	-	5.90	-	44.55	-	35.12	-	20
-	4.12	-	0.32	-	20.23	-	10.02	-	30
Mean of level Biochemical changes=0.98	10.53	-	3.60	-	36.15	-	26.71	-	Average
	L.S.D. (0.05)								Mean of Drought levels =0.69

Recently plants treated with bio-regulators and antioxidants in order to alleviate the biotic and abiotic stresses(42).However, The essential oils represent valuable sources for active molecules natural antioxidant(6). (7) observed that total phenolic content of *N. sativa* essential oil revealed that a prominent antioxidant activity.

Finally, (64) found that treatment of cotton plants grown under drought stress with ascorbic acid (ASC), glutathione (GSH) and/or α -tocopherol (α -TOC)and their interaction caused enhancement of growth and yield characters and increased pigments content, total soluble sugars, total carbohydrates and total free amino acids. Finally, it can be concluded that foliar application of ASC, GSH, α -TOC and their interaction improved the drought tolerance of cotton plants.

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