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The Effect Of 2,4-D And Kinetin On Trigonella Foenum-Graecum L. Callus Using Hypocotyl Segments

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Abstract

The present study aimed to induce callus from fenugreek plant Trigonella foenum-graecum L. in vitro on (Murashige and Skoog) MS medium after supplying with different combinations of auxin (2,4 Dichlorophenoxy acetic acid) at concentrations (1,2,3) mg/L and cytokinin(kinetin) at concentrations (1,2,3) mg/L. The results showed that kinetin 2mg/L +2,4-D 2mg/L was the best combination which gave the highest fresh weight of callus compared with other treatments.

The results also showed that enzymatic activity of CAT and SOD increased by increasing the concentrations of hormones . The highest catalase activity was at kinetin concentrations and the highest value of SOD was at 2,4-D concentrations . It was also found that the proline concentrations in callus increased by increasing the concentrations of auxin and cytokinin .

Introduction

Fenugreek, **Trigonella foenum-groecum L** is an annual herb indigenous to the countries bordering on the eastern shores of the Mediterranean and largely cultivated in India, Egypt and Morocco. Scientists have reported several medicinal uses of fenugreek seeds such as remedies for diabetes and hypercholesterolemia ,hepatoprotective protection against free radicals, and protection against breast and colon cancer (Al-Oqail et al., 2013). The seeds are considered useful in heart disease and aphrodisiac and as a galactogogue promoting lactation (Tiran, 2003). ” Importance of tissue culture In a relatively short time period and space a large number of plantlets can be prepared starting from the single explant. It is easy to select popular traits directly from the culture setup (in-vitro) thereby decreasing the amount of space required, for field trials. In vitro growing plants usually free, from the bacterial and fungal diseases. Virus eradication and maintenance of plants in virus free state. This facilitates movement of plant across international boundaries. Plant tissue banks can be frozen and then renewed through tissue culture. It preserves the pollen and cell collections from which plants may be propagated (Rohan et al ., 2019) Callus induction Exogenous

application of auxin and cytokinin induces callus in various plant species. Generally, speaking, an intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively. Since the discovery of this regeneration system, it has been widely used, for example, in the propagation of economically important traits and the introduction oftransgenes. However, auxin and cytokinin have been by far the mostextensively used and studied hormones in the context of callus formation and subsequent organ regeneration (Ikeuchi et al, 2013)2,4-D (2,4-dichlorophenoxyacetic acid) is a synthetic auxin and is the most commonly used growth regulator in cereal tissueculture (Maliket al ., 2004) Although 2,4-D has been found to elicit rapid cell proliferation and callus formation, the reduction or removal of 2,4-D from the regeneration medium is essential for plant development from callus (ZhengandKonzak, 1999) .Kinetin, one of the commonly used cytokinins in plant tissue culture, has important effects on callusdevelopmentandregenerationIt appeared that a certain concentration of kinetin in the callus initiation medium was essential for callus to regenerate plants after transfer to a hormone-free regeneration medium Some studies showed that kinetin enhanced callus proliferation and regeneration by influencing mitosis, cytokinesis, total protein synthesis, lignin biosynthesis, vascular differentiation, the differentiation of maturechloroplastsfromprotoplastids,etc.(Wan,1988)

Materials and methods :

Preparation of Seeds Culture Medium :

Murashige and Skoog (MS) medium free form plant growth regulators were used in this experiment to culturing seeds of **Trigonella foenum graecum L.**Melt 4.41 g (one pack) of MS medium in a certain amount of distilled water and add a sugar at concentration of 30 g/l. The solution was placed on hot plate stirrer for 5 minutes to insure full dissolving of sugar and medium components. Medium was adjusted to pH 5.8 with 0.1N NaOH or HCl before autoclaving and solidified with 0.7% agar, then complete the final volume to 1 liter of distilled water. Medium was heated up to boiling to melt the agar. When the solution became transparent about 15 ml medium per culture tubes was dispersed into sterile culture tubes. The culture tubes containing medium were autoclaved at 121°C and pressure at 15 lb/sq inch for 15 min. After cooling down tubes were taken out and kept in culture room .

Preparation ofTrigonella foenum graecum L.

Seeds were collected from the Babylon province market and were diagnosed in the lush College of Science, University of Babylon.

Seeds sterilization :

A suitable amount of seeds were placed in flask and washed with distilled water three times to eliminate dust and suspended solids and sterilized by immersing them in the solution of 2% sodium hypochlorite and shake for 15 minutes. Washed with sterile distilled water for one minute at three times, and then added 70% ethyl alcohol and Shake for one minute and then washed with sterile distilled water for one minute at three times to eliminated of ethyl alcohol, then the seeds were placed in a glass petri dish containing sterile filter paper in order to absorb stuck water (Awika and Rooney, 2004).

Preparation of growth regulators solutions :

A suitable basic solution for all plant growth regulators used in the experiments was prepared by dissolving 50 mg of 2,4-D in 1 ml absolute ethyl alcohol to ensure the full dissolving and complete volume to 50 ml with distilled water to become our fundamental solution concentration of 1 mg/ml, while benzyl adenine (kinetin) prepared by dissolving 50 mg of kinetin in 1 ml HCl to ensure the full dissolving and complete volume to 50 ml with distilled water to become our fundamental solution concentration of 1 mg/ml.

Growth Regulator 2,4-D

Plant tissue culture technique was used according to the system of Hirata et al (1990) for the purpose of callus induction from hypocotyl of **Trigonella** callus were cultured in fresh medium for the purpose to study some of the vital signs of the plant such as; catalase enzyme activity, proline content and superoxide dismutase. hypocotyl were cultured on MS supplemented with different concentrations of 2,4-D (1, 2 and 3 mg/l) and by one vegetarian portion of each tube at a rate of 12 replicates for each concentration of 2,4-D for the purpose of callus induction. Tubes were incubated in a growth chamber at a temperature of 25 ± 1°C in the dark.

Determination of Callus Fresh weight (g) : After 15 days of hypocotyl cultivation in MS medium, callus started to emerge and after eight weeks the callus growth was harvested. Where fresh weight of callus induced from the seeds was calculated for all concentrations of 2,4-D used in experiment. 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 and placed on blotting paper.

Determination of Callus dry weight (mg) : Fresh callus was placed in a glass Petri dishes by two dishes each containing 6 pieces of callus tissue for each concentration of 2,4- D, Callus dry weights were determined after dried in oven at 40°C for 24 hours and then get out and calculated.

Accumulation phase:

Growth Regulator Kinetin : After a period of 8 weeks of cultivation of **Trigonella** hypocotyl were selected developing callus on MS medium containing 2 mg / l 2,4- D, which represents the best concentration for the induction of callus in terms of fresh and dry weight. Callus cut into parts up to 250 mg and sub-cultured again in same MS media containing growth regulators 2,4-D at concentration of 2 mg/l and kinetin at concentrations of (1, 2 and 3 mg/L) with same growth conditions. Callus cultured at 12 replicates for each concentration of kinetin, taking into account the cutting callus process and weighted using sterilized scalpels and sensitive balance device sterilized with absolute ethyl alcohol and the overall operation carried out inside laminar air flow device and tubes were incubated in the incubator at a temperature of 25°C±1°C in the dark.

Callus fresh weight (g) determination : Callus fresh weight was measured for all concentrations of kinetin added to the MS medium that was mentioned in the previous step to identify the impact of kinetin in the developing callus vital in the growth medium.

Callus dry weight (mg) determination : Followed the same steps mentioned in determination of callus dry weight (mg).

Estimation of Catalase Enzyme Activity

Catalase enzyme activity was estimated by the method mentioned by Aebi (1974), where this method depends on the amount of change in absorbance at 240nm wavelength of hydrogen peroxide solution (30mM) and phosphate buffer solution (50mM) at pH= 7.

Preparation of Solutions: phosphate buffer solution (50mM) at pH= 7

Solution A: Prepared by dissolving 0.871 gm of $K_2 HPO_4$ in a little volume of distilled water and then complete the volume to 100 ml distilled water.

Solution B: Prepared by dissolving 0.6804 gm of $KH_2 PO_4$ in a little volume of distilled water and then complete the volume to 100 ml distilled water. To get a phosphate buffer solution (50mM at pH=7). Added a given volume of solution B to (50 ml) of A solution until reaching pH value to 7.

2-Hydrogen peroxide solution H2O2 at a concentration of 30 mM Prepared by adding 0.34 ml of 30% $H_2 O_2$ in the volume of phosphate buffer solution at a concentration of 50mM at pH=7 and then completed the volume to 100 ml of phosphate buffer solution.

Method

Crush 0.5 g of callus fresh weight with 5 ml phosphate buffer solution and added 0.15 g Polyvinylpyrrolidone during the crushing using ceramic mortar above the amount of ice for 10-15 minutes, then filtered the extract through two layers of gauze and then took the filter and centrifuged at 10000 r/min for 10 minutes at 4 ○C . Took 2 ml phosphate buffer solution at a concentration of 50 mM and PH = 7 then add 2 ml of hydrogen peroxide solution $H_2 O_2$ at a concentration of 30 mM and 40 micro letters of enzymatic extract, the volume of the interaction shall be 4.04 ml. incubated at 25 ˚C for one minute then read the absorbance at 240 nm wavelength, were observed the absorbance decrease with time with the use of blank solution that composed of the same materials with the replacement material foundation by the phosphate buffer solution. Calculation the activity of catalase enzyme by the following equation:

$$\text{catalase activity(unit)} = \frac{\Delta\,\text{bs/min} \times \text{reaction volume}}{0.01}$$

Where: Δbs= absorbance difference within a minute 0.01 = constant, Min = reaction time , 4.04 ml = reaction volume

Estimation of Proline Content

According to Bates et al. (1973) method is used to measure the amount of proline, through the addition of 0.1 ml of callus extract with 2 ml of ninhydrin acid and glacial acetic acid, and then read the absorbance at a wavelength of 520nm.

1-Preparation of ninhydrin acid solution:

Prepared by dissolving 1.25g ninhydrin acid in 30 ml glacial acetic acid and 15 ml of distilled water with 5 ml of orthophosphoric acid in a concentration of 6M.

2-A sulfosalicylic acid solution at a concentration of 3%:

Prepared by dissolving 30 gm of Sulfosalicylic acid material in the amount of distilled water then completed the volume to one liter with distilled water.

Method

Took 0.5 g of fresh callus sample and added 5 ml of a 3% sulfosalicylic acid and crush well using mortar casserole even the mixture homogeneity. Centrifuged by centrifuge devise at 2000 r/min for 10 minutes, Added 2ml of ninhydrin acid to 2ml of glacial acetic acid and 100 microliters of filtrate and placed in glass test tubes and heated in a water bath at a temperature of 100 ° C for 30 minutes and leave to cool and then added to it 1 ml of toluene with shaking for 20 seconds. Estimated the amount of proline by measuring red toluene layer by a spectrophotometer at a wavelength of 520 nm and used 3 replicates per treatment and compared with the standard curve of proline, while blank solution composed of 5 ml of toluene.

Proline Standard Curve

Standard solution of proline prepared at concentration 10µg/ml by dissolving 0.01gm of standard proline in a small amount of distilled water and then completes the volume to one liter using distilled water. Prepared graded concentrations of proline from standard solution of proline (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4) µg/ml. Then added 2 ml of each of glacial acetic acid and ninhydrin acid solution to 100 microliters of standard solution and blending the mixture well and incubated tubes in a water bath at a temperature of 100 ° C for 30 minutes and then cooled and added 5 ml of toluene and then read absorbance by spectrophotometer at 520 nm. The last tube was used to reset the spectrophotometer device and recorded data to draw a standard curve by represent the concentrations of proline on the x-axis and absorbance on the y-axis, then calculate the amount of proline in samples by using the proline standard curve through the followed equation:

$$\mu \text{ moles per gm fresh weight} = \frac{\text{proline } \mu\text{g/ml} \times 5 \text{ ml toluene}}{115.5 \times \text{G sample}}$$

G sample = weight of sample

115.5 = constant

5 ml toluene: volume of toluene

proline µg/ml : the concentration of proline

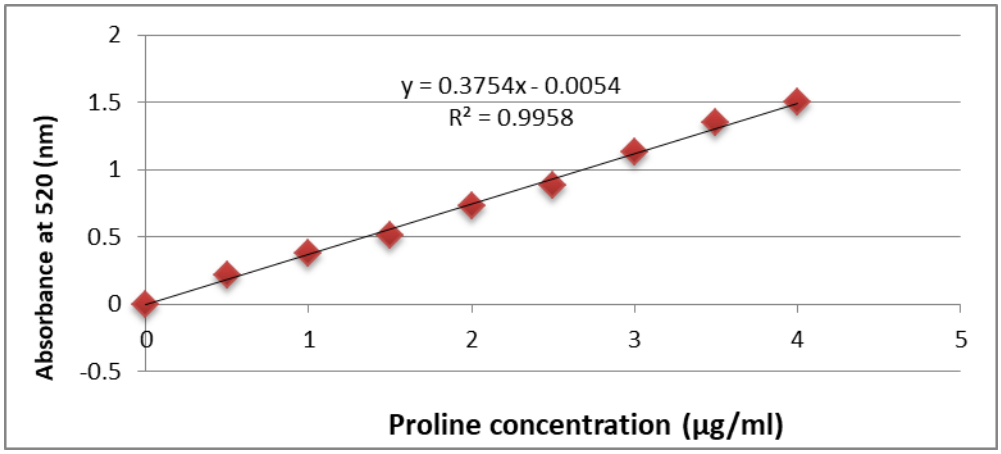

Figure (1) Proline standard curve at 520 nm wavelength

Estimation of superoxide dismutase (SOD) activity

The efficacy of the SOD enzyme indicated by Marklund and Marklund (1974) was estimated as the interaction mixture of 50 ml of the extraction solution added to 2 ml of tris-buffer solution and 0.5 ml of pyragallol solution (0.2 mM) This solution absorbs light at a wavelength of 420 nm

Preparation of solutions for estimation of SOD activity

1. The solution of phosphate buffer solution (pH = 7.2-7.4) prepares gaseous (1.1 g) Na_2HPO_4 and 0.27 g of KH_2PO_4 in 100 ml of distilled water.

2. Tris buffer pH = 8.2 (0.288 g) of tris and 0.111g of EDTA in 100 ml of distilled water.

3. pyragallol solution: Prepared by dissolving 0.252 g of pyragallol in 10 microliters of HCl in 100 ml distilled water.

Method

0.5 g of callus were milled and mixed with 5 ml of the phosphate buffer (pH = 7.2-7.4) using ceramic mortar above the amount of ice for 10-15 minutes and filtered through gauze cloth and centrifugal deposition at 10,000 rpm for 15 min (4 C˚), then take 50 µl of the extract plus 2 ml of the tris buffer solution and 0.5 ml of pyragallol solution for the test solution, then compare with the change in absorption of the control solution containing distilled water instead of the enzyme. Distilled water was a blank solution. One unit of the unit (U) is defined as the amount of enzyme capable of inhibition of pyragallol oxidation by 50%. The following equation determinate the enzyme efficacy:

SOD activity was calculated using the following equation

$$\text{SOD Activity (units)} = \frac{\frac{\%\textbf{inhibition of pyragallol reduction}}{50\%} \times \textbf{reaction volume}}{\textbf{total test period (10min)}}$$

So:

Inhibitory of pyragallol reduction % = percentage of pyragallol reduction inhibition.

Reaction volume = abstract size.

Total test period (10 min) =total reaction period 10 min.

Results and Discussion :

Callus Induction Phase

Different levels of growth regulator 2,4-D (1, 2, and 3) mg/l were studied in callus induction from the hypocotyl of Trigonella. The results in Table (1) and Figure (1) showed the significant effect of 2,4-D concentrations added to the MS medium in the callus fresh weight, where the 2,4-D with concentration (2 mg/l) gave a higher fresh and dry weight (1.563 gm) and (147mg) respectively . The lowest fresh and dry weight was found in the concentration (3 mg/l) of 2,4-D (0.917 g) and (89 mg) respectively . Data in Table (4.1) also indicate to decrease in fresh and dry weight of callus induced a concentration of (1mg/l) 2,4-D (1.214 gm) and (89mg) respectively .

The development of callus from immature hypocotyl explants is directly related to the presence of 2,4-D which is a suitable growth hormone responsible for callus induction in most plant Species in plant tissue culture work. This is similar to the findings of Mamun et al. (2004) and Baskaran et al. (2005). The production of a yellowish, compact and nodular callus at cut edge of explant may be due to the wound caused during the process of cutting which resulted in a synchronous cell division**.**(Tahiret al ., 2011) .

Table (1): Effect of the 2,4-D hormone onthe fresh and dry weight of callus of Trigonella foenum-graecum L.

2,4- D (mg/l)	Fresh weight(gm) (Mean ± SD)	Dry weight (mg) (Mean ± SD)
1	1.214 ± 0.551	112±53.014
2	1.563 ± 0.667	147±65.873

3	**0.917 ± 0.344**	**89±34.488**
L.S.D. (0.05)	**0.609**	**38.433**

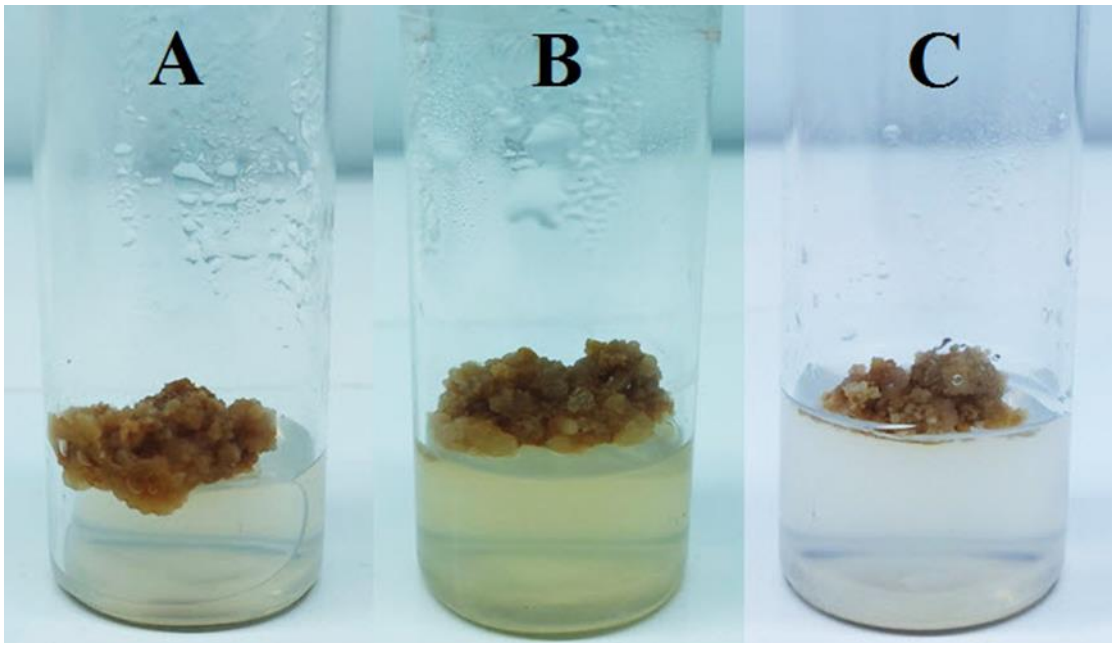

Figure (1) Callus of Trigonella foenum-graecum L. grown in test tube containing MS medium with different concentration of 2,4-D incubated at 25 C˚± 1 C˚ for eight weeks, (A): 1 mg/l 2,4-D (B): 2 mg/l 2,4-D (C): 3 mg/l 2,4-D

Accumulation Phase

The kinetin growth regulator was added to callus culture at concentrations of (1, 2, and 3) mg/l to study the effect of cytokinin (kinetin) in the growth of callus tissues induced from hypocotyl of **Trigonella**by using (2mg/l) 2,4-D. Results in the Table (2) and Figure (2) Indicate the presence of the significant effect of kinetin concentrations on fresh and dry weight of callus tissues, where the concentration (2mg/l) of kinetin + (2mg/l) of 2,4-D added to culture medium gave the higher fresh and dry weight of callus (1.385g) and (134.133mg) respectively. while the less fresh and dry weight was recorded with (3mg/l) kinetin+ (2mg/l) 2,4-D (0.718g) and (68mg) respectively while the treatment with (1mg/l) kinetin gave fresh and dry weight (1.035gm) and (68mg)respectively.2,4-D is a potent auxin stimulating callus induction , it is found to be more effective in association with kinetin, as their combination is essential for DNA synthesis and mitosis (Narasimhulu and Reddy, 1983) Well-grown, watery, granular, and greenish callus without necrosis developed in both auxin concentrations, in the presence of kinetin in hypocotyl. The initiation and the developmental stage of differentiation of the embryoids on the PGR-free medium were affected by the PGR

supplements in the preceding callus induction/maintenance medium. Larger number of the globular shaped embryoids and the heart or cotyledonary stage embryoids were found in cultures derived from callus induced with 2,4-D in the presence of kinetin(Tawfik and Noga, 2002) .

Table (2): Effect of 2,4-D and kinetin hormones on the fresh and dry weight of callus of Trigonella foenum-graecum L.

2,4- D (mg/l)	kinetin(mg/l)	Fresh weight (gm) (Mean ± SD)	Dry weight (mg) (Mean ± SD)
2	1	1.035 ± 0.544	92 ±52.082
	2	1.385 ± 0.926	134.133 ±92.679
	3	0.718 ± 0.433	68 ±42.183
L.S.D. (0.05)		0.492	48.660

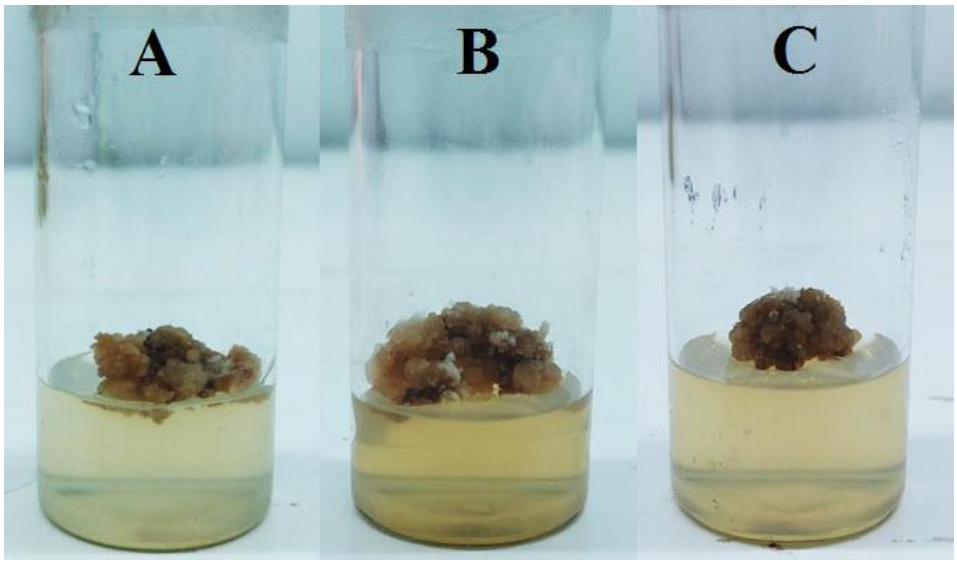

Figure (2) Callus of Trigonella foenum-graecum L. grown in test tubes containing MS medium with different concentration of kinetin incubated at 25C˚±1 C˚ for eight weeks, (A):1 mg/l kinetin +2mg/l 2,4-D (B): 2 mg/l kinetin+2mg/l 2,4-D (C): 3 mg/l kinetin+2mg/l 2,4-D

Effect of 2,4-D on proline , catalase and superoxide dismutase activity in callus of Trigonella foenum graecum L.

Table (3) shows that the different concentrations of 2,4-D significantly influenced the proline activity . Proline activity at higher concentrations (3mg/l) of 2,4-D was recorded maximum (0.284µ moles/g) as compared to (1mg/l) and (2mg/l) (0.135µ moles/g) (0.147µ moles/g) respectively. The level of catalase increased by 2,4-D, the catalase activity was increased in all three concentrations of 2,4-D Table (3) shows that catalase activity at 3mg/l of 2,4-D concentration (3.662u/mg) is elevated as compared with the treatment 1mg/l and 2mg/l (1.247u/mg) (1.626 u/mg) respectively, The data in Table (3) an increase of 2,4-D cause a significant increase in SOD activity and the higher activity of SOD recorded in (3mg/l) (0.206) unit/ml, whereas (1mg/l) gave less SOD activity was about (0.117) unit/ml. 2,4-D at (3 mg/l) is considered the best treatment correlation with SOD activity in callus tissue while (2mg/l) (0.135) unit/ml. Table 3shows that the different concentrations of 2,4-D significantly influenced the proline content. Thismay be due to induction of oxidative stress which has been shown to protect plantsagainst free radical induced damage **(Singh et al., 2006).** Proline synthesis from glutamatemight be an adaptive mechanism to reduce the accumulation of NADH during stresscondition **(Alia and Saradhi, 1991)**. And it has been proposed that proline accumulationcould play a role of redox buffer by storage of excess reductants in a non toxic form**(Bellinger and Laher, 1987)**The enzyme SOD activity increased as concentration of 2,4-D increases.Superoxide dismutase catalyses the disproportionation of superoxide anion O2 to molecularO2 and H2O2. SOD scavenges superoxide anion and hence decreases the risk of hydroxylradical formation from superoxide and protects the cell against oxidative stress **(Kuramaet al., 2002)**, simultaneously SOD is involved in H2O2 detoxification **(MitlIer, 2002).**thereby indicatingthe enhanced generation of reactive oxygen species (ROS) **(Singh et al., 2006).** Thissuggests that plant species use different enzymes as primary defense against H2O2 andthere may require different regulatory systems **(Mitller, 2002**). H2O2 is also convertedto water and oxygen by two electron dismutation of CAT in peroxisomes **(Willekens etal., 1997).**

Table (3) Effect of 2,4-D on proline , catalase and superoxide dismutase activity in callus of Trigonella foenum-graecum L.

2,4-D(mg/l)	Proline content µ mole/g (Mean ±SD)	Catalase activity Unit/ml (Mean ± SD)	Superoxide dismutase Unit/ml (Mean ± SD)
1	0.135± 0.030	1.247 ± 0.425	0.117± 0.033
2	0.147± 0.015	1.626 ±0.791	0.135± 0.028
3	0.284± 0.086	3.662 ±1.365	0.206± 0.026
L.S.D.(0.05)	0.107	1.885	0.059

Effect of 2,4-D and Kinetin on proline, catalase and superoxide dismutase activity in Trigonella foenum- graecum L.

Table (4) shows that the different concentrations of 2,4-D significantly influenced the proline activity . Proline activity at higher concentrations (2mg/l 2,4-D+3mg/l kinetin) was recorded maximum (0.316µ moles/g) as compared to (2mg/1 2,4-D+ 1mg/l kinetin) and (2mg/l 2,4-D+ 2mg/l Kinetin) (0.153µ moles/g) (0. 171µ moles/g) respectively. The level of catalase increased by kinetin, the catalase activity was increased in all three concentrations of kinetin Table (4) shows that catalase activity at 2mg/l 2,4-D+3mg/l of kinetin concentration (3.872unit/ml) is elevated as compared with the treatment 2 mg /l 2,4-D + 1mg/l kinetin and 2mg/l 2,4-D+ 2mg/l kinetin (1.542unit/ml) (1.965unit/ml) respectively, The data in Table (4) an increase of 2,4-D cause a significant increase in SOD activity and the higher activity of SOD recorded in (2mg/l 2,4-D +3mg/l kinetin) (0.185) unit/ml, whereas (2mg/l 2,4-D+1mg/l kinetin) gave less SOD activity was about (0.099) unit/ml.(2mg/l 2,4-D +3 mg/l kinetin) is considered the best treatment correlation with SOD activity in callus tissue while (2mg/l2,4-D+2mg/l kinetin) (0.111) unit/ml.A way of cytokinin to combat reactive oxygen species (ROS) is to increase the activity of antioxidant enzymes (Rashotte et al., 2014). One of the most important mechanisms exerted by higher plants under environmental-stress conditions is the accumulation of compatible solutes such as proline. The environmental stress induces an increase in proline concentration in plants. Proline accumulation under environmental stress may contribute to osmotic adjustment, protecting cell structure and function or may serve as metabolic or energetic reserve in plants (Vamilet al ., 2011). the results in this study may be justified by the level of catalase (CAT) present in peroxisomes, which is inefficient at removing low concentrations of H2O2 (Petit paly et al .,

1999) .He et al. (2005) observed that in hybrid corn exhibiting delayed senescence, an increase in cytokinin content is accompanied with an increase in SOD activity.

Table (4) Effect of 2,4-D and kinetin on proline, catalase and superoxide dismutase activity in callus of Trigonella foenum-graecum L.

2,4-D (mg/l)	Kinetin mg/l	Prolinecontent µmole/g (Mean ± SD)	Catalase activity Unit/ml (Mean ± SD)	Superoxide dismutase Unit/ml (Mean ± SD)
2	1	0.153± 0 .039	1.542±0.979	0.099 ± 0.013
	2	0.171± 0.045	1.965±0.871	0.111 ± 0.029
	3	0.316± 0.057	3.872±0.880	0.185 ± 0.051
L.S.D.(0.05)		0.095	1.821	0.070

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