



## EVALUATION OF WATER STRESS EFFECT ON SOME CHEMICAL PROPERTIES OF SEVERAL SUNFLOWER VARIETIES CALLUS TREATED WITH COLOCYNTH FRUIT EXTRACT

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### Abstract

The study was carried out in plant tissue culture laboratory, University of Baghdad during the period 2017-2019, as factorial experiment in complete randomized design, to study the effect of PEG at (0, 2, 4, 6 and 8%) on physiological and chemical changes in callus of three sunflower (Ishaqi 1, Aqmar and Al-haja) induced by the cultivation of the young stem in vitro under water stress. The content of callus cells of SOD, POD, CAT and APX enzymes as well as total dissolved carbohydrate were determined as indicators to determine the effect of PEG in callus tissue cells cultivated on medium equipped with the PEG concentrations. The results showed that cultivars were differs significantly, and A-haja variety was superior in increasing SOD to 12.26 absorption unit  $\text{mg}^{-1}$  protein, and the variety Ishaqi was superior in increasing POD, CAT and APX to 7.25, 11.54 and 0.4285 absorption unit  $\text{mg}^{-1}$  protein respectively. Aqmar variety was superior in increasing the total dissolved carbohydrate content to 0.34980  $\text{mg}^{-1}$ . PEG concentrations significantly affected enzyme activity, and 6% gave the highest efficacy of SOD and APX reached to 13.78 and 0.2976 absorption unit  $\text{mg}^{-1}$  protein respectively, while 8% PEG gave the highest effective of POD and CAT reached to 5.54 and 7.73 absorption unit  $\text{mg}^{-1}$  protein respectively. The total dissolved carbohydrate content achieved the highest mean at 8% PEG and reached to 0.31,128  $\text{mg.g}^{-1}$ . The interaction caused significant effect and the highest SOD enzyme was obtained from Al-haja variety at 6% PEG, while POD, CAT, and APX enzymes (11.33, 16.30, and 0.5208 unit absorption  $\text{mg}^{-1}$  protein) were obtained with Ishaqi 1 variety at the concentrations of 2, 8 and 8% PEG respectively. Aqmar variety at 8% PEG gave the highest average of total dissolved carbohydrate content reached to 0.35357  $\text{mg g}^{-1}$ . It was appeared that (by using plant tissue culture technique) Ishaqi 1 was superior in increasing the effectiveness of POD, CAT and APX enzymes compared to other two varieties, which was important indicator to increase plant water stress tolerance.

**Keywords :** Sunflower, callus, colocynth, Ishaqi, Al-haja, Aqmar.

### Introduction

Sunflower is important crop as feeding and industrial crops, its oil is one of the best healthy vegetable oils for nutrition with high proportion of unsaturated fatty acids as well as its containing of vitamins that prevent the oxidation of oil. Plant tissues culture is a forerunner of the emergence of genetic modification and biotechnologies which uses in plant breeding. The researchers have contributed to the concept of plant tissue culture and its effective role in the conservation of genetic resources, micro-propagation, breeding and plant improvement (Anis, 2016). The world's climatic changes in arid and semi-arid regions, including Iraq, lead to loss large agricultural land area annually due to failure in managing water and sewage, which cause the problem of soil water logging and thus reduce the productivity of this field crops on the one hand, and the lack of water which began to unfold day after day in most world countries due to lack of water resources and mismanagement of water properly. World food production is based on the expansion of the cultivation of high-yielding varieties and its depended on traditional breeding methods as well as biotechnology, which depend on the existence of genetic variability and thus the starting point of any plant breeding and improvement program, including oil crops that haven't all genetic differences needed for varieties improvement. Mutations are one of breeding methods, but the natural mutations are few and difficult to rely mainly in plant breeding program (Al-Khayri, 2015), so using of colocynth fruit extract plays an active role in the possibility of producing and improving promising new varieties. Studies indicated that it is possible to use colocynth fruit extract in inducing genetic variations (Zaidan, 2017). This crop was

selected in this study and included in in-vitro breeding programs in order to obtain drought-tolerant genotypes under water stress in Iraq.

### Materials and Methods

The study was carried out in the plant tissue laboratory for postgraduate studies at the Faculty of Agriculture, University of Baghdad, during the period 2017-2019, in order to stimulate the genetic variations in callus of several sunflower varieties to drought tolerance using tissue culture technology. The dietary medium (Murashige and skoog, 1962) prepared by Himedia company was used at a rate of 4.91  $\text{g L}^{-1}$  and added sucrose and growth regulators (2,4-D at 1  $\text{mg L}^{-1}$  and BA at 0.5  $\text{mg L}^{-1}$ ) for sunflower varieties. The pH after the complete size to 1000 ml and then added agar 7  $\text{g L}^{-1}$ , then heating the mixture medium, then distributed in vials (10 ml) and sterile with sterilizer at 121 C and pressure 1.04  $\text{kg cm}^2$  for 15 minutes and left to cool and harden at room temperature and kept for use. The seeds were fertilized with 70% alcohol for 60 seconds and then submerged in 6% NaOCl (commercial minor solution) for 30 minutes. The seeds were then washed three times with distilled water for 3-5 minutes each time to remove the residual effect of the sterilized material. The seeds were then transferred to 250 mL sterile bottles and added 10 ml of distilled water to ensure that the seeds were soaked, then closed the bottles and incubated the seeds in the dark for 24 hours at  $25 \pm 2$  C to stimulate the embryo. The seeds were then placed in the center of MS media free of growth regulators. The seeds were incubated at  $25 \pm 2$  ° C for 10 days, 16 hours light and 8 hours of darkness.

### Treatment with colocynth fruit extract

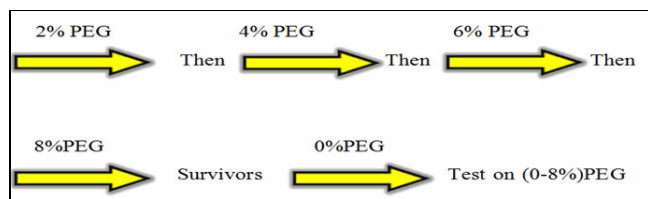
The modified method of Fleming (2000) was used in the preparation of the colocynth fruit extract at the concentration of 100 ml l<sup>-1</sup> to use in varieties seed soaked for 24 hours before surface sterilization (Zaidan, 2017), then the seeds were sterilized as before. Seeds were cultivated in a medium free hormones for seed germination. Callus induction media containing 2,4-D (1.0 mg L<sup>-1</sup>) and BA (0.5 mg L<sup>-1</sup>), with colocynth fruit extract (100 mL L<sup>-1</sup>) at pH 5.7 and completed the size to 1000 ml and add the agar 7 g l<sup>-1</sup> and sterilized and then planted the seedling treated with colocynth fruit extract on the media with 10 replicates for each Sunflower variety.

### Cultivation of treated Callus with colocynth fruit extract under levels of PEG

A new medium containing 100 mL L<sup>-1</sup> of colocynth extract was prepared with the interaction of pEG concentrations (2, 4, 6, 8 and 10%), as well as control treatment, then taking a constant weight of callus and planting at a rate of 10 replications per variety and concentration, then incubating under the previous conditions.

### - Gradual selection of treated callus cells with colocynth fruit extract

The Callus which was treated with 100 ml L<sup>-1</sup> in fixed weight was planted in MS provided with PEG at (0-10%) for 4 weeks. After determined the LD<sub>50</sub> (4% of the PEG for 4 weeks), the callus was transferred in a medium with concentrations PEG gradually increased by 0, 2, 4, 6, and 8% respectively for 5 reculture processes for two weeks for each concentration and for all cultivars as in Fig. 1. Then cultivating callus in induction media free of PEG; to refresh cells growth and thus increase the divisions for 4 weeks and then the callus transplantation to the same PEG concentrations mentioned earlier as follow.



**Fig. 1:** The method of gradual selection under different levels of PEG for the treated kidneys with the extract of 100 mg l<sup>-1</sup>

After the series of selection, the experiment was carried out for each variety with 10 replicates. The cultures were incubated under the same conditions. In order to determine their tolerance to drought, the following traits were studied after 4 weeks of planting and as follows:

### Enzymes Effectiveness in Callus

To estimate the enzymes effectiveness, mash 1 g of soft callus with 10 ml of Potassium Phosphate pH = 7.8. Then, the solution was centrifuged at 4 °C at 4000 cycle min<sup>-1</sup> for 30 minutes. The leachate was then evaluated for enzymatic efficacy: Determination of Superoxide dismutase enzyme effectiveness (SOD) according to Beyer and Fridoich (1987).

Determination of the effectiveness of Peroxidase enzyme according to Nezh (1985).

Determination of Catalase Effectiveness according to Aebi (1984). Determination of the efficacy of ascorbate peroxidase (APX) according to Nakana and Asada (1981). Estimate percentage of total dissolved carbohydrates (mg g<sup>-1</sup>), according to phenol-sulfuric acid (Herbert *et al.*, 1971).

### Statistical Analysis

The results were analyzed according to genstat program, and the means were compared by least significant difference (LSD 0.05), (Steel and Torrie, 1980).

### Results and Discussion

#### Effect of Drought on the Effectiveness of SOD Enzyme in Callus Sun Flower

Table 1 showed a significant increase in the effectiveness of the SOD enzyme, where the varieties treated with the extract in were differ significantly in their behavior and Al-haja variety was superior by giving the highest mean of 12.26 unit absorption mg<sup>-1</sup> protein compared to Ishaqi and Aqmar varieties that gave 5.11 and 9.56 unit absorption mg<sup>-1</sup> protein. This differences may be due to differences in their susceptibility to stress and also the genetic variation of the varieties. This may be due to the effect of the colocynth extract in stimulating the defense system due to its content of some Toxic and mutagenic compounds like phenols, alkaloids, terpens. PEG treatment also affected SOD efficacy, where 6% of the PEG achieved the highest mean of 13.78, while the concentrations of 0 and 2% did not differ significantly. This is due to that the production of free radicals under the normal conditions of the plant grows are at low levels and do not harm the cell. These free radicals, such as hydrogen peroxide, hydroxyl radicals and superoxide, are responsible for the damage of the cell under water stress and that this stress leads to product high amounts of free radicals working to damage cellular membranes that increase the effectiveness of SOD enzyme (Table 1). The increase in free radical levels may be attributed to the presence of high concentrations of PEG in the media. This can be explained by the fact that water stress and treatment with colocynth extract may have stimulated the genes responsible for the production of this enzyme like *sod4* and *soda*, (Sofa *et al.*, 2015). Table 1 showed significant interaction between cultivars treated with colocynth extract and the PEG coefficients, and Al-haja variety + 6% PEG gave the highest value of 16.39 unit absorption mg<sup>-1</sup> protein, while Ishaqi 1 variety at control gave the lowest value of 1.34 unit absorption mg<sup>-1</sup> protein.

**Table 1:** Effect of varieties treatment with colocynth extract and PEG concentrations in the efficacy of the SOD (unit absorption mg<sup>-1</sup> protein) after four weeks of cultivation in media MS.

Mean	Varieties			PEG concentrations%
	Ishaqi 1	Aqmar	Al-haja	
6.91	1.34	11.88	7.51	0
5.84	1.37	5.71	10.42	2
10.77	11.12	9.24	11.93	4
13.78	9.83	15.13	16.39	6
7.61	1.90	5.85	15.07	8
	5.11	9.56	12.26	Mean
	Interaction =4.492	Concentrations =2.594	Varieties =2.009	LSD 0.05

### Effect of Drought on the Effectiveness of POD Enzyme in Sunflower Callus

Showed a significant increase in the effectiveness of the POD enzyme, and Ishaqi 1 variety was superior by giving the highest mean of 7.25 unit absorption  $\text{mg}^{-1}$  protein compared to Aqmar and Al-haja varieties with percentage decrease of 84.55 and 60% for cultivars respectively, Which may be due to the fact that the cultivar Ishaqi 1 treated with of the colocynth extract has the ability to withstand drought by increasing the effectiveness of this enzyme, and the active substances contained in the fruits of the colocynth may have increased the drought tolerance to contain the secondary compounds as well as containing a high percentage of phenolic compounds (Abbas *et al.*, 2012). PEG treatment also affected POD efficacy, where 8% of the PEG achieved the highest mean of 5.54 unit absorption  $\text{mg}^{-1}$  protein, The increase in the enzyme peroxidase in the callus increases the concentration of PEG to a method of resistance to stress conditions that stimulate the oxidative stress of increasing the formation of reactive oxygen species (ROS) harmful to plants, which increase production when the cell to stress where the work of peroxidase to eliminate by removing hydrogen peroxide (Gara *et al.*, 2003). These oxygen radicals are located in specific components of the cell such as chloroplast, Peroxisomes, Glyoxysomes and cytoplasm (Xiong and Zhu, 2003). Table 2 showed significant interaction between cultivars treated with colocynth extract and the PEG coefficients and Ishaqi 1 variety + 2% PEG gave the highest value of 11.33 unit absorption  $\text{mg}^{-1}$  protein, while Aqmar variety +6% PEG gave the lowest value of 0.10 unit absorption  $\text{mg}^{-1}$  protein

**Table 2:** Effect varieties treated with colocynth extract and PEG concentrations in the efficacy of the POD (unit absorption  $\text{mg}^{-1}$  protein) after four weeks of cultivation in media MS.

Mean	Varieties			PEG concentrations%
	Ishaqi 1	Aqmar	Al-haja	
2.88	6.11	0.48	2.04	0
4.98	11.33	3.18	0.43	2
4.63	10.88	0.76	2.25	4
0.76	1.64	0.10	0.55	6
5.54	6.30	1.07	9.24	8
	7.25	1.12	2.90	mean
	Interaction =1.889	Concentrations =1.090	Varieties =0.845	L.S.D 0.05

### Effect of Drought on the Effectiveness of the CAT Enzyme in Callus

Table 3 showed that varieties had significant effect on the effectiveness of the CAT enzyme, and Ishaqi 1 variety was superior by giving the highest mean of 11.54 unit absorption  $\text{mg}^{-1}$  protein compared to Aqmar and Al-haja varieties, which did not differ significantly. This may be due to their genetic variation and their adaptability and tolerance to stress conditions. PEG treatments also influenced the efficacy of the CAT enzyme, where 8% PEG achieved the highest mean of 7.73 unit absorption  $\text{mg}^{-1}$  protein, while control gave the lowest value of 3.01 unit absorption  $\text{mg}^{-1}$  protein. Many researchers have focused on the key role of the CAT enzyme in drought stress conditions and its

effectiveness varies with the severity and type of stress, thus affecting the total tissue content of absorbed carbon as it decreases. This is accompanied by inhibition of growth and also affects the state of carbohydrates and hormonal ratios during stress as it allows the removal of  $\text{H}_2\text{O}_2$  from tissues that suffer from drought, especially in high temperatures thus photovoltaic respiration acts as a source of energy to prevent over-limiting of electron photon transfer chain and photoinhibition (De Pinto *et al.*, 2013). Adaptation of plant tissues to drought is often associated with increased levels of ROS free radicals that are toxic to cells such as superoxide anion ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), hydroxyl radical (HO) and singlet oxygen ( $^1\text{O}_2$ ). ROS is secondary products of aerobic metabolism and its production is enhanced during stress conditions by disrupting the electronic transport system and oxidation of metabolic activities occurring in green plastids, mitochondria and microbodies. Under Non-stressful conditions, ROS is efficiently eliminated by non-enzymatic and enzymatic antioxidants, while drought conditions, ROS production exceeds the ability of antioxidant systems to be removed, causing oxidative stress (Ajithkumar and Panneerselva, 2014). The symmetric forms of CAT are iron porphyrin enzymes that act as an effective system to clean ROS to avoid oxidation caused by stress (Sofa *et al.*, 2015). Table 3 showed significant interaction between cultivars treated with colocynth extract and the PEG coefficients and Ishaqi 1 variety + 8% PEG gave the highest value of 16.30 unit absorption  $\text{mg}^{-1}$  protein, while Aqmar variety at control gave the lowest value of 1.71 unit absorption  $\text{mg}^{-1}$  protein.

**Table 3:** Effect varieties of the treatment with colocynth extract and PEG concentrations in the efficacy of the CAT (unit absorption  $\text{mg}^{-1}$  protein) after four weeks of cultivation in media MS.

Mean	Varieties			PEG concentrations%
	Ishaqi 1	Aqmar	Al-haja	
3.01	3.86	1.71	3.46	0
5.62	12.65	2.45	1.75	2
5.32	10.32	2.98	2.66	4
6.53	14.57	2.56	2.47	6
7.73	16.30	4.16	2.72	8
	11.54	2.77	2.61	mean
	Interaction =3.848	Concentrations =2.222	Varieties =1.721	LSD 0.05

### Effect of Drought on the Effectiveness of APX Enzyme in Callus

Table 4, showed that Ishaqi 1 was superior, with an mean of 0.4285 unit absorption  $\text{mg}^{-1}$  protein while the two cultivars Aqmar and Al-haja did not differ significantly. PEG treatments also influenced the efficacy of the APX enzyme, where 6% of the PEG achieved the highest mean of 0.2976 unit absorption  $\text{mg}^{-1}$  protein, while 2% of the PEG gave the lowest value of 0.1871 unit absorption  $\text{mg}^{-1}$  protein. The increased efficiency of this enzyme is due to the fact that the course of glutathione ascorbate is a major system for the removal of hydrogen peroxide from plant cells under abiotic stresses. Ascorbate peroxidase (APX) plays a key role in stimulating the conversion of  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$ , using Ascorbate as a specific donor of the electron (Correa-Aragunde *et al.*, 2013), especially in chloroplast. The expression of APX genes is precisely regulated in response to drought (Caverzan

*et al.*, 2012). Genes APXs are particularly important in maintaining the balance of ascorbate (ASA) and glutathione (GSH), as non-enzymatic antioxidants. ASA is involved in other functions such as plant growth, gene regulation, modification of certain enzymes, oxidation regulation of antioxidant compounds associated with the membrane in plants under water stress. RNA molecules are involved in the organization of different pathways associated with stress conditions, including drought and are particularly involved in the antioxidant defense. APX-coding was found to be the highest regulated in water and salt stress conditions, indicating the pivotal role of molecular regulatory mechanisms resulting from this type of abiotic stress (Xie *et al.*, 2015).

Table 4 showed significant interaction between cultivars treated with colocynth extract and the PEG coefficients, and Ishaqi 1 variety + 8% PEG gave the highest value of 0.5208 unit absorption  $\text{mg}^{-1}$  protein, while Aqmar variety at control gave the lowest value of 0.0192 unit absorption  $\text{mg}^{-1}$  protein.

**Table 4 :** Effect varieties of the treatment with colocynth extract and PEG concentrations in the efficacy of the APX (unit absorption  $\text{mg}^{-1}$  protein) after four weeks of cultivation in media MS.

Mean	Varieties			PEG concentrations%
	Ishaqi 1	Aqmar	Al-haja	
0.2137	0.3639	0.0192	0.2580	0
0.1871	0.3497	0.0935	0.1181	2
0.2205	0.4242	0.1380	0.0994	4
0.2976	0.4838	0.2497	0.1593	6
0.2734	0.5208	0.1792	0.1201	8
	0.4285	0.1359	0.1510	Mean
	Interaction =0.06941	Concentrations =0.04007	Varieties =0.03104	LSD 0.05

#### The Effect of Drought on Total Carbohydrate Content in Callus

Table 4, showed that cultivar treated with colocynth extract caused significant effect, where Aqmar was superior to the rest of the varieties, with an mean of 0.3498  $\text{mg g}^{-1}$  with percentage decrease of 14.74 and 22.80% for the two cultivars Ishaqi 1 and Al-haja respectively. PEG treatments also influenced total carbohydrate content where 8% of the PEG achieved the highest mean of 0.31128  $\text{mg g}^{-1}$ , while 2% of the PEG gave the lowest value of 0.30172  $\text{mg g}^{-1}$ . This increases in carbohydrate content with increasing water stress was due to reduce the susceptibility and efficiency of callus cells in the transformation of simple sugars into complex sugars (Hanafy-Ahmed *et al.*, 2002). It may also as methods of endurance water stress is to increase the amount of soluble sugars then increase the osmotic, and therefore these organic compounds will play an important role to bear the drought as it works with proline to increase the osmotic potential in the cell

Table 5 showed significant interaction between cultivars treated with colocynth extract and the PEG coefficients and Aqmar variety + 8% PEG gave the highest value of 0.35357  $\text{mg g}^{-1}$ , while Al-haja variety + 8% PEG gave the lowest value of 0.26662  $\text{mg g}^{-1}$ .

**Table 5:** Effect varieties of the treatment with colocynth extract and PEG concentrations in total carbohydrate content ( $\text{mg g}^{-1}$ ) after four weeks of cultivation in media MS.

Mean	Varieties			PEG concentration %
	Ishaqi 1	Aqmar	Al-haja	
0.30775	0.29610	0.35154	0.27562	0
0.30172	0.29277	0.34214	0.27024	2
0.30470	0.29242	0.35209	0.26959	4
0.30467	0.29624	0.34964	0.26814	6
0.31128	0.31365	0.35357	0.26662	8
المتوسط	0.29823	0.34980	0.27004	Mean
	Interaction =0.004379	Concentration =0.00252	Varieties =0.00195 8	LSD 0.05

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