EFFECT OF THE HYDROGEN PEROXIDE AND SALICYLIC ACID ON INDUCTION THE SOD GENE EXPRESSION OF DATE PALM (*Phoenix dactylifera* L.) AS DEFENSE FACTOR AGAINST SALINITY STRESS

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ABSTRACT

This experiment was conducted in order to determine the effect of spraying acid salicylic (SA) at concentrations of 250, 500 mg L^{-1} and hydrogen peroxide H_2O_2 at 3% and 6% compared with water as a control treatment on three cultivars of date palm tree (Barhi, Hilali and Majhoul), which are propagated via tissue culture under salt stress conditions, to estimate the gene expression of Superoxide dismutase (SOD), The SA 250 and 500 mg L^{-1} for Folding values achieved the highest gene expression for the cultivar Barhi and Hilali, which reached 2.549121, 3.363586, 5.098243, and 4.924578, respectively, While the highest gene expression of this enzyme for the Majhoul when treated with 6% hydrogen peroxide, which was recorded at 2.828427.

Keywords: abiotic stress, cultivars, reactive oxygen species, superoxide dismutase

تأثير بيروكسيد الهيدروجين وحامض السالسليك في تحفيز جين SOD لنخيل التمر كعامل محفز لتحمل ظروف الاجهاد الملحي

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المستخلص

اجريت التجربة بهدف تحديد تأثير الرش بحامض السالسليك (SA) بتركيزي 250 و 500 ملغم لتر$^{-1}$ وبيروكسيد الهيدروجين H_2O_2 بتركيزي 3% و6% مقارنة مع الماء كمعاملة سيطرة لأجل المقارنة في ثلاثة اصناف (برحي , هلالي و مجهول) من اشجار نخيل التمر المكثر نسيجيا والتداخل بينهما تحت ظروف الاجهاد الملحي لتقدير التعبير الجيني Gene Expression لإنزيم Superoxide dismutase (SOD) فقد حققت كل من المعاملة SA 250 و 500 ملغم لتر$^{-1}$ لقيم Folding اعلى تعبير جيني للصنف برحي وهلالي بلغت 2.549121 و3.363586 و5.098243 و4.924578 على التوالي بينما سجل اعلى تعبير جيني لهذا الانزيم للصنف مجهول عند المعاملة ببيروكسيد الهيدروجين 6% قيمة بلغت 2.828427.

الكلمات المفتاحية: الاجهاد اللاحيائي، اصناف، الاوكسجين الفعال، انزيم سوبر اوكساید دسميوتيز

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INTRODUCTION

Date Palm (*Phoenix dactylifera* L.), especially those that are of propagated via tissue culture techniques, are exposed to many unfavorable conditions for growth, which are known as stresses, whether they are biotic or abiotic stresses that may affect the growth of these trees and may lead to their death later. Therefore, these trees were treated with some growth regulators that lead to giving characteristics of resistance and tolerance against these stresses (12). Many plant growth regulators have been used of trees, which are those non-nutritious organic chemical compounds that work in very low concentrations to activate, inhibit or alter physiological processes inside cells, in addition to proteins (amino acids) that conduct most of the biotic processes in the cell. In addition to being active as antibodies for the purpose of maintaining cell continuity in activity and growth (10), one of the growth regulators that use in this field is Salicylic Acid (SA), which is known to stimulate plants to resist stress, especially abiotic stresses, which belongs to the group of phenols (Phenolic Compound), which is found in plants and has a various of roles for plant growth and development, increasing the efficiency of carbon representation, transpiration, ion absorption and transporting (29). Previous studies showed that the use of salicylic acid as a spray on the shoot of the plant stimulates the growth of the palm leaf (9,4). Another application in increasing the tolerance of plants to stress is spraying with a solution of Hydrogen Peroxide (H_2O_2), which is a double-edged factor as, in low concentrations, it acts as a complex molecular signal that causes the plant to endure against stress, whether biotic or abiotic (26). As for high concentrations of SA, it results in the release of the factors that stimulate programmed cell death (14), and this compound contributes to many mechanisms of resistance by strengthening the cell wall through the synthesis of lignin, which is a means of protection and defense against pathological injuries and the production of defense materials. And improving resistance, as the presence of hydrogen peroxide inside the plant works to directly kill pathogens or stimulate defense genes to limit infection (16). It also works as a regulatory key for a number of physiological processes such as aging, photosynthesis, respiration, and stomatal movement (11). Salt stress is considered among the abiotic stresses that most plants are exposed, about 870 million hectares are affected by salinity, which is often natural causes, and these lands constitute about 6% of the land in the world. High evaporation-transpiration and the attendant impairment in soil and water management (8, 1). High is toxic and exerts additional stress on physiological and biochemical processes in plant cells (7). Date palm varieties differ among themselves in tolerance and resistance to these stresses (3). Salt stress, like other abiotic stresses, causes oxidative damage in plant cells by inducing the emergence of Reactive oxygen species (ROS), such as superoxide, single oxygen, hydroxyl radical and hydrogen peroxide, which leave different effects in plants. When the plant is subjected to stress and if it was in high concentrations, it caused the oxidation of the lipids and the denaturation of the proteins (6, 27). The most of important types of stress is hydrogen peroxide. When plants feel stressed, a series of physical and chemical indicators are stimulated at the molecular and cellular levels to protect the plant from stress (17, 21), and most plants have developed specialized mechanisms at the physiological and molecular levels for survival and endurance under stress biotic or abiotic (18, 31, 33). Stress often leads to the formation of ROS, which breaks down unsaturated fats, which if high in concentrations leads to toxic stress in cells and thus causes cellular dysfunction and plant tissue damage (28, 19). ROS formation also leads to the expression of the gene for the production of the enzyme superoxide dismutase SOD and its increase in activity (5, 25). In a study carried out by AL Kharusi *et al.* (2) to determine the resistance to salt stress of two varieties of date palm Umsila (tolerant to salinity) and Zabad (sensitive to salinity), which were treated with sodium chloride (240 mmol) with irrigation water. The results indicated that the salinity treatments had a negative effect on growth and photosynthesis of the salt-sensitive Zabad cultivar is more

than the salt-tolerant variety due to its ability to accumulate less sodium and more potassium to maintain the normal concentration of ROS and produce enzymatic antioxidants (SOD), (CAT) and (APX). Real time-PCR technology is considered one of the modern applications as it is usually used in both diagnostic and basic research. This technique has spread as a way to detect modern diseases such as influenza strains in diagnostic tests or examinations and in recent research studies of this technique that has been used mainly to provide quantitative measurements of copies On the basis of which the genetic expression is determined for the environmental changes of certain genes over time, for example, through tissue culture of plant cells, a specific drug was identified, monitoring the development of differentiated cells, and responding to environmental changes (13). We can say that Real time-PCR is a multiplication and increase in the number of copies of the target DNA pieces, with the presence of a camera or detector (radioactive dyes) recording and monitoring these processes in order to give a detailed report on the number of cycles and the corresponding increase and amplification of copies of the target gene. Studies of gene expression have become extremely important in obtaining insights into gene function and understanding molecular mechanisms. QRT-PCR is the gold standard for quantifying gene expression due to its specificity, accuracy, measurable results, and sensitivity; however, its accuracy is greatly influenced by the integrity of the extracted RNA and the quality and efficiency of cDNA amplification (23). Recent studies on several fruit trees have indicated that there are changes in gene expression that occur during the fruit development stages or when exposed to stresses (15). For that, this study aimed to determinate the genetic behavior under salt stress conditions using SA and H_2O_2 of date palm.

MATERIALS AND METHODS

The experiment was carried out during the growing season of 2019 using three varieties of date palm trees (Barhi, Hilali and Majhoul or Madjoul), which resulted from textile cultivation and imported from the Arab Gulf. The study included 45 trees, 15 trees for each variety at the age of 8 years in 5 x 5 m distance between them. Samples of soil were taken randomly before performing two-depth treatments (0-30 and 30-60 cm), and analyzed for their physical and chemical characteristics (Table 1.) in the laboratory of the Wasit Agriculture Directorate.

Table 1. Showed some physical and chemical characteristics of soil used in the study

Factors	Unit	Value
Soil reaction degree (pH)	-	7.8
Electrical conductivity (EC 1:1)	0 – 30 ds.m^{-1}	11.4
	30 – 60	9.3
The electrical conductivity of irrigation		0.95
Cation exchange capacitance (CEC)	cmol (+) kg $^{-1}$	26.3
Dissolved potassium		3.7
Dissolved sodium		8.8
Dissolved calcium		24.3
Magnesium	Mmol. Liter $^{-1}$	5.6
Bicarbonate		13.7
Chloride		14.8
Sulfates		4.1
Potassium exchange		4.3
Sodium Exchanged	Centimol. Kg $^{-1}$. Soil	7.9
Calcium Exchange		7.2
Bulk density	mcg. 3-1	1.2
Field capacity	%	27.8
Organic matter		5.71
Sand		194
Silt		510
Clay		296
Texture	———	SiCL

Study treatments

The variety: the treatments were carried out on three varieties of date palms (thickened tissue and imported from the Emirates), which are Barhi, Hilali and Majhou.

Spray Treatments

-Salicylic acid

Salysalic Acide ($C_7H_6O_3$) was added in two concentrations (250 mg L^{-1} and 500 mg L^{-1}).

- Hydrogen Peroxide

Hydrogen Peroxide, a concentration of 50% of Turkish origin, was added in two concentrations (3% and 6%). Both treatments were used as a spray on the fronds and in three batches (5/2, 15/2 and 25/2) in addition to the control treatment, so the treatments become as follows:

1- Control treatment (spraying water only)
2- Salicylic acid 250 mg L^{-1}
3- Salicylic Acid 500 mg L^{-1}
4- Hydrogen peroxide 3%
5- Hydrogen peroxide 6%

Thus, we have 15 treatments and each treatment is repeated three times, and thus we have 45 palm trees in the study. The spraying treatments were carried out using a 12 liter spray on the back to ensure the use of the necessary concentration for each experimental unit and reaching the point of complete wetting, with the addition of the diffuser Tween 20 (0.1%) to the prepared solutions to reduce the surface tension of water and increase the adhesion of the material to the leaves and thus increase the absorption. Spraying early in the morning. **Molecular study**

1- Plant material 2- Converting RNA to cDNA, Total RNA isolation, cDNA synthesis and gene identification 3- Real time qPCR amplification

Extraction of RNA genetic material

The botanical samples (Wicker) were taken from the palm fronds (young leaves). The wicker was cleaned and washed to get rid of the dirt. Then the palm frond wicker was crushed and grinded using liquid nitrogen in a ceramic jar until it became powdery. The finely ground (150 mg) plant samples were placed into a ZR BashingBead ™ Lysis Tubes. RNA was extracted according to the method described with the ZRPlant RNA Mini Prep ™ Catalog No. R 2024.

Converting RNA into Cdna

RNA was converted into cDNA according to the method provided with the PrimeScriptTM RT Reagent Kit (Perfect Real Time) # RR037A (Table 2).

Table 2. Materials included in the Prime Script TM RT Reagent Kit (Perfect Real Time) # RR037A for converting RNA into cDNA

Reagents	Volumes
1- 5 × PrimeScriptTM mix	2 µl 1
2- total RNA	
3- RNase Free dH2O	up to 10 µ l
Total	12 µ l

Real time qPCR amplification Two types of prefixes were used (primer) (Table 3,4) the target gene and Reference gene. According to the gene responsible for resistance to stress conditions (from the NCBI website) SOD gene primers (LOC103718751). The instantaneous polymerase chain reaction was performed using the American-made KAPA SYBR® FAST qPCR Master Mix (2X) Kit which contains Sybr green that radiates in the presence of a double DNA strand (Table 5)

Table 3. SOD gene primers (Target Gene)

Primer	Sequence
Forward	TGGTTTGGGATTACTCGCCC
Reverse	GCTCTTTGCCAGCCAGAGTA

Table 4. Elongation factor gene primer (Reference Gene)

Primer	Sequence
Forward	TCTTCTGCCCCTATCGCACG
Reverse	TGCTGTGAGCCTGTGAGAAG

Table 5. Stages of operation of the qpcr

Step	Temp. (°C)	Time	Cycle
Enzyme activation	95 °C	05:00 min	Hold
Denaturation	95.0 °C	00:20 sec	
Annealing	60.0 °C	00:20 sec	40
Extension	72.0 °C	00:20 sec	

Calculations of results

The results were taken from the device after the end of the last cycle and a doubling curve was drawn for all samples under study. The value of Cycle threshold was determined for each sample using the first starter with the constant expression primer. The value of Delta ct was calculated from the equation.

ΔCt (sample) = Ct $(target)_{mean}$ – Ct $(reference)_{mean}$

Ct (Controls) = Ct $(controls)_{mean}$ – Ct $(reference)_{mean}$

$\Delta\Delta$ Ct = Δ Ct (sample) – Ct (controls)

Normalized target gene expression level $=2^{(\Delta\Delta Ct)}$

RESULTS AND DISCUSSION

The results are indicated in Table 6. that refer to the gene expression values represented by the value of Cycle threshold (ct), which indicates the degree of gene expression inversely (where the lower the value of ct the more the gene expression process increases), it was found that the highest gene expression (Fig 1.& Fig2.) at the Barhi of the enzyme SOD is when Spray treatment SA 500 mg L^{-1} in which the mean value of ct was 29.9 followed by SA 250 mg L^{-1}, when it recorded 30.2 compared to the control of the treatments for the same cultivar, which recorded the lowest expression of the target gene with close values. As for the Hilali cultivar, it was found that spraying with salicylic acid SA 500 mg L^{-1} and SA 250 mg L^{-1} and hydrogen peroxide solution at concentrations of 3 and 6% increased the gene expression value according to the mean of ct values of 28.1, 28.15, 29.1 and 29.25 respectively compared to the control treatment.

Table 6. Shows the gene expression results and values

Treat.	Ct target	Ct reference	ΔCt	ΔΔCt	Folding
Control _Barhi	**31.55**	**18.15**	**13.4**		**1**
SA 250 _ Barhi	**30.2**	**18.15**	**12.05**	**-1.35**	**2.549121**
SA 500 _ Barhi	**29.9**	**18.25**	**11.65**	**-1.75**	**3.363586**
H_2O_2 3%_Barhi	**31.3**	**18.05**	**13.25**	**-0.15**	**1.109569**
H_2O_2 6%_Barhi	**31.85**	**18.2**	**13.65**	**0.25**	**0.840896**
Control _ Hilali	**30.35**	**17.95**	**12.4**		**1**
SA 250 _ Hilali	**28.15**	**18.1**	**10.05**	**-2.35**	**5.098243**
SA 500 _ Hilali	**28.1**	**18**	**10.1**	**-2.3**	**4.924578**
H_2O_2 3% _Hilali	**29.1**	**18.05**	**11.05**	**-1.35**	**2.549121**
H_2O_2 6% _Hilali	**29.25**	**18.1**	**11.15**	**-1.25**	**2.378414**
control_ Majhoul	**31.6**	**18.2**	**13.4**		**1**
SA 250 _ Majhoul	**30.2**	**18.1**	**12.1**	**-1.3**	**2.462289**
SA 500 _ Majhoul	**30.25**	**18.1**	**12.15**	**-1.25**	**2.378414**
H_2O_2 3% _Majhoul	**30.9**	**18.35**	**12.55**	**-0.85**	**1.802501**
H_2O_2 6% _Majhoul	**29.85**	**17.95**	**11.9**	**-1.5**	**2.828427**

As for the Majhoul cultivar, all treatments had increased gene expression for the SOD enzyme compared to the control treatment, and it was maximum for spraying with a 6% hydrogen peroxide solution, which reached 29.85 ct. When compared with the cultivars, it was found that the Hilali cultivar had the highest gene expression (Control) 30.35, followed by the cultivar by Barhi and Majhoul with values of 31.55 and 31.6 for the mean values of (control) ct.However, if folding values compared to the highest response of each cultivar to the effect of exposure with salicylic acid and hydrogen peroxide, it was found that the Hilali cultivar at SA 250 mg L^{-1} was more responsive, which were 5.098243 than both Barhi and Majhoul, Which arrived at SA 500 mg L^{-1} (3.363586) and H_2O_2 6% (2.828427) respectively.

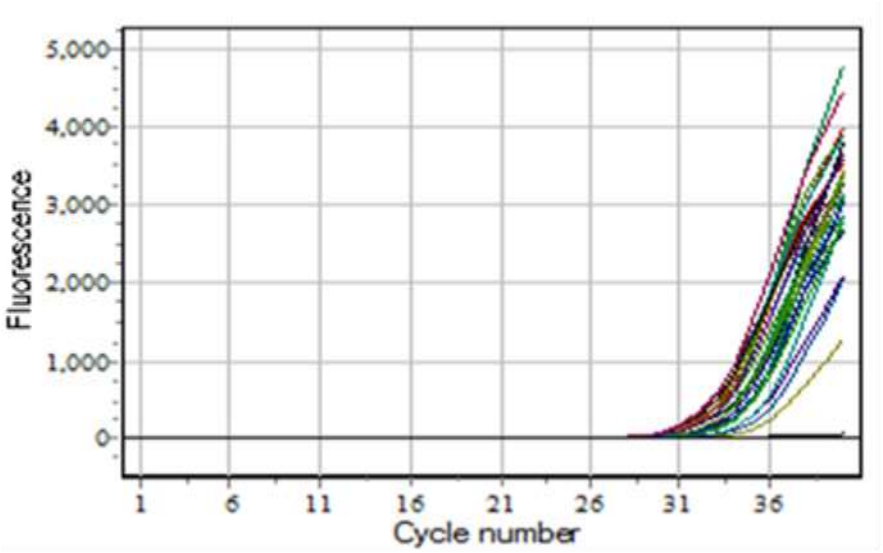

Fig. 1. A plot representing amplification plot curves of the parameters in the qPCR device for the SOD gene in date palm (Target gene).

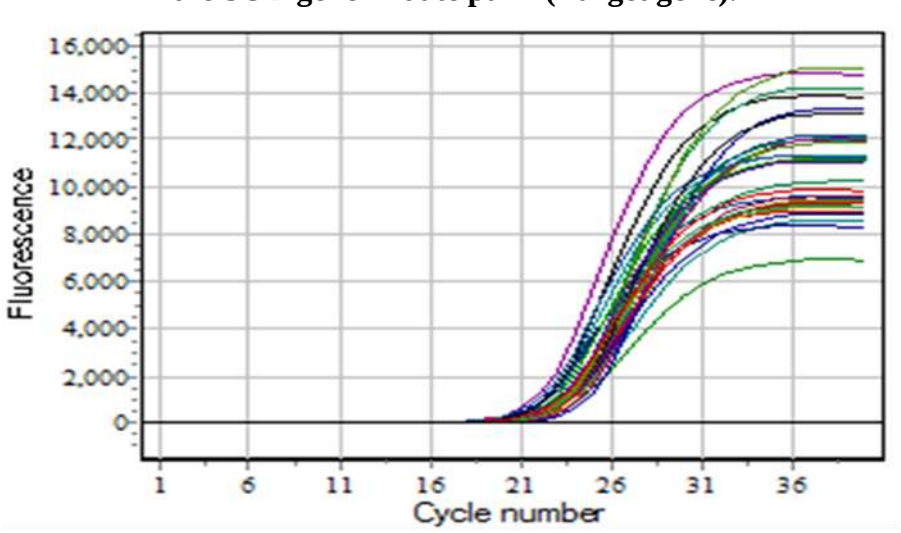

Fig. 2. A plot representing the amplification plot of the parameters in the qPCR device for the elongation gene in the date palm (Reference gene).

A series of physical and chemical indicators are triggered when plants are under stress and this response is at the molecular and cellular levels to protect the plant from stress (17, 18). Stress often leads to the formation of ROS compounds which in turn leads to increased gene expression. Some ROS synthesizers, including Superoxide dismutase SOD, have a role in increasing the susceptibility to stress conditions (25). These enzymes can be catalyzed by certain compounds such as salicylic acid, which generates the signal that leads to the production of proteins and defense enzymes to reduce the damage caused by plant exposure to salt stress conditions (22, 24, 34), which explains the increased gene expression of the SOD enzyme.The explanation for the increase in gene expression when spraying with hydrogen peroxide (H2O2) may be due to the action of this compound sending molecular signals that counteract stress through its regulation of pathways. This can be collected in a phrase "Multiple stress response and one gene expression" (20, 26,30 , 32). As for the reason for the increase in gene expression in the Hilali cultivar on the two cultivars of Al-Barhi and Majhoul, it may be due to its rapid response to the added spraying agents due to the genetic variation between them.

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