

Oxidative responses and genetic stability of date palm *Phoenix dactylifera* L. Barhi cv. under salinity stress.

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Abstract

This study was aimed to investigate the oxidative responses and genetic stability of date palm *Phoenix dactylifera* L. under different irrigation water salinity, date palm off shoots cv. Barhi subjected to different concentrations of NaCl (100, 200, 300 and 400 μ M) for 180 days. The obtained results showed that, the date palm responses to salinity stress, this responses was increased of H₂O₂ level, Peroxidase activity and Malondialdehyde MDA concentration with increase NaCl concentration, while opposite trend with membrane stability index, which H₂O₂ was increased from (0.73 μ M/g) in control to (2.20 μ M/g) in 400 μ M treatment, MDA was increased from (2.35 nmole/g) in control to (nmole/g) in 400 μ M treatment, also peroxidase activity was reached to (39.59 U/min/g) in 400 μ M treatment, while was (20.73 U/min/g) in control and Membrane stability index reduction significantly from (81.36%) in control to (64.13%) in 400 μ M treatment. In terms of genetic stability of date palm under salinity stress, the ISSR markers analysis showed that, the high concentrations of NaCl (200, 300 and 400 μ M) produced more polymorphic fragments comparison to control treatment, while the DNA profile was identical between control and 100 μ M treatment. Dendrogram was generated using similarity indices of ISSR markers showed, the lowest genetic similarity was found between 400 μ M NaCl concentration and control and 100 μ M treatment, followed with both 200 and 300 μ M treatments, which the control and 100 μ M treatment was grouped in one cluster, also treatments with 200 and 300 μ M grouped in one cluster, while the treatment with 400 μ M NaCl separated in cluster.

Keywords: ISSR, Genetic stability, Oxidative stress, Peroxidase, MDA, membrane peroxidation

1-Introduction.

Date palm (*Phoenix dactylifera* L. 2N=36) belongs to the family Aracaceae, it is believed that the date palm originated in the ancient Mesopotamia area (Wrigley, 1995). Date palm trees are widespread in different regions worldwide, particularly in the Middle East, North Africa, and less intensity in North and South America, Southern Europe, Pakistan and India (Zaid, 2002). This tree is a typical urban tree in Iraq and cultivated over large areas, usually growing by roadsides in industrial, rural, residential and agricultural areas (Al-Jabary *et al.*, 2016).

Soil and water salinity considered as a major agricultural problem in many countries including Iraq (Zowain and Ismail, 2013). Salinity can effect of plants through the adverse osmotic effect (Munns and Tester, 2008) and toxic effect led to cellular damage (Munns, 2005), and affects several physiological and biochemical processes, which Abdulwahid (2012) reported that, the activity of antioxidant enzymes superoxide dismutase (SOD), Catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD), as well as proline was increased in date palm Hillawi cultivar leaves when irrigated with 200 mM of NaCl, while soluble protein decreased under salinity stress, also *in vitro* responses of date palm Hillawi cv. callus to NaCl treatments were investigated (Abbas, 2016), the growth of callus was negatively affected and decreased of catalase activity while salinity treatments lead to accumulation of proline and Hydrogen peroxide (H₂O₂). Despite date palm considered as a relatively salt tolerance species (Ramoliya and Pandey, 2003), but the molecular basis of salt tolerance is poor understood in this tree (Yaish and Kumar, 2015), and previous work focused on the genetic differentiation of adaptive varieties using DNA marker analysis, such as random amplified polymorphic DNA (RAPD) (Sedra *et al.*, 1998; Kurup *et al.*, 2009; Abbas, 2016) and microsatellites (Elshibli and Korpelainen, 2008).

Inter simple sequence repeat (ISSR) markers successfully used for estimating of genetic diversity in plants when exposed to environmental stress (Khalil, 2013). However, this technique has not been used previously to determine the genetic diversity of date palm under salinity stress. The objectives of the present study were determined some oxidative responses and genetic stability by using ISSR markers of date palm Barhi cultivar under different salinity concentrations.

2-Materials and methods

This study was conducted in date palm research centre, Basra University, tissue culture derived date palm offshoots Barhi cultivar at two years old were selected, these offshoots were cultured in pots, each pot filled with 15 kg of soil, and soil chemical characteristics were: pH=7.22, Ec=4.12, Cation Exchange Capacity (CEC) = 8.5 cmole, Organic Matter (OM) = 0.86 % and Soil texture was Loam (silt=45.65, sand=32.44 and clay=21.91). Date palm offshoots subjected to four concentrations of NaCl were 100, 200, 300 and 400 mM, as well as control treatment, treatments were applied by irrigation using RO water (Reverse Osmosis) which pH of water was 7.6 and Ec 1.3 ds/m, plants were irrigated with treatments for 180 days, during the period from 1/6/2019 to 30/152/2016. The experiment was laid according to Complete Randomized Block Design (RCBD), with triplicate for each treatment and results analyzed by using SPSS program V.21. Mean was statistically compared by LSD test at $p < 0.05$.

Oxidative stress responses of treated date palm

The responses of date palm to salinity stress were evaluated at 180 days after treatments:

H2O2 content

H2O2 content in date palm leaves was determined according to Sergiev *et al.* (1997), briefly. A 500mg sample of fresh leaves was homogenized with 5 mL of trichloroacetic acid (TCA; 0.1%: w/v). The homogenate was centrifuged at 13,000 rpm for 15 min, then, 1 mL of the supernatant was added to 0.5 L of potassium phosphate buffer (10 mM, pH =7.0) and 1 mL of potassium iodide(1 M). The absorbance of the supernatant was measured at 390 nm. Hydrogen peroxide was used to establish a standard curve.

Malondialdehyde (MDA) content. MDA content was estimate according to Heath and Packer (1968); in date palm leaves, 500 mg of leaf tissue was homogenized in 5 mL TCA (0.1%, w/v). Then the homogenate was centrifuged at 10,000 rpm for 5 min, 4 mL of thiobarbituric acid (TBA; 0.5% w/v) and 1 mL of supernatant prepared in 20% TCA was boiled, after 30 min the reaction was terminated by placing the mixture on ice, then another centrifugation at 10,000 rpm for 15 min was done. The supernatant absorbance was measured at 532 and 600 nm, the MDA content was calculated using an extinction coefficient of 155.

Membrane stability index (MSI).

Membrane stability index (MSI) was measured in date palm leaves. 0.25 g of fresh palm leaves in each heavy metal stressed and control treatments were cut into small parts and homogenate using 10 mL of deionized water and incubated at room temperature on a rotary shaker for 24 h. Subsequently, the initial electrical conductivity of the medium (C_1) was measured. The samples were placed in an oven at 90°C for 2 h to expel all electrolytes, and cooled at 25°C, and then the second electrical conductivity (C_2) was measured. Leaf membrane stability index was calculated using the following formula (Lutts *et al.*, 1996):

$$\text{Membrane Stability Index (MSI \%)} = \left[1 - \frac{C_1}{C_2} \right] * 100$$

Peroxidase activity:

peroxidase activity in date palm leaves was estimated according to the method of Kim and Yoo (1996). every single unit of peroxidase catalyzed the oxidation of guaiacol in 1 min (U/min/g). The absorption stability as a result of tetraguaiacol formation was measured at 470 nm.

Genetic stability in treated date palm.

DNA of date palm Barhi cv. leaves was extracted according to Doyle and Doyle (1990), by using CTAB (cetyltrimethyl ammonium bromide) method, and DNA was quantified in 1.5% agarose gels to check out the DNA integrity. Electrophoresis was conducted in a 1X TBE buffer [100 mL 10X TBE (0.89 M Tris base, 0.89 M Boric acid, 20 mM EDTA pH 8.0) and 900 mL distilled water] at 60 V for 30 min and then at 120 V for 1.5 h. The DNA was stained with 0.5 mg/mL of ethidium bromide. DNA concentration was measured by Nano-Drop spectrophotometer (Bio-Rad, USA) at A 260 and 280, DNA templates were diluted to 30 ng/μL with TE buffer.

ISSR primers description and PCR amplification

Five different primers manufactured by Bioneer – South Korea were used for ISSR analysis (Table 2). Each polymerase chain reaction (PCR) was carried out in a 25 μL volume, contain 30 ng DNA template, 1.5 mM MgCl₂, 0.32 mM dNTPs, 1X Taq DNA polymerase buffer, 10 pmol oligonucleotide primer and 2 unit of Taq

DNA polymerase. Amplification was performed in a thermal cycler using the following conditions: denaturation at 95 °C for 5 min.; 35 cycles of 1 min denaturation at 94 °C, 2 min annealing at 36°C and 2 min extension at 72 °C, and a final extension at 72 °C for 7 min.

ISSR markers data analysis

DNA markers of Promega 100 DNA Ladder (100-3000, twelve fragments) were used to detect the precise molecular sizes of each individual fragment using the PhotoCapt MW software (VilberLourmat) and photographed under UV light. Total bands number, polymorphic and monomorphic fragments were scored visually. The following primer parameters were measured according to Alansari *et al.* (2014).

1- Primer efficiency (%)

$$= \frac{\text{the total number of fragments amplified by primer}}{\text{the total number of obtained fragments}}$$

2- Polymorphism (%)

$$= \frac{\text{the number of polymorphic fragments}}{\text{the total number of bands amplified by the same primer}}$$

3- Discrimination power (%)

$$= \frac{\text{the total number of polymorphic fragments amplified by primer}}{\text{the total number of obtained polymorphic fragments}}$$

The change observed in ISSR profiles, and scored as appearance of new band (1) or disappearance of bands (0) to create the binary matrix, and genetic similarity index for all pairs treatments was calculated according to Nie and Li (1979) formula.

$$\text{Genetic Similarity Index (GSI)} = \frac{2A}{(B + C)}$$

Where (A) number of similar fragments in both treatments, (B) and (C) the total number of bands in the first and second treatments.

Table (1) ISSR primers sequence, length and GC content.

| Primer ID | Primer sequence | Primer length | G+C content (%) |
|-----------|------------------------------|---------------|-----------------|
| 815 | CTC TCT CTC TCT CTC TG | 17 | 52.94 |
| 818 | CAC ACA CAC ACA CAC AG | 17 | 52.94 |
| 822 | TCT CTC TCT CTC TCT CA | 17 | 47.05 |
| 834 | AGA GAG AGA GAG AGA G (CT) T | 19 | 47.36 |
| 855 | ACA CAC ACA CAC ACA C (CT) T | 19 | 47.36 |

3-Results and Discussion

Oxidative stress responses in date palm leaves under salinity stress.

H₂O₂ and Peroxidase activity

The results in Fig (1) showed the date palm responses to salinity stress by accumulation more H₂O₂ in leaves, the level of H₂O₂ was 0.73 μM/g in control plant without significant difference compared to 100 and 200 μM NaCl treatments, while the level of H₂O₂ increased significantly in high concentrations, which the high level was found in 400 μM NaCl treatment, which 2.20 μM/g with significant difference to other treatments, followed by 300 μM treatment (1.31 μM/g). Similar trend of results were observed for peroxidase activity, which the salinity treatments induced more activity of peroxidase, and this increase was gradual with increased NaCl concentration, the resulting in Fig (2) showed that, the lowest activity was found in control treatment which

20.73 U/min/g without significant difference of 100 μM treatment (22.18 U/min/g), while the highest activity was found in 400 μM treatment (39.59 U/min/g) with significant difference to other treatment, followed by treatments 300 μM (32.12 U/min/g) and 200 μM (26.85 U/min/g), with a note that, no significant difference between 200 and 300 μM treatments.

The results showed that, H_2O_2 production increased up to 3 fold in date palm leaves by increasing NaCl concentration to 400 μM compared to those in control treatment. H_2O_2 is one kind of reactive oxygen species (ROS), with different role in plants (Abass and Morris, 2013), among them, it has been suggested that, H_2O_2 act as cellular signal (Prasad *et al.*, 1994), and accumulation of H_2O_2 may be due to increasing of Superoxide dismutase (SOD) enzyme activity, in which this enzyme catalyzes the dismutation of anion free radical to O_2 and H_2O_2 (Meloni *et al.*, 2003). The increase of hydrogen peroxide levels was accompanied with an increase in peroxidase activity, Lin and Kao (2002) stated that, peroxidase enzyme protecting cell from the destructive influence of H_2O_2 . The increase of peroxidase activity in date palm leaves under salinity stress reported in previous works for other cultivar, such Hillawi (Abdulwahid, 2012) and Bartomouda (Darwesh, 2013).

Malondialdehyde (MDA) and Membrane stability index (MSI).

Malondialdehyde content was measured in date palm leaves as indicator of lipid peroxidation, the results in Fig (3) revealed that, no significant effect of 100 μM of NaCl in MDA content, however, the increase of NaCl induced MDA accumulation in date palm leaves, MDA content was 2.35 nmole/g in control treatment, while reached to 3.95 nmole/g in 400 μM as highest value with significant difference to other treatments, also results showed, the MDA content in 300 μM was 2.45 nmole/g without significant difference with 200 μM which 2.39 nmole/g, but these treatments superior significantly of 100 μM and control treatments. The high lipid peroxidation in salinity stressed date palm plants was accompanied with a significant reduction in membrane stability, the obtained results showed in Fig. (4) was indicated to reduction MSI from 81.30 % in non-stressed plant to 64.15 % in 400 μM treatment with significant difference to other treatments, while treatment 300 μM recorded 73.48 % without significant difference compared 200 μM treatments which 75.23 %.

MDA is considered as a cytotoxic end product of lipid peroxidation (Agadjanyan *et al.*, 2006), as well known biotic and abiotic stress including salinity induced ROS production and these molecules attack on phospholipid molecule present in polyunsaturated fatty acids (PUFA), and product MDA which is responsible of membrane damage (Sharma *et al.*, 2012), also Howladar (2014) reported a well correlate between increase of H_2O_2 level and lipid peroxidation. Salt stress has also been related to lipid peroxidation and membrane damage (Azooz, 2009). Membrane damage and lipid peroxidation was reported in date palm leaves under other abiotic stress such as heavy metals (Abass *et al.*, 2016; Zouari *et al.*, 2016).

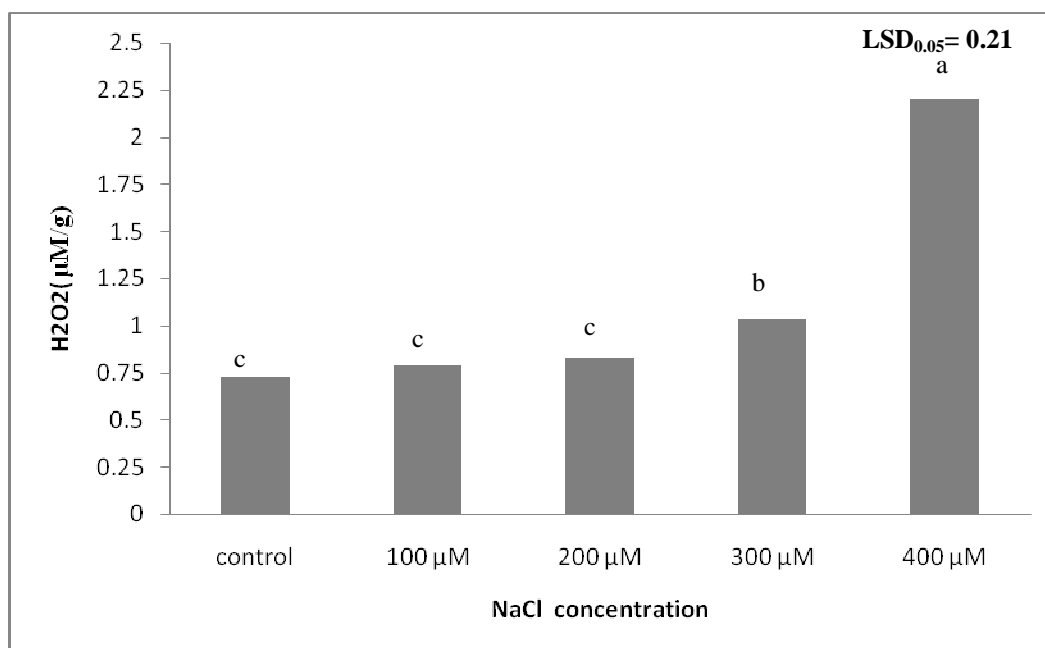


Fig (1) H_2O_2 concentration in ($\mu\text{M}/\text{g}$) date palm Barhi cv. leaves under different salinity treatments.

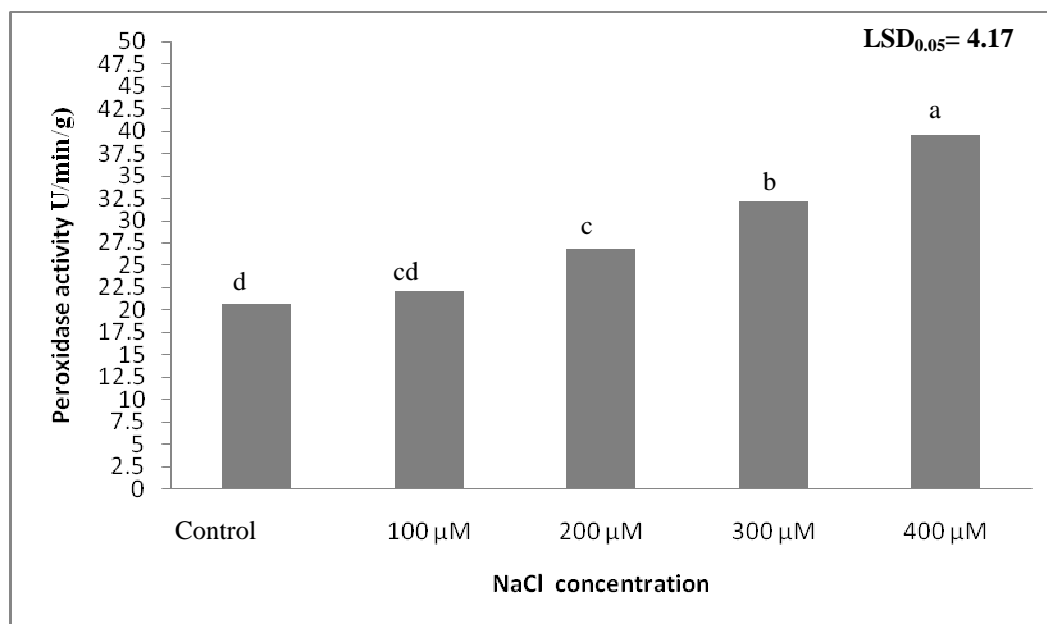


Fig (2) Peroxidase activity (U/min/g) in date palm Barhi cv. leaves under different salinity treatments.

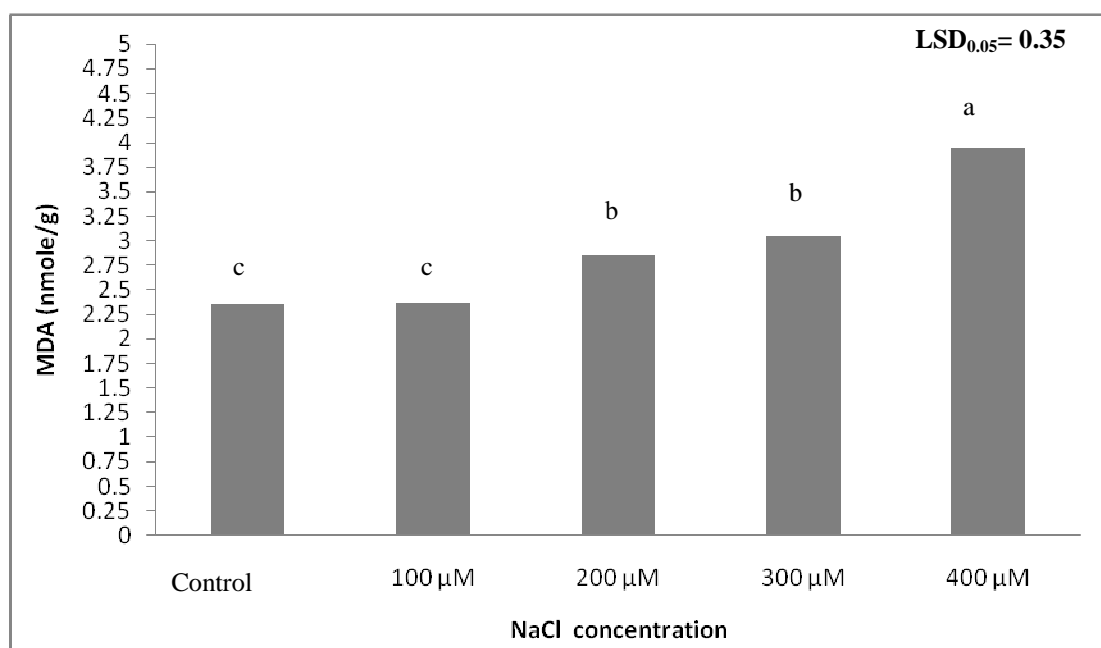


Fig (3) MDA (nmole/g) concentration in date palm Barhi cv. leaves under different salinity treatments.

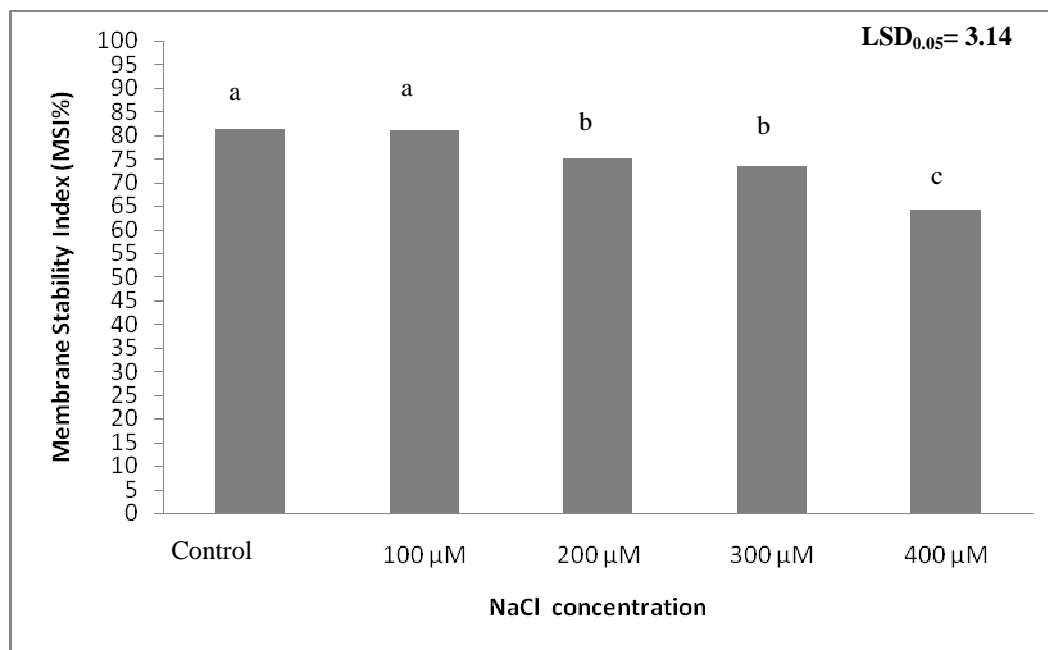


Fig (4) MSI (%) in date palm Barhi cv. leaves under different salinity treatments.

Genetic stability in date palm leaves under salinity stress.

ISSR primers amplification

Using five ISSR primers, one could not amplify DNA, and four gave clear results (818, 822, 834, 855), and these primers were generated a total of 97 reproducible and clear fragments, with average 24.25 bands for each primer, 19 out of them were polymorphic, and size of fragments ranging from 200 to 2100 bp. The results of primers parameters presented in table (2) showed that, the primer 818 produced 19 fragments; it is considered the lowest compared to other primers, with 7 polymorphic fragments, and the size of generated fragments between 560-2000 bp, with efficiency of 19.58% and 36.84% for polymorphism and discrimination power, also the results showed the primer 822 recorded the highest number of a total fragments compared to other primers, which this primer was generated 32 fragments, and the size of fragments ranging from 56 to 2100 bp, with 4 polymorphic, and recorded an efficiency 32.98%, with 12.5 and 21.05 % for polymorphism and discrimination power, respectively. Additionally, the tested primer 834 produced 21 fragments as a total number of fragments, only one fragment was polymorphic, and the size of fragments ranging between 540 and 200 bp, with 21.64, 4.76 and 5.26 % as efficiency, polymorphism and discrimination power, respectively, while the primer 855 generated 25 fragments, 7 out of them as polymorphic fragments, with 25.77 % efficiency, 28 % polymorphism and 36.84% discrimination power.

According to ISSR analysis, the results in Fig. (5 a-d) showed the profile of control and 100 μM NaCl treatments was identical, also the 200 and 300 μM profiles was identical, but with different fragment profile compared to control treatment, while the increase of NaCl concentration to 400 μM showed a difference compared to other fragments profiles of other treatments, a new appearance and disappearance was distinguishable of fragments profile for this treatment.

Genetic Similarity Index

The results presented in table (3) showed similarity indices results of ISSR markers analysis, and the data revealed that, the highest average GSI was recorded between control and 100 μM treatments, which was 1, followed by 200 and 300 μM treatments, which was 0.86, while the lowest GSI was recorded between 400 μM and control treatments, which was 0.72.

The UPGAM dendrogram Fig (6) indicated that, the treatments grouped in three clusters, the first cluster had control and 100 μM treatments, second cluster had 200 and 300 μM treatments, while 400 μM treatment was separated in third cluster.

Results of this work was revealed the suitability of ISSR technique to detect the genotoxicity of salinity stress, this is indicated by previous works, that conducted on other plants, such as wheat (Lang *et al.*, 2001), rice

(Kaushik *et al.*, 2003), barely (Khatab and Samah, 2013) and sugarcane (Markad *et al.*, 2014). Change in DNA profile under high concentrations of NaCl compared to non-stressed plant could be attributed to point mutation; this mutation (including insertion or deletion) led to appearance new amplicons or disappearance exiting fragments (Atienzar and Jha, 2006; Liu *et al.*, 2012).

Table (2) ISSR primers parameters

| Primer ID | Range of bands size (bp) | Total bands | Polymorphic bands | Primer efficiency (%) | Primer polymorphism (%) | Discrimination power (%) |
|-----------|--------------------------|-------------|-------------------|-----------------------|-------------------------|--------------------------|
| 818 | 560-2000 | 19 | 7 | 19.58 | 36.84 | 36.84 |
| 822 | 560-2100 | 32 | 4 | 32.98 | 12.5 | 21.05 |
| 834 | 540-2000 | 21 | 1 | 21.64 | 4.76 | 5.26 |
| 855 | 200-1700 | 25 | 7 | 25.77 | 28 | 36.84 |
| Total | | 97 | 19 | | | |

Table (3) Similarity indices of Nie and Li coefficient of date palm produced by different NaCl concentrations obtained from ISSR markers.

| Treatment | Control | 100 μ M | 200 μ M | 300 μ M | 400 μ M |
|-------------|---------|-------------|-------------|-------------|-------------|
| control | 1 | | | | |
| 100 μ M | 1 | 1 | | | |
| 200 μ M | 0.86 | 0.86 | 1 | | |
| 300 μ M | 0.86 | 0.86 | 1 | 1 | |
| 400 μ M | 0.72 | 0.72 | 0.70 | 0.70 | 1 |

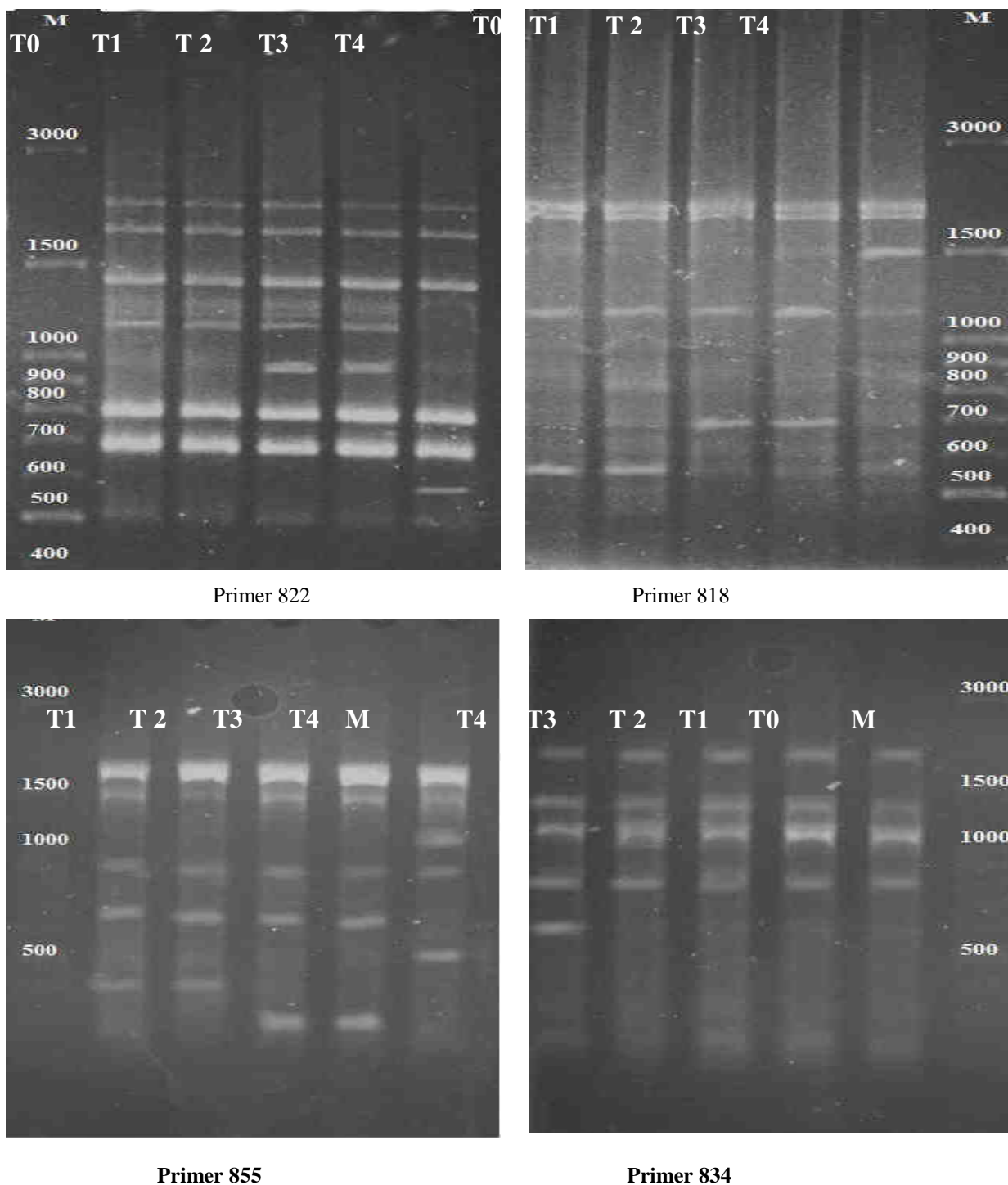


Fig (5): ISSR markers analysis of date palm produced of different NaCl concentrations: M: DNA marker, T0: control; T1: 100 μ M; T2: 200 μ M; T3: 300 μ M; T4: 400 μ M.

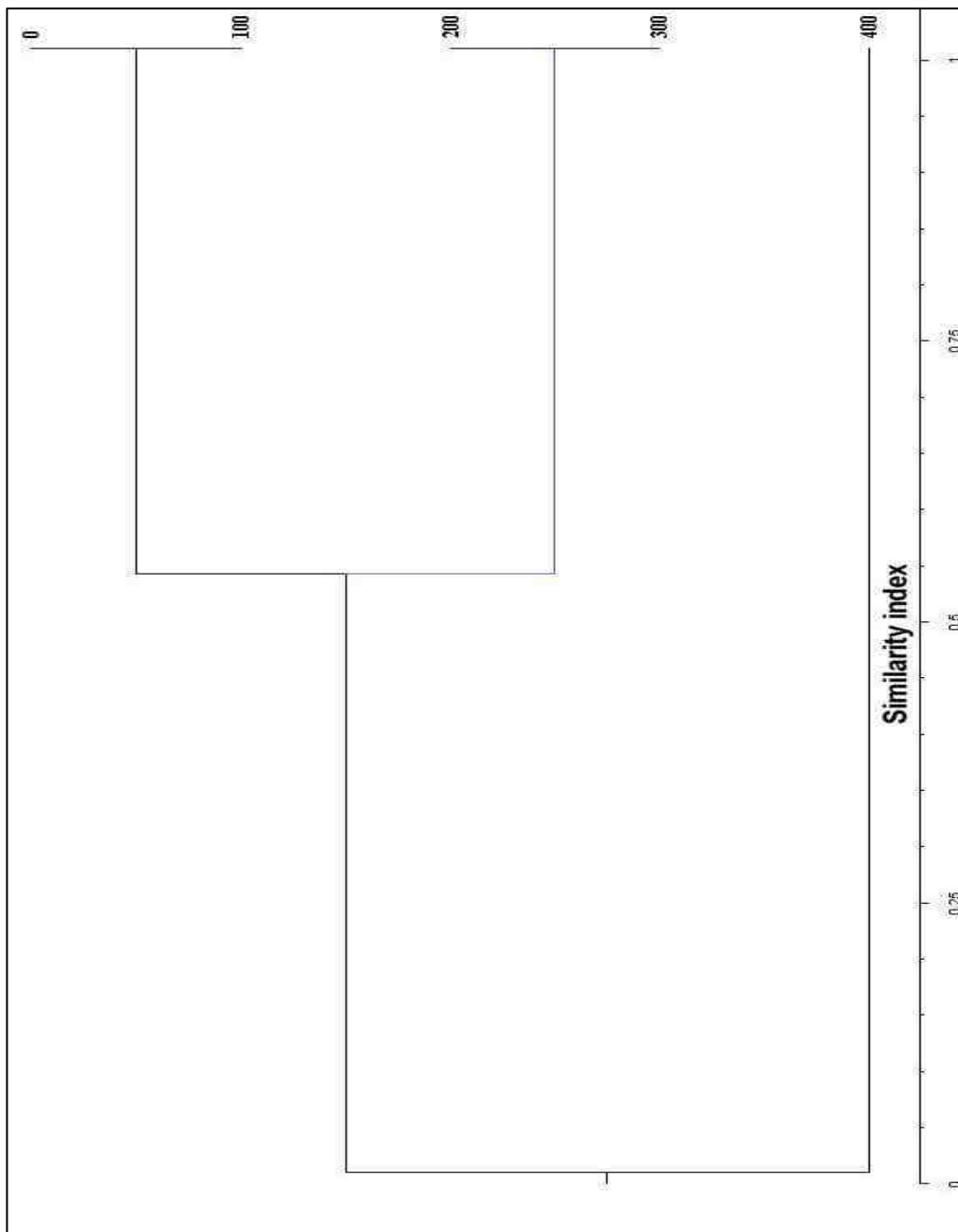


Fig (6) Dendrogram generated by UPGAM cluster method based on ISSR markers of date palm produced by different concentrations of NaCl treatments.

4-Conclusion

The results of this study demonstrated, date palm responded to high concentrations of NaCl (200-400 μ M) by accumulation of H₂O₂ and increase peroxidase activity, this indicates to oxidative stress, also salinity stress caused damage to membranes, and that through increase of MDA concentration and reduction of membrane stability index. Also the results showed the possibility of using ISSR markers to detect DNA damage in date palm under salinity stress.

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