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# Biochemical and Molecular Genetic Markers Associated with Salinity tolerance in Flax (*Linum usitatissimum* L.)

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**ABSTRACT**

Flax is considering one of the most important oil crops where its seeds contribute to bridging the gap in the production of healthy oils such as hot oil. Further, it is considered an important source for manufacturing of textiles and fabrics besides, the manufacture of particleboard, banknote and other strategic industries. This investigation was launched with great effectiveness in view of the high level of soil salinity in lands prepared for cultivating of flax and its dangerous effect on all stages of its germination and final yield. Also, the present investigation was conducted to know the genetic behavior associated with salt-stress tolerance in some flax cultivars with various response to salt stress. Yield and its components and some physiological traits related to salinity tolerance were the most measurements evaluated under control experiment and both salinity treatments. Molecular genetic markers were used to identify the molecular genetic differences among the five flax cultivars. As well as, identifying the impact of salinity- stress on water soluble protein and enzymes important for all biological and biochemical processes such as peroxidase (POD) and polyphenol oxidase (PPO) isozymes in flax cultivars. Results confirmed that the three flax cultivars; Giza 5, 6 and 7 exhibited highly rank of salinity tolerance for all studied traits under both salinity levels compared to the standard experiment. Further, the first salinity level was the safest limit that plants can tolerate with a slight loss in the final yield. Profile of ISSR analysis succeed in comparing among the previous flax cultivars through generating a total of 11 polymorphic bands with 37.93% polymorphism. Also, results of biochemical studies proved that the salt stress had a clear effect on protein and both types of isozymes in all flax cultivars under study, especially the second level of salting.

Keywords: - Flax, Salinity Tolerance, Yield and its components, Biochemical Markers, ISSR primers.

## Introduction

Flax is considered one of the strategic oil crops, which is of great importance on the medical, food and industrial levels as well. Flax seed is multi-use and is grown for commercial purposes. After extracting the oil, a set of treatments are performed to increase its nutritional and export efficiency **[1-3].**In addition, the oil extracted from flax has many uses in cooking**[4].**High salinity level in soil and irrigation water is considered one of the most serious environmental obstacles that limits crop production especially flax which in turn leads to decay in the final yield of agricultural production and reflected in the countries' economy significantly **[5].**Saline stress is causing negatively impacts on all physiological, biochemical, and biological processes besides, metabolism processes that all participate in the final plant growth process **[6].** For all these reasons combined, the main trend of scientists in this regard is genetic improvement to raise salinity tolerance in flax besides, high yielding through using traditional plant breeding programs along with modern scientific trends of biotechnology and genetic engineering programs. As well as, screening a large number of flax genotypes and knowing their reaction to salinity tolerance in order to use the most tolerant genotypes in breeding programs for improving the Egyptian flax crop and increase salinity tolerance in it. Seven flax genotypes were evaluated under various salinity stress levels in both experiment by **[7]** and revealed that the flax entries Sakha 1 and 2 exhibited the highest limit of salinity tolerance in this regard. Exogenous implementation of ascorbic acid for attenuation the counteractive impacts of salt- stress in some flax genotypes were showed by **[6].** Results revealed that salt-stress inspired significant and imperceptible damaged in all growth traits in the leaves of 3 flax accessions with increasing salinity limits except proline which confirmed an increase compared to the normal plants. As well as, data of UPGMA cluster analysis using SRAP markers had clustered all flax genotypes into two main cluster where each cluster contains the highest closed accessions together according their response to salinity stress. Also, salinity stress caused highly moral impacts on the content of measured biochemical parameters in flax **[8].**Thus, plants have neutralized reactive oxygen species (ROS) produced under salinity stress conditions using a complex antioxidant system which consists of antioxidant enzymes such as superoxide dismutase, catalase, polyphenol oxidase and peroxidase and non- enzymatic antioxidants including ascorbic acid carotenoids and glutathione **[9].** After all that has been presented, the objectives of

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this study can be summarized in determining the biochemical and molecular genetic markers responsible for salinity tolerance in some flax genotypes. In addition, sifting all flax accessions and knowing their reaction to salinity tolerance as an attempt to use it in plant breeding programs for the advancement and genetic improvement in Egyptian flax varieties for salt-stress tolerance through transferring tolerance genes to new flax lines high sensitive to salt stress.

## Materials and Methods

### Plant Materials:-

The present investigation was carried out in lyzimeters units at Sakha Research Station including two salinity levels (4dsm$^{-1}$ and 6dsm$^{-1}$) besides, the control experiment (Tap water) in Agriculture research centre, Sakha city, Kafrel-sheikh governorate, Egypt. A split plot design with three replicates was used in this investigation during 2017/2018 and 2018/2019 seasons using 5 flax cultivars with various response to salinity tolerance, table (1). The three salinity levels were in main plot and the 5 flax cultivars in sub-plot in both growing seasons, respectively.

**Table 1: Classification of parental flax cultivars used in this study and their origin.**

| Parental Cultivars | Origin | Reaction to Salinity |
|---|---|---|
| **Giza 5** | **Local Variety** | **Tolerant** |
| **Giza 6** | **Local Variety** | **Tolerant** |
| **Giza 7** | **Local Variety** | **Tolerant** |
| **Sakha 1** | **Local Variety** | **Moderate** |
| **Sakha 2** | **Local Variety** | **Moderate** |

### Soil analysis:-

Before conducting all experiments, soil samples were taken from different sites of each experiment. Each sample was taken from a depth of 0-30 cm from each treatment. The chemical analysis was carried out for each soil extract 1:5 to estimate the soluble anions, cations and total dissolved salts (TDS). The electrical conductivity (EC) was estimated in the extract of the soil saturate paste. The procedure for preparation and measurements of the soil extract was taken according to the method **of [10].**The methods of **[11]** of soil chemical analysis were followed. The description of the three soil experiments used in this investigation after salting are shown in table (2).

**Table: 2 Chemical Classification of normal and both levels of salinity soils during two growing seasons.**

| Characteristics | Normal soil (Tap water) | | Level I of Salinity (4 dsm$^{-1}$) | | Level II of Salinity (6 dsm$^{-1}$) | |
|---|---|---|---|---|---|---|
| | 2017/2018 season | 2018/2019 season | 2017/2018 season | 2018/2019 season | 2017/2018 season | 2018/2019 season |
| **EC (dS/m)** | 0.5 | 0.5 | 4.0 | 4.0 | 6.0 | 6.0 |
| **pH (1:2.5)** | 8.0 | 8.0 | 8.07 | 8.06 | 8.14 | 8.11 |
| **TDS mg/ litre (ppm)** | 378.0 | 396.0 | 3745.0 | 3755.0 | 5014.23 | 5026.34 |
| **Ca++** | 2.49 | 2.33 | 11.67 | 10.69 | 13.87 | 13.04 |
| **Mg++** | 1.58 | 1.53 | 10.34 | 10.07 | 12.95 | 11.53 |
| **Na+** | 10.68 | 10.17 | 21.56 | 20.79 | 35.87 | 32.47 |
| **K+** | 0.24 | 0.19 | 0.32 | 0.37 | 0.71 | 0.68 |
| **$CO3^{--}$** | 0.07 | 0.04 | 0.14 | 0.11 | 0.08 | 0.05 |
| **$HCO3^{-}$** | 3.56 | 3.75 | 1.88 | 1.73 | 1.63 | 1.58 |
| **$Cl^{-}$** | 12.37 | 11.93 | 25.08 | 25.21 | 38.14 | 36.74 |
| **$SO4^{-}$** | 3.64 | 3.61 | 15.16 | 14.85 | 27.94 | 27.66 |
| **Texture** | Clay | Clay | Clay | Clay | Clay | Clay |

**EC = Electrical conductivity, TDS = Total dissolved salts, * Measure of soil saturation, ** Measure of soil water extract 1:5**

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**System of salinization in lyzimeter units:**-1):- Normal experiment (Tap water):- Irrigation was done using normal water at E.C (0.5) ds/$m^{-1}$ or 500 ppm, 2):- Level I of salinity: - It was irrigated with saline water at E.C (4) ds/$m^{-1}$ or 4000 ppm, 3):- Level II of salinity: - It was irrigated with saline water at E.C (6) ds/$m^{-1}$ or 6000 ppm and stock solution was prepared using NaCl and CaCl2 at the ratio of (2:1), respectively. Lyzimeter unit was 10 m long and 5 m width in addition, 120 cm depth.

**Measured traits**

Fifty plants were taken randomly at maturity from each replicate of each experiment for each genotype to evaluate the following attributes:1):- Seed yield/plant (g), 2):- 1000-seed weight (g), 3):- Number of capsules/plant, 4):- Number of seeds/capsule, 5):- The proline content: - it was determined according to **[12]** and modified method by**[13].** 6):- Glycine betaine contents: - It was carried out according to the method of [**14]** and 7):- Osmotic adjustment: - It was determined by the formula of **[15].** Fresh leaf samples were taken after 45 days of planting, given that irrigation with saline water for each experiment was carried out from the first day of planting.

**Statistical analysis**

The obtained data were subjected to analysis of variance according to **[16].** Treatment averages were compared by Duncan's Multiple Range Test**[17]**. All statistical analyses were performed using analysis of variance technique employing the "COSTAT" computer software package.

**Salinity tolerance indices:-**

All salinity tolerance indices for the five flax cultivars were estimated using seed yield/plant trait only according to **[18-24].**

**Molecular Markers:-**

**ISSR-PCR technique**

**DNA Extraction**

DNA extraction for fresh leaves of the 5 flax cultivars was performed as described by **[25].**

**Inter Simple Sequence Repeat ISSR-PCR Analysis**

ISSR analysis for 6 ISSR primers (Table 5) were performed as described by **[26, 27].**

**Gel documentation:**

Gels were photographed scanned, analyzed using Gel Doc Vilber Lourmat system (Vilber Company, France) to capture the image and to calculate band intensities.

**Data handling and Phylogenetic tree (Cluster analysis)**

It was performed according to **[28, 29].**

**Biochemical Studies**

**Protein Determination:** Protein concentration was measured according to **[30].**

**SDS-protein electrophoresis**: SDS-PAGE was according for the five flax cultivars according to the method of **[31]** as modified by **[32].**

**Isozymes electrophoresis**

It was conducted according to **[33-35].**

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## Results

### Mean performance

Mean values of all studied traits for the control experiment and both salinity stress treatments was viewed in table (3).Initially, most of flax cultivars showed significant and highly significant differences for all treatments of all attributes under study in both growing seasons (2017/2018 and 2018/2019) through using duncan's test. For tap water treatment; the flax cultivars; Giza (5, 6 & 7) were recorded the highest mean values for all studied traits in both seasons and combined analysis. Whereas, the osmotic adjustment trait did not have any calculated data in the standard experiment for the two growing seasons. This is the normal case in calculating this trait because it is calculated under both salinity stress compared to the normal conditions. With respect to both salinity levels (4 $dsm^{-1}$ and 6 $dsm^{-1}$), the flax cultivars; Giza 5, Giza 6 and Giza 7 exhibited the highest mean values for the traits; seed yield/plant, 1000-seed weight, number of capsules/plant, number of seeds/capsule and proline content in both seasons besides, the combined analysis. While, the cultivars; Giza 5, Giza 6 and Sakha 1 were recorded the highest rank in glycine betaine content and osmotic adjustment traits under the same treatments. It is worth noting that, the negative effect of the second level of salinity stress (6 $dcm^{-1}$) on the five flax cultivars was higher than the first salinity stress (4 $dcm^{-1}$) compared to the control treatment. Also, the most salinity-tolerant cultivars which were recorded high yielding under the first salinity treatment conditions were Giza 5, 6 and 7 in all studied attributes. While, Sakha 1 and 2 were coming in the second rank in this regard. Accordingly, it can be seen that the first salt stress level was the safest limit compared to the second salinity stress level.

### Salinity tolerance indices parameters:-

Data viewed in table (4) revealed that the flax cultivars; Giza 5, Giza 7 and Sakha 2 for both levels of salinity stress in the first growing season were exhibited the highest mean values for YSI parameter. While, Giza 5, Sakha 1 and Sakha 2 for the first salt stress level in the first season besides, Giza 5, Giza 6 and Giza 7 for the second salinity level in the second year were recorded also the same results for the same parameter, respectively. In the same track, the flax cultivars; Sakha 5 and 6 were recorded the highest mean values for both salinity stress levels in the two growing seasons for MP and GMP parameters. The flax cultivars Giza 5, 6 and 7 for both salt stress levels in both seasons were recorded mean values higher than the unity for YI parameter. In the same context, Giza 5 and 6 were exhibited mean values higher than one for both salinity levels in the two growing season for STI parameter except, the flax cultivar Giza 5 for the second level of salinity in the first season where it was lower than one, respectively. On the other hand, all flax cultivars were exhibited mean values lower than one in the two salinity stress levels for the two growing seasons for YR parameter. Also, the cultivars; Giza 5, Giza 7 and Sakha 2 for salinity stress level one besides, Giza 5 and Giza 7 for salinity level two in the first season were recorded mean values lower than one for SSI parameter. While in the second season, Giza 5, Sakha 1 and Sakha 2 for the first salinity stress level and Giza 5 and Giza 7 only for the second salinity stress were exhibited the same results for SSI parameter in (Table 4), respectively.

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**Table 3: Mean values of all studied attributes for the five flax cultivars under the control and both salinity levels during the two growing seasons.**

| Salinity levels | Cultivars | 2017/2018 and 2018/2019 seasons | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Attributes | | | | | | | | | | | | | | | | | | | |
| (Main plot) | (Sub-pot) | Seed yield/plant (gm) | | | 1000-seed weight (gm) | | | Number of capsules/plant | | | Number of seeds/capsule | | | Proline Content | | | Glycine betaine contents | | | Osmotic adjustment | | |
| | | F.S | S.S | Comb. | F.S | S.S | Comb. | F.S | S.S | Comb. | F.S | S.S | Comb. | F.S | S.S | Comb. | F.S | S.S | Comb. | F.S | S.S | Comb. |
| (Tap water) | Giza 5 | 1.23 bc | 1.36a | 1.29b | 12.04b | 12.32a | 12.18b | 12.56b | 13.23ab | 12.89b | 12.23ab | 12.05a | 12.14ab | 34.16b | 28.25c | 31.20b | 48.10a | 39.43a | 43.76a | __ | __ | __ |
| | Giza 6 | 1.45 a | 1.48a | 1.46a | 14.43a | 13.98a | 14.20a | 14.05a | 14.67a | 14.36a | 13.54a | 12.97a | 13.25a | 45.33a | 39.04a | 42.18a | 37.05b | 33.12b | 35.08b | __ | __ | __ |
| | Giza 7 | 0.95 c | 1.03bc | 0.99c | 11.67bc | 12.01ab | 11.84bc | 10.31c | 10.56b | 10.43c | 10.52bc | 10.48b | 10.50b | 26.07cd | 25.0d | 25.53cd | 22.06d | 19.67d | 20.86d | __ | __ | __ |
| | Sakha 1 | 0.74 cd | 0.78cd | 0.76cd | 8.66cd | 8.55bc | 8.60c | 8.44cd | 8.69d | 8.56d | 7.22d | 7.05cd | 7.13c | 29.05c | 32.11b | 30.58bc | 26.15c | 23.44c | 24.79c | __ | __ | __ |
| | Sakha 2 | 0.55 d | 0.57d | 0.56d | 8.04d | 8.13c | 8.08cd | 9.15d | 9.23cd | 9.19cd | 9.01cd | 8.94c | 8.97bc | 22.14d | 24.13de | 23.13d | 18.76e | 15.32e | 17.04e | __ | __ | __ |
| 4 $dsm^{-1}$ | Giza 5 | 0.94 a | 1.06a | 1.0a | 10.77ab | 10.84ab | 10.80ab | 10.07b | 10.04ab | 10.05ab | 10.08ab | 9.56b | 9.82b | 67.23b | 69.44b | 68.33b | 55.89b | 51.03b | 53.46b | 0.48bc | 0.30cd | 0.39c |
| | Giza 6 | 0.87 bc | 0.93ab | 0.90a | 11.14a | 11.22a | 11.18a | 12.09a | 11.78a | 11.93a | 11.34a | 11.67a | 11.50a | 78.13a | 73.86a | 75.99a | 61.0a | 57.33a | 59.16a | 0.62b | 0.57c | 0.59bc |
| | Giza 7 | 0.73 c | 0.71bc | 0.72ab | 9.22bc | 9.43b | 9.32bc | 9.23bc | 9.05bc | 9.14bc | 9.07bc | 9.23bc | 9.15bc | 41.67c | 44.01c | 42.84c | 17.34e | 14.28e | 15.81e | 1.23a | 1.18ab | 1.20b |
| | Sakha 1 | 0.51 cd | 0.58cd | 0.54bc | 7.01cd | 7.42bc | 7.21cd | 6.93d | 6.83d | 6.88d | 6.28d | 6.37cd | 6.32d | 37.83d | 40.28d | 39.05d | 35.32c | 39.40c | 37.36c | 0.86ab | 0.75bc | 0.80ab |
| | Sakha 2 | 0.41 d | 0.43d | 0.42cd | 6.45d | 6.39c | 6.42d | 7.33c | 7.12cd | 7.22cd | 7.44cd | 7.16c | 7.30cd | 30.08e | 27.18e | 28.63e | 24.25d | 27.19d | 25.72d | 1.42a | 1.53a | 1.47a |
| 6 $dsm^{-1}$ | Giza 5 | 0.78 a | 0.85a | 0.81a | 8.02ab | 8.19ab | 8.10ab | 8.64b | 8.54b | 8.59b | 8.76b | 8.55b | 8.65ab | 82.15b | 85.19b | 83.67b | 71.19a | 74.02b | 72.60a | 0.71bc | 0.35cd | 0.53bc |
| | Giza 6 | 0.69 bc | 0.74ab | 0.71a | 9.34a | 9.28a | 9.31a | 10.47a | 10.43a | 10.45a | 10.02a | 9.79a | 9.90a | 94.27a | 91.48a | 92.87a | 68.89ab | 78.11a | 73.50a | 0.48c | 0.18d | 0.33c |
| | Giza 7 | 0.52 cd | 0.57bc | 0.54ab | 7.78bc | 7.93bc | 7.85bc | 7.43bc | 7.15bc | 7.29c | 8.31b | 8.04bc | 8.17bc | 52.32c | 54.85c | 53.58c | 41.06cd | 48.93c | 44.99b | 1.36a | 1.11ab | 1.23a |
| | Sakha 1 | 0.28 d | 0.32cd | 0.30bc | 5.35cd | 5.41cd | 5.38cd | 5.14cd | 5.07cd | 5.10d | 5.83d | 5.76d | 5.79cd | 43.0d | 46.11d | 44.55d | 42.67c | 46.45d | 44.56b | 1.09ab | 0.94bc | 1.01ab |
| | Sakha 2 | 0.28 d | 0.26d | 0.27c | 4.92d | 5.03d | 4.97d | 6.04d | 5.78c | 5.91cd | 6.19c | 6.15cd | 6.17c | 36.87e | 34.07e | 35.47e | 31.05d | 36.83e | 33.94c | 1.54a | 1.39a | 1.46a |
| Interactions C X S.L | | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| F. test | | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |

**F.S:- First season, S.S:- Second season, Comb.:- Combined among two seasons, C: - Cultivars and S.L. Salinity levels.**

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**Table 4: Estimation of salinity tolerance indices for the five flax cultivars especially for seed yield trait under normal and both levels of salinity during the two growing season.**

| Cultivars | Salinity Tolerance Indices (4 $dsm^{-1}$) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Season 2017/2018 | | | | | | | | | Season 2018/2019 | | | | | | | | |
| | GYP | GYS | YSI | YI | MP | STI | GMP | YR | SSI | GYP | GYS | YSI | YI | MP | STI | GMP | YR | SSI |
| Giza 5 | 1.23 | 0.94 | 0.76 | 1.36 | 1.08 | 1.19 | 1.07 | 0.24 | 0.82 | 1.36 | 1.06 | 0.77 | 1.43 | 1.21 | 1.33 | 1.20 | 0.23 | 0.79 |
| Giza 6 | 1.45 | 0.87 | 0.60 | 1.26 | 1.16 | 1.30 | 1.12 | 0.40 | 1.37 | 1.48 | 0.93 | 0.62 | 1.25 | 1.20 | 1.27 | 1.17 | 0.38 | 1.31 |
| Giza 7 | 0.95 | 0.73 | 0.76 | 1.05 | 0.84 | 0.71 | 0.83 | 0.24 | 0.82 | 1.03 | 0.71 | 0.68 | 0.95 | 0.87 | 0.67 | 0.85 | 0.32 | 1.10 |
| Sakha 1 | 0.74 | 0.51 | 0.68 | 0.73 | 0.62 | 0.38 | 0.61 | 0.32 | 1.10 | 0.78 | 0.58 | 0.74 | 0.78 | 0.68 | 0.41 | 0.67 | 0.26 | 0.89 |
| Sakha 2 | 0.55 | 0.41 | 0.74 | 0.59 | 0.48 | 0.23 | 0.47 | 0.26 | 0.89 | 0.57 | 0.43 | 0.75 | 0.55 | 0.50 | 0.22 | 0.49 | 0.25 | 0.86 |
| Cultivars | Salinity Tolerance Indices (6 $dsm^{-1}$) | | | | | | | | | | | | | | | | | |
| | Season 2017/2018 | | | | | | | | | Season 2018/2019 | | | | | | | | |
| | GYP | GYS | YSI | YI | MP | STI | GMP | YR | SSI | GYP | GYS | YSI | YI | MP | STI | GMP | YR | SSI |
| Giza 5 | 1.23 | 0.78 | 0.63 | 1.52 | 1.0 | 0.99 | 0.97 | 0.37 | 0.77 | 1.36 | 0.85 | 0.62 | 1.57 | 1.10 | 1.06 | 1.07 | 0.38 | 0.80 |
| Giza 6 | 1.45 | 0.69 | 0.47 | 1.35 | 1.07 | 1.03 | 1.0 | 0.53 | 1.10 | 1.48 | 0.74 | 0.50 | 1.37 | 1.11 | 1.0 | 1.04 | 0.50 | 1.06 |
| Giza 7 | 0.95 | 0.52 | 0.54 | 1.01 | 0.73 | 0.51 | 0.70 | 0.46 | 0.95 | 1.03 | 0.57 | 0.55 | 1.05 | 0.80 | 0.53 | 0.76 | 0.45 | 0.95 |
| Sakha 1 | 0.74 | 0.28 | 0.37 | 0.54 | 0.51 | 0.21 | 0.45 | 0.63 | 1.31 | 0.78 | 0.32 | 0.41 | 0.59 | 0.55 | 0.22 | 0.49 | 0.59 | 1.25 |
| Sakha 2 | 0.55 | 0.28 | 0.50 | 0.54 | 0.41 | 0.15 | 0.39 | 0.50 | 1.04 | 0.57 | 0.26 | 0.45 | 0.48 | 0.41 | 0.13 | 0.38 | 0.55 | 1.17 |

GYP: Yield under control conditions, GYS: Yield under stress conditions, YSI: Yield stability index, YI: Yield index, YR: Yield reduction, MP: Mean of normal and stress yield, GMP: The square of the yield under control conditions and the yield under salinity treatment, STI: Salinity tolerance index and SSI: Salinity susceptibility index.

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## Molecular and biochemical studies

## Molecular Characterization

## Profile of ISSR analysis

The six ISSR primers namely; 98 A, HB-10, 11, 12, 13 and 14 generated a total of 29 bands (18 of them were monomorphic and 11 polymorphic) with 37.93 % polymorphism as presented in table (5) and (Fig.1). The average numbers of polymorphic ISSR markers were 1.83 fragments for each primer. Polymorphic fragments number ranged from 1 to 3 and molecular size ranging from 915-200 bp. The highest number of polymorphic bands (3) were showed in primers 98 A and HB-12 primer for each one of them followed by HB-10 and HB-11 primers where they recorded (2) fragments for each one of them and then followed by HB-14 primer (only one band), respectively. Further, HB-13 primer no recorded any polymorphic bands in this regard. Data shown in (Table 5) confirmed that the highest polymorphism % (50.0%) was observed in primers 98 A, HB-10, HB-11 and HB-12 for both of them. Whatever, the lowest polymorphism % (16.66%) was obtained in HB-14 primer. In the same context, the highest total number of bands (6) were observed in primers 98 A, HB-12 and HB-14 for each one of them, followed by primers HB-10 and HB-11 (4) and then followed by the primer HB-13 where it exhibited (3) bands. Results presented in (Table 6) revealed that the flax cultivars Giza 5 and Giza 6 exhibited the highest number of amplified fragments (25) for both of them, followed by Sakha 1 (24), followed by Sakha 2 (23) and then followed by Giza 7 (22), respectively. It is noted that, primers 98 A, HB-12 and HB-14 exhibited the highest number of fragments (23, 22 and 27) for each one of them in all flax cultivars under study. While HB-10 and HB-13 primers generated the lowest number of amplicons (15) for the same flax materials.

**Table 5: Band variation and polymorphism percentage in 5 flax cultivars using 6 ISSR primers**.

| No | ISSR Primers | Total Bands | Molecular Siza (bp) | Monomorphic bands | Polymorphic bands | Polymorphism % | **Sequence** |
|---|---|---|---|---|---|---|---|
| 1 | 98 A | 6 | 815-280 | 3 | 3 | 50% | 5` CAC ACA CAC ACA AG 3` |
| 2 | HB-10 | 4 | 735-200 | 2 | 2 | 50% | 5` GAG AGA GAG AGA CC 3` |
| 3 | HB-11 | 4 | 670-230 | 2 | 2 | 50% | 5` GTG TGT GTG TGTTGT CC 3` |
| 4 | HB-12 | 6 | 915-240 | 3 | 3 | 50% | 5`CAC CAC CAC GC 3` |
| 5 | HB-13 | 3 | 480-230 | 3 | - | - | 5´ GAG GAG GAG C 3` |
| 6 | HB-14 | 6 | 875-420 | 5 | 1 | 16.66% | 5` CTC CTC CTC GC 3` |
| Total | | 29 | 915-200 | 18 | 11 | 37.93% | |

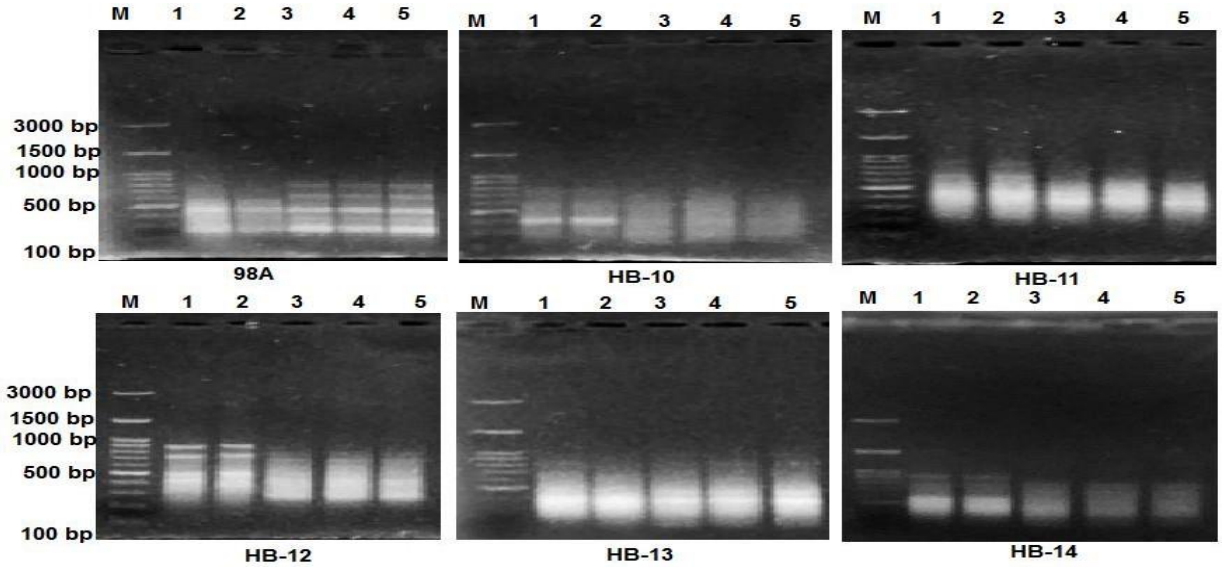


**Figure 1: The inter-simple sequence repeat (ISSR) amplification pattern observed in the five flax cultivars namely; (a): primer 98A, (b): primer HB-10, (c): primer HB-11, (d): HB-12, (e): HB-13 and (f): HB-14 and the numbers from 1 to 5 are meaning the five flax cultivars namely; Giza 5, Giza 6, Giza 7, Sakha 1 and Sakha 2, respectively.**

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**Table 6: Total bands obtained from the 6 ISSR primers of 5 flax cultivars and all amplified fragments for each genotype.**

| Cultivars | Primers | | | | | | |
|---|---|---|---|---|---|---|---|
| | 98 A | HB-10 | HB-11 | HB-12 | HB-13 | HB-14 | Total |
| Giza 5 | 4 | 3 | 4 | 5 | 3 | 6 | 25 |
| Giza 6 | 4 | 3 | 4 | 5 | 3 | 6 | 25 |
| Giza 7 | 5 | 3 | 2 | 4 | 3 | 5 | 22 |
| Sakha 1 | 5 | 4 | 3 | 4 | 3 | 5 | 24 |
| Sakha 2 | 5 | 2 | 4 | 4 | 3 | 5 | 23 |
| Total Bands | 23 | 15 | 17 | 22 | 15 | 27 | 119 |

**Proximity matrix analysis (Genetic Similarity):-**

Data showed in (Table 7) recorded (10) pairwise comparisons to debate the genetic relationships among 5 flax cultivars detected in terms of similarity. The genetic similarity ranged from (0.620 to 1.0) with an average of (0.810) where, the biggest value of genetic similarity was (1.0) between (Giza 5&Giza 6). While the lowest value of similarity (0.620) was observed between each of (Giza 5 & Giza 7) and (Giza 6 & Giza 7), respectively. Also, high genetic similarity values were observed within (Giza 7&Sakha 1) (0.916), (Giza 7 & Sakha 2) (0.954) and (Sakha 1&Sakha 2) (0.875), respectively.

**Table 7: Genetic similarity %in 5 flax cultivars using 6 ISSR Primers.**

| Similarity | Giza 5 | Giza 6 | Giza 7 | Sakha 1 | Sakha 2 |
|---|---|---|---|---|---|
| **Giza 5** | 1 | | | | |
| **Giza 6** | **1** | 1 | | | |
| **Giza 7** | **0.620** | **0.620** | 1 | | |
| **Sakha 1** | 0.689 | 0.689 | 0.916 | 1 | |
| **Sakha 2** | 0.642 | 0.642 | 0.954 | 0.875 | 1 |

**Cluster analysis (Phylogenetic tree):-**

Data of cluster analysis which viewed in (Fig.2) partitioned all flax cultivars into two main cluster. The cluster I contained Giza 5 and Giza 6 only. While cluster II contained two sub-cluster. The sub-cluster one included one group (Giza 7 and Sakha 2). Whatever the sub- cluster II included Sakha 1, respectively.

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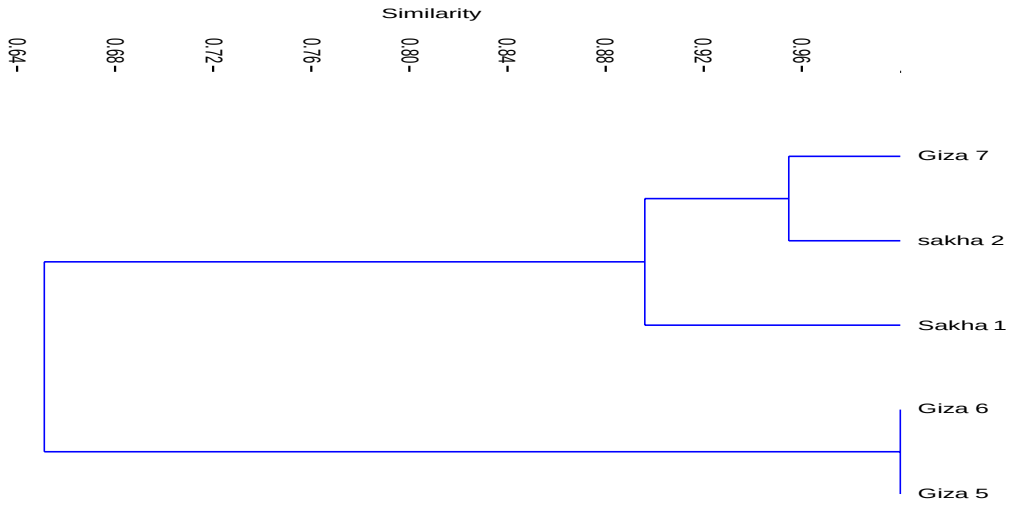


**UPGMA**

**Nei-Li's similarity coefficient**

**Figure 2: Dendrogram representing the genetic relationship among the five flax cultivars using UPGMA cluster analysis of Nei-Li's similarity coefficient generated from the 6 ISSR markers.**

## Protein electrophoretic pattern

(Table 8;Fig.3) show the changes in electrophoretic patterns of equal concentration (20 µg) for protein extracted from the leaves of 5 flax cultivars (Giza 5, Giza 6, Giza 7, Sakha1 and Sakha 2) under normal and two levels of salinity stress conditions. Salinity stress caused an induction of some bands and disappearance of others depending on the flax variety. A total number of 7 bands with different MWt ranged from 34 to 120 KDa can be detected under 2 levels of salinity stress conditions. Both bands with MWt of 120 and 34 KDa are induced under level 2 of salinity stress conditions only.Bands with MWt of (110 & 44 KDa) and (80 & 55 KDa) are completely almost disappeared under level 1 and level 2 of salinity stress conditions, respectively. Further, the intensity of band with M Wt 60 KDa increases by increasing salinity level for all flax cultivars.

**Table 8: Electrophoretic pattern of protein extracted from Giza 5, Giza 6, Giza 7, Sakha1 and Sakha 2 flax cultivars under control and 2 levels of salinity stress conditions.**

| Band No. | M. Wt. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 120 | - | - | - | - | ++ | - | - | - | - | - | - | + | ++ | + | ++ |
| 2 | 110 | - | + | + | + | + | - | - | - | - | - | + | + | ++ | + | ++ |
| 3 | 80 | + | ++ | + | + | - | + | + | + | + | - | - | - | - | - | - |
| 4 | 60 | - | ++ | - | + | +++ | - | - | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ |
| 5 | 55 | + | - | ++ | - | +++ | +++ | +++ | - | + | + | - | - | - | - | + |
| 6 | 44 | + | + | + | - | +++ | - | - | - | - | - | + | + | ++ | + | + |
| 7 | 34 | - | - | - | - | - | - | - | - | - | - | + | + | + | + | + |

**(+): very faint (++): faint (+++): very dark (-): absence of bands**

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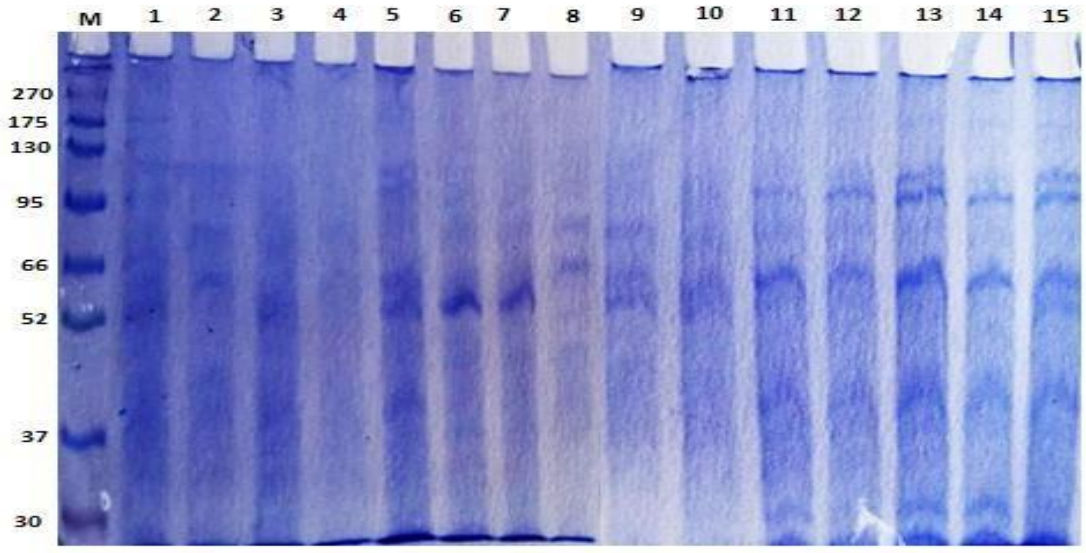


**Figure 3: Protein banding patterns in 5 flax cultivars (Giza 5, Giza 6, Giza 7, Sakha1 and Sakha 2) under normal conditions (lanes 1-5), 1st salinity level (lanes 6-10) and 2nd salinity level (11-15), respectively.**

**Antioxidant isozymes electrophoresis**

**Polyphenol oxidase (PPO) isozymes**

Under normal and two levels salinity stress conditions, only two major bands can be detected for PPO in all flax cultivars, while, under both salinity stress treatments, the intensity and density of all bands increase and this may be related to the elevated PPO activity under salinity stress conditions (Table9; Fig.4).

**Table 9: Effects of normal and 2 levels of salinity stress conditions on polyphenol oxidase (PPO) isozymes for 5 flax cultivars (Giza 5, Giza 6, Giza 7, Sakha 1 and Sakha 2).**

| Conditions | Normal | | | | | 1st salinity stress level | | | | | 2nd salinity stress level | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Band No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| 1 | ++ | ++ | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ | +++ | +++ | +++ | +++ |
| 2 | + | + | + | + | + | + | ++ | ++ | ++ | + | + | ++ | ++ | ++ | ++ |
| Total | 3 | 3 | 3 | 3 | 3 | 4 | 5 | 5 | 5 | 4 | 3 | 5 | 5 | 5 | 5 |

**(+): very faint (++): faint (+++): very dark (-): absence of bands**

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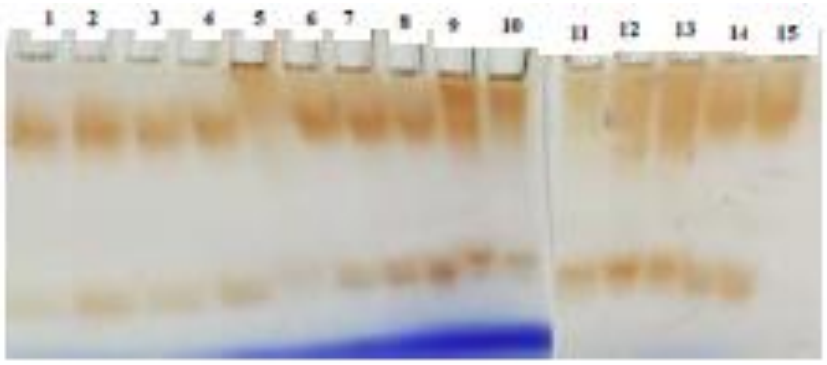

**Figure 4: Electrophoretic patterns of the polyphenol oxidase (PPO) isozymes for the 5 flax cultivars under normal conditions (lanes 1-5), 1st salinity level (lanes 6-10) and 2nd salinity level (11-15), respectively.**

**Peroxidase (POD) isozymes**

Under normal conditions, only one band with different degrees of intensity can be detected for all flax cultivars (Giza 5, Giza 6, Giza 7, Sakha 1 and Sakha 2; lanes 1, 2, 3, 4 and 5).While, under salinity stress conditions, the intensity of all bands increases with the appearance of two additional bands for Sakha 1 and Sakha 2cultivars (lanes 9 and 10) and Giza 5, Giza 6 and Giza 7 cultivars (lanes 11, 12 and 13) under the first and second salinity levels, in (Table10;Fig.5), respectively. Further, these bands can't be detected in other cultivars under salinity conditions. This may be attributed to severe salinity conditions which in turn induce POD to reach its maximum activity for neutralization of reactive oxygen species (ROS) resulted under salinity stress conditions.

**Table 10: Effects of normal and 2 levels of salinity stress conditions on peroxidase (POD) isozymes for 5 flax cultivars; (Giza 5, Giza 6, Giza 7, Sakha 1 and Sakha 2).**

| Conditions | Normal | | | | | 1st salinity stress level | | | | | 2nd salinity stresslevel | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Band No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| 1 | + | ++ | ++ | ++ | + | +++ | +++ | +++ | +++ | + | + | ++ | +++ | +++ | +++ |
| 2 | - | - | - | - | - | - | - | - | + | + | + | + | ++ | - | - |
| 3 | - | - | - | - | - | - | - | + | + | + | + | + | + | - | - |
| Total | 1 | 2 | 2 | 2 | 1 | 3 | 3 | 4 | 5 | 3 | 3 | 4 | 6 | 3 | 3 |

**(+): very faint (++): faint (+++): very dark (-): absence of bands**

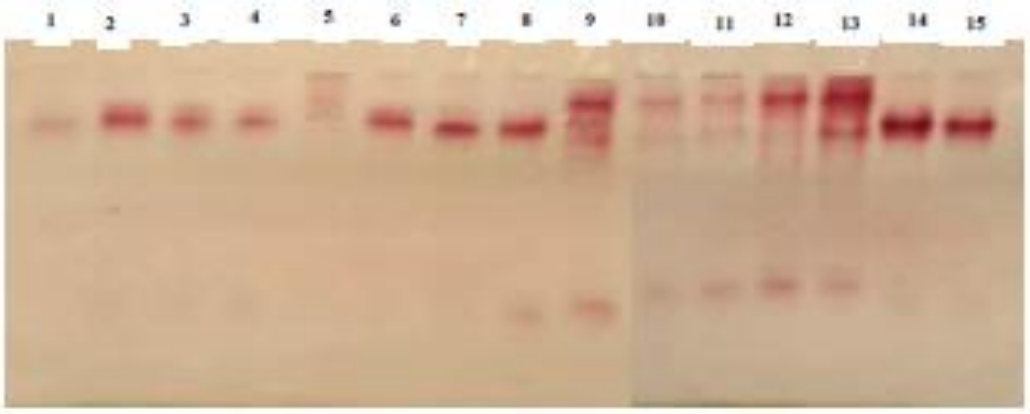

**Figure 5: Electrophoretic patterns of the peroxidase (POD) isozymes for the 5 flax cultivars under normal conditions (lanes 1-5), 1st salinity level (lanes 6-10) and 2nd salinity level (11-15), respectively.**

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## Discussion

Data obtained in Table 3confirmed that the first level of salinity stress was high impact in genetic improvement for salinity tolerance in flax cultivars compared to the second level of salt stress. Because, it is considered the safest limit that plants can handle with a slight loss rate in all studied traits, especially seed yield/plant. While, the second salinity stress level caused a large rate of loss for all studied traits in the five flax cultivars in this context. The highest mean values of all attributes under study were observed in Giza 5, 6 and 7 in the three treatments especially the control experiment followed by the first salinity level and followed by the second salinity stress. This fact confirmed that the previous three flax cultivars were recorded highly rank of salinity tolerance followed in by the second rank of endurance Sakha 1 and 2, respectively. Further, the three physiological traits in this regard, proline, glycine betaine contents, and osmotic adjustment were the normal screening for flax cultivars to salinity tolerance. Because of tolerant plants can increase the production of proline and glycine betaine contents under saline stress conditions, **[36, 37].** Further, these superior materials were able to physiological modification by reducing the osmotic pressure and transferring it to the minimum levels (osmotic adjustment). This mechanism enables tolerant- flax cultivars to complete and carry out all the vital processes necessary for growing to the fullest without affecting yield and its components or at least decreasing the impact of salt stress without affecting on the final output, **[37].** This of course does not occur in other salt-sensitive cultivars such as Sakha 1 and 2. In other words, in the past, the Egyptian flax cultivars, especially the tolerant ones, were not able to grow strongly and give a crop under the first salinity level and there are many researches and an approved publication by the Agricultural Research Center confirming that the flax was sensitive to growth at the level of salt stress up to 2500 ppm. But, the new in this study the tolerant cultivars were able to break this rule and gave a good yield at the first level of saline stress of 4 $dsm^{-1}$. In addition, the waste percentages of the final yield and plant death were small compared to the second level of saline stress (6.0 $dsm^{-1}$). This explains the proven fact that the tolerant plants were unable to grow and give good production in the past. Also, this translates into a positive genetic change that occurred in favor of increasing the level of tolerance to high salinity and this represents the apex of the genetic response in the positive direction of tolerant flax cultivars. These results were agreed with **[38]** who succeed to clarify the genetic and physiological response associated with salinity tolerance in the flax cultivar Sakha 3. Also, **[39]** was also able to prove that the flax cultivars; Sakha 1 and Sakha 2 were among the most tolerant cultivars to high salinity level in irrigation water. The sensitivity of salinity stress varies when examining all results of all studied traits under both salinity levels compared to the standard experiment. It has already proved that the second level of industrial salting had a significant and clear effect on all the studied traits. Also, it had a negative effect on all biological, physiological and biochemical processes, and then on the final output. While, the first dose of industrial salting was considered the safest dose that all flax cultivars were able to tolerate and give an acceptable yield compared to their peers under standard experiment conditions. Further, its negative impact was not significant after reviewing the data for all traits calculated in this regard. These results are in agreement with both **[6, 7, 40]** who confirmed that the salt stress had a very negative effect on the stages of germination and growth of a number of flax cultivars. On the same side, it also affects the delay in the emergence of seedlings, **[41].** But, this effect differed in severity from one variety to another according to the degree of tolerance. Also, it affects the efficiency of physiological processes, which negatively affects the productivity of flax and its quality level, **[5, 42].** In light of the different effects of salt stress on the rate, percentage and speed of germination **[43]** explained that flax seeds can germinate at a concentration of 200 Mm of NaCl, while the rate of germination is very low if the concentration of CaCO3 reaches 50 Mm. This confirms that flax cultivars do not have the same level of physiological response to salt stress. Also, the physiological traits evaluated at both levels of salt stress namely; proline and glycine betaine contents besides, osmotic adjustment were the most important indicators that demonstrated salinity tolerance in the three flax cultivars, Giza 5, 6 and 7, respectively and these results agreed with **[37]** which confirmed that the high level of proline and glycine betaine contents parallel to decrease in osmotic adjustment comes as a natural response to water stress tolerance in barely. This fact indicated that the three flax cultivars Giza 5, 6 and 7 were highly tolerance to salt stress. Because, these materials were able to increase their production of proline and glycine betaine contents under saline stress conditions compared to the normal treatment and this enhances their ability to tolerate salt stress in this context. Further, the losing rate in yield and its components was slight especially when it was exposed to the first level of salt stress. In the same context, the osmotic modification by reducing

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the frequency of osmotic adjustment responsible for controlling the opening and closing of the stomata, reducing the amount of water lost during photosynthesis and carrying out the vital processes necessary for producing of dry matter is a great leap in gene expression and the physiological evolution of salinity tolerance in flax and goes in the context of genetic improvement for salinity tolerance in this regard **[44].**

Salinity tolerance indices is considering one of the most remarkable physiological quiz's exercised to find out the gauge of the progress achieved in high toxicity- salinity tolerance in the flax varieties under studying, in relation to the final seed yield/plant only, Table 4. Because of, the salt-tolerant cultivars of flax were able to effectively reduce the losing rate in the final yield under saline stress compared to the standard experiment. In addition, giving final output that is close to the ideal situation, unlike the rest of the flax cultivars which were sensitive and moderate to salinity tolerance in this regard, **[45-47].** Results presented in (Tables 5 and 6) and (Fig. 1) had the greatest merit in finding the molecular genetic differences among the five flax cultivars. Where, the six ISSR primers generated 11 polymorphic fragments with 37.93 % polymorphism. Further, these markers were as evidence for the differentiation and comparison among the five various flax cultivars. Therefore, the superiority of all tested traits of the two cultivars, Giza 5 and Giza 6 over the rest of flax cultivars under studying was not accidental. Rather, it was based on mechanisms and molecular genetic differences that were the classification evidence not only for these superior varieties, but also strong evidence for their tolerance to multilevel salt stress compared to the standard experiment. In addition, the four ISSR primers namely; 98A, HB-10, HB-11 and HB-12 were responsible for the actual molecular genetic comparison between the five flax cultivars, giving the highest genetic differences because of actually managed to record the highest polymorphism percentage (50.0%). Therefore, this will successfully assist in identifying cultivars tolerant or sensitivity to salt stress. This step may help maximize their utilization in flax genetic improvement programs to tolerant biotic and abiotic stresses in the future,**[36, 47-53].**In the same context, we find that it was necessary to determine the degree of genetic similarity, i.e. the genetic divergence and convergence between the various flax cultivars under studying. This of course, will help in determining which of them are environmentally and genetically compatible together by drawing the cluster tree or phylogenetic tree for this purpose and this is what has already been accomplished in the (Table 7; Fig. 2). Accordingly, the strongest and correspondences genetic similarities appeared between (Giza 7 and Sakha 2) followed by (Giza 7 and Sakha 1) followed by (Sakha 1 and Sakha 2) and then followed by each (Giza 5 and Sakha 1) and (Giza 6 and Sakha 1), respectively. The other genetic similarities were come at the second rank in this regard, **[54-59].**

The final aspect of this study, which is no less important than the previous parts is biochemical and molecular studies such as protein electrophoretic pattern and antioxidant isozymes electrophoresis through determining PPO and POD isozymes. Because in fact it was able to show the direct effects of salt stress on the total protein content and the activity of the previously mentioned enzymes which has been explained in detail in (tables 8, 9 and 10). Where, results of the biochemical parameters confirmed that the salt stress induces the tolerant flax varieties to produce certain proteins. This indicates that the use of these tolerant varieties in flax breeding programs will be a great leap in the field of genetic improvement for flax tolerance not only to salt stress but also to abiotic stresses that are a major reason for destroying the final production of crops and this is what we will explain in some detail. As shown in (Table 8; Fig. 3) bands with M Wt 120 and 34 KDa appear only under level 2 of salinity stress conditions, but can't be detected under level 1 of salinity or normal conditions. These induced polypeptides may play a crucial role in flax resistance against elevated salt stress conditions. Gene expression of polypeptides was regulated genetically depending on the various salt concentrations besides, the genetic differences between the flax accessions, **[60, 61].** The present results are in accordance with **[62]** who observed that drought was responsible for manufacturing two new types of proteins based on the wheat variety. Induction of proteins during drought stress can help in comprehension of the molecular detection of the alterations in gene expression of flax varieties. Thus, proteins accumulation under drought stress conditions could protect the plants from dehydration damage. Some investigator found that plants under water stress stimulated expression of protein not to neutralize this stress, but for protection the plant against cell damage **[63, 64]**.On the other hand, bands with M Wt of 110 & 44 KDa and 80 & 55 KDa are almost completely vanished under level 1 and 2 of salinity stress conditions, respectively. Moreover, bands disappearance under stress may be due to the denaturation of enzymes involved in protein synthesis. Our results are similar with **[65]** who reported that polypeptides disappearance under stress compensates the synthesis of other proteins. In this respect, under salt stress despite depletion in protein levels **[66].** Cells synthesize a specific group of proteins that are known as stress proteins **[42, 60,**

**67].**These proteins may possess an osmo-protection function to protect the cellular structure, so these proteins can be considered as a biomarker to characterize flax tolerance against salt stress conditions. Several studies on different flax species have showed that salt stress alters the quantity and the activity of antioxidant enzymes which causes diversity in number and intensities of anti-oxidative enzymes isoforms involved in salinity tolerance **[68, 69].** As shown in (Fig. 4 and 5) and (Table 9 and 10), the bands intensity of both PPO and POD for all flax genotypes increases under salinity stress conditions. This demeanor may be attributed to the impact of salt stress to alter the gene expression of both isozyme **[70].** These results are in accordance with **[71]** who reported that POD and PPO intensities increased upon lead treatment as a result of their role in synthesis of phenolic compounds to detoxify heavy metals. Also similar results proved by **[72]** on flax cultivars and **[73]** who found that the combination between gamma rays and salt stress not only increased the activity of POD and PPO enzymes, but also increased the detected number of isozyme bands. All in all, the induction of numerous antioxidant enzymes is positively correlated with elevated tolerance levels against stresses conditions which leading to the protection of plants against oxidative damage **[74, 75].**On contrary, it was found a decrease in the activity of both PPO **[76]** and POD **[77]** after roasting treatment**.** This decrease in isozymes activity after roasting may be due to protein denaturation **[61, 78].** For POD isozymes, it was noticed the appearance of additional two bands in some flax cultivars and their absence from other genotypes; the absence of some bands may be related to the suppression of the genes responsible for the synthesis of proteins by salinity stress **[79].** The appearance of additional bands may be attributed to the tolerance of flax cultivars to salinity **[80].**

## Conclusion

This investigation was a serious attempt to study the genetic and physiological responses of salt stress tolerance in Egyptian flax cultivars using two salinity stress levels besides, the control experiment. The important results concluded that the most desirable flax cultivars recorded highly rank of salt-stress tolerance under both salinity levels compared to the control in all studied traits were Giza 5, Giza 6 and Giza 7. Also, the activity of both polyphenol oxidase (PPO) and peroxidase (POD) isozymes varied according to flax variety and salinity stress level. These results can be applied as biochemical parameters for screening salinity tolerant flax cultivars in the salt lands. Further, these findings prove the importance of the relationship between the development of salt tolerance and antioxidant activity which may cause some shift in gene expression.

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