Stimulation of LOS5/ABA3 and antioxidant enzymes of half diallel crosses of maize under water stress conditions

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Abstract
Water stress affects maize especially during the reproductive stage. LOS5/ABA3 expression and levels of antioxidant enzymes were studied in half diallel crosses and their parents in both well-watered and drought treated plants. The arrangement of split plot with RCBD was used where the irrigation intervals (each 5 and 10 days) occupied the main plots while the genotypes (5 inbreds and their crosses) occupied the sub plots. Results showed that plant growth and yield were hindered because of the expanding of irrigation interval up to 10 days.

However, all hybrids significantly performed better than their parents and this confirmed the positive role of hybrid vigour in all studied traits. Moreover, LOS5/ABA3 expression was significantly upregulated in drought treated plants and associated with worthy behaviours in terms of growth and yield. The role of antioxidant enzymes evident under drought conditions since SOD and CAT activities was obviously noticed in plants when drought treatment was applied. Moreover, these enzymes activity levels were shown to be hybrid vigour dependant. In sum, plant machinery system of defence in terms of gene expression and level of antioxidant enzymes were clearly stimulated when plants were exposed to water stress as well as they were increased in hybrids due to the hybrid vigour. Therefore, the use of more divergent inbreds is highly recommended in plant breeding programmes for obtaining tolerant genotypes associated with good field performance.

Keywords: Gene Expression, LOS5/ABA3, Inbred lines, Superoxide dismutase, Catalase, drought.

Introduction

Maize (Zea mays L.) contributes together with wheat in more than 50% from worldwide cereals production since 2013 which compromise 1016 and 713 million tonnes[10] respectively. This production should be redoubled by 60 to 110% in 2050 in order to cope with population requirements. Maize has many uses starting with being a cereal crop that can be used in human and animal nutrition as well as its use as a source of biofuel as ethanol gas and environmental friendly trash boxes. However, its worldwide production increased to 1670 million tonnes per annum in 2015, but the need of production increases still yet necessary because of the increase of its uses as well as the population expansion.[27]

The production of enough food covers the population growth in the next 50 years, is the biggest challenge. Abiotic stresses are the factors that can affect maize productivity. Drought loss has increased as climate changes such as the elevation of earth temperature, lack of rain and no uniformity in distribution in key production areas.

The achievement in the last 50 years in improving drought tolerance in maize formed a base for producing drought tolerant genotypes.[8] The use of genetics in improving drought tolerance and yield stability under stress is an effective solution for the stability of worldwide maize production. Drought tolerance trait is very complex; hence it depends not only on the drought severity but also on the growth stage and the duration of exposure to it. In order to know the molecular mechanisms of drought tolerance in maize inbred, the genome tools should be used.[13] The methods that determine the gene expression and some molecular parameters are directing the breeding programs towards drought tolerance in the plant.[7]

Genetic engineering is the most updated method in improving water stress tolerance in crops in comparison with conventional plant breeding methods. Abscisic acid (ABA) plays an effective role in abiotic stress e.g. drought; therefore, all genes involved in synthesis and degradation of ABA, as well as its signalling pathways, are important candidates genes for plant breeders in order to improve drought tolerance of which *LOS5/ABA3*.[19] For the importance of producing maize tolerant genotypes, this study came to shed light on the physiological and biochemical responses and gene expression of *LOS5/ABA3* in five maize lines and their crosses under two irrigation intervals.

Material and Methods

Plant material: The study included five inbred lines that were locally produced and subjected to half diallel hybridization according to the second method of Griffing[16] in order to get 10 crosses in the autumn season of 2015. In the spring season of 2016, the 15 genotypes (5 inbreds and their 10 crosses) were sown on 17th March 2016 in Baghdad (Abu-Ghraib). The seeds of genotypes were sown in 70*20

cm plant density and standard agricultural practices were applied to the field. The experiment was laid out in a split plot arrangement in a randomized complete block design (RCBD) where the main plots were occupied by irrigation intervals (each 5 and 10 days respectively) while the genotypes occupied the subplots and each treatment was replicated three times. When the field emergence got to more than 90% and plants got to 3-4 leaf stage (3-4 ZCK),[29] the irrigation intervals treatments were applied. When plants reached to 7-9 leaf stage (31-32 ZCK), samples were taken in the early morning for molecular studies.

Studied traits were plant height, number of leaves per plant, Total dry matter (TDM), stem diameter and relative electrical conductivity (REC). Samples for measuring REC were taken simultaneously with samples for molecular studies. Leaf samples were placed into tubes and equal sufficient amount of distilled water was added to each of them (20 ml) and covered with lids and then incubated at room temperature (approx. 25°C) overnight (24 hours) for slow interaction with leaf tissues and then the electrical conductivity (EC1) of the solution was measured. Tubes were then autoclaved at 121°C for 15 min and again incubated at room temperature overnight, then EC2 was measured. Relative Electrical Conductivity (REC) was calculated as REC%=EC1/EC2*100.[3]

Antioxidant Enzymes activity: The sample was prepared by grinding 1 g from fresh leaf tissue and then adding 10 mL of 0.1M K_2HPO_4 at pH=7.8. The solution was filtered by using a clean piece of cloth and then was centrifuged by using cooling centrifuge (4°C) at 4000 rpm for 30 min. The produced supernatant was taken in order to estimate the activity of SOD and CAT enzymes.[21]

Assay of Superoxide dismutase (SOD - EC 1.15.1.1): Superoxide dismutase (SOD) activity was determined as mentioned in Bayer and Fridoich.[5] Nitro Blue Tetrazolium (NBT) and Riboflavin in order to estimate SOD activity were used. The following solutions were used:

Solution A: 82.4 mM of K_2HPO_4 in 18.75 mL.
Solution B: 14 mM of L-Methionine in 1.5 mL.
Solution C: 1% Triton X-100 in 0.75 mL.
Solution D: Nitro Blue Tetrazolium (NBT) (14.4 mg+10 mL dH_2O) in 1 mL.
Solution F: 47.7 micromoles of Riboflavin (0.0018 g in 100 mL of dH_2O).

1.5 mL of the working mixture was added to each test tube and then 500 µL of dH_2O was also added. 40 µL from the prepared sample (supernatant) was added to the mixture. The control treatment was prepared in the same way except adding 40 µL from solution F instead of a sample. Tubes were then subjected to 650 nm (wavelength) in a spectrophotometer (UV-spectrophotometer- SP30000 nm Optima- Japan). The tubes were also subjected to light for 7 min as this instrument has two lamps (18 watt each). The standard curve was made by using 10, 20, 40, 60, 80, 100, 120, 140 µL of the samples and the highest inhibition rate was estimated according to the aforementioned curve. The activity was calculated according to:

%SOD inhibition = (A1B-A2B)/(A1B-A2)-(A1S-A2S)

where A1B = Absorbance of Blank before turning light on, A2B = Absorbance of Blank after turning light on, A1S = Absorbance of Sample before turning light on and A2S = Absorbance of Sample after turning light on.

The unit of SOD is defined as sample volume that causes 50% reduction in NBT while the activity of SOD was estimated according to:

SOD activity (unit mL^{-1}) = (Sample inhibition/highest inhibition rate) × (Dilution factor/Sample volume)

where dilution factor is 2000 µL and sample volume is 40 µL.

Assay of Catalase (CAT - EC 1.11.1.6): The activity of CAT was estimated according to Aebi[1] by using spectrophotometer at a wavelength of 240 nm. The solution of potassium phosphate (K_2HPO_4) (50 mM, pH=7) was prepared. 30 mM of H_2O_2 was prepared by taking 0.34 ml from 30% H_2O_2, then the volume was completed to 100 mL by adding a solution of phosphate buffer. The mixture consisted of 0.1 mL from the sample (supernatant), 1.9 mL from the buffer solution and 1 mL from H_2O_2 solution. The mixture was mixed thoroughly and subjected to 240 nm wavelength in a spectrophotometer (UV-Spectrophotometer, SP3000 nm Optima-Japan). Changes were checked each 30 sec for 3 min. the control was prepared in the same way of preparing samples except no adding of samples (supernatant). Finally, the activity of CAT was estimated using the following equation:

CAT activity (Unit mL^{-1}) = Δ Spectrophotometer reading/Δ time/0.1×0.01.

Molecular studies: Samples for molecular studies were taken early morning from the apical meristems from each treatment. Samples (≈ 200 mg) were put in Eppendorf containing 1 ml of Trizol (Life technologies- California) and then directly transferred to the lab. The samples were ground and homogenized with micro-pestle for 2 min. After that 400 ml of chloroform was added and centrifuged. 0.5 ml of 100% isopropanol was added to the samples which were then centrifuged at 12000 rpm for 1 min. The liquid was discarded and the pellets containing RNA were kept. Pellets were washed with 1 ml of 75% ethanol. Samples were then vortexed for short time and then centrifuged at 7500 rpm for 5 min in order to get rid of air bubbles.

RNA pellets were rehydrated with RNase-free water by using micropipette tips. Finally, incubate the samples at 55-

60ºC for 15 min and then samples were kept in deep freezing (-70ºC) before using them in RT-qPCR. In order to investigate LOS5/ABA3 expression in plants under the two irrigation intervals, two types of primers were used as shown in table 1. Primers for target and housekeeping gene were designed according to NCBI GenBank.

cDNA libraries were made by using One-Step RT-qPCR (Smart cycler- Cepheid, USA). The reaction consisted of 10 µl of master mix, 2µl of each primer (Forward and Reverse of either LOS5/ABA3 or Actin-1), 1.6 µl Nuclease-free water and 4 µl of each sample. All these components were put in smart tubes for qPCR and then were vortexed and put in thermal cycler according to the program shown in table 2.

Statistical analysis: Data were subjected to ANOVA by using SPSS software and comparison of means was made using least significant differences test (LSD) at 5% level of probability. Results were presented as mean±SE.

Results and Discussion

Drought is one of the main environmental stresses that faces plant production in most agricultural lands worldwide. It prevents plants from being showing their genetics potentials.[24,30] Drought or water stress affects maize productivity as a final function of its growth. Presented results in table 3 and 4 indicated that all growth and yield traits were extremely affected when the field was irrigated each 10 days (stressed) in comparison with irrigation every 5 days (control) based on Iraqi environmental conditions in the fall season of 2017.

Maize water requirements are less in the early growth stages and start increasing as its growth approaching reproductive stage and declines up to complete maturity. Metabolic processes inhibition is the first response of maize to moderate water stress for long periods followed with stomatal closure and gas exchange inhibition.[18] The differences between genotypes (inbred lines and hybrids) are attributed to the differences in genetic factors; therefore, this requires continuous study of genetic behaviors in order to know the gene action that controls traits under study.

Hybrid vigor is a phenomenon that can be utilized by plant breeders and a most important genetic parameter for plant breeders that rely on in most crops. This phenomenon becomes clear in divergent inbred lines. Based on current study results, hybrids were generally superior compared to their parents. This superiority is attributed to the increase in SCC (system capacity constant) that is related strongly to the hybrid vigor.[23] Elaloosy and Elsshookie[12] found same results as their hybrids were faster in growth and more dry matter aggregation and this, in turn, enhances the relationship between the source and sink and then give higher plants in hybrids in comparison with inbred lines. The hybrid vigor values of Erdeaan and Al-Issawi[14] strongly supported this result.

Drought tolerance includes first stress signal perception followed by signal transduction which then leads to several of the physiological and metabolic processes.[11] Hundreds of genes and their signal pathways have been characterized as important keys for drought tolerance and overexpressing of some of them made plant drought tolerant.[9,22] LOS5/ABA3 is considered very important to the biosynthesis of ABA through regulation of the final step by AO (aldehyde oxidase).

Table 1
The primer sequences of the target gene (LOS5/ABA3) and housekeeping gene (Actin-1).

Primer name	Primer sequence	Primer length
LOS-F	5´-CTGATAGCTTCTCAGGGTTCAC-3´	103 bp
LOS-R	5´-GGTGGCATCCATCCACTAAA-3´	
Actin-1-F	5´-CCCAAAGGCCAACAGAGAGAAG-3´	137 bp
Actin-1-R	5´-CACCAGAGTCCAGCACAATACC-3´	

Table 2
The applied thermal cycle program

Step			Cycle	Temperature (c)	Time (min : sec)
1	Reverse Transcription		1	≥37°C	15:00 minutes
2	RT inactivation/Hot-start activation		1	95°C	10:00 minutes
3	qPCR steps	a. Denature	40	95°C	00:10 seconds
		b. Anneal/Collect Data		60°C	00:30 seconds
		c. Extend		72°C	00:30 seconds
4	Dissociation		1	60–95°C	

Table 3
Growth characteristics of inbred lines and their half diallel hybrids under two irrigation intervals.

Genotypes	Plant height (cm)		Dry weight (g plant-1)		Ear height (cm)		Stem diameter (mm)		Electrical conductivity (EC%)	
	5 days	10 days	5 days	10 days	5 days	10 days	5 days	10 days	5 days	10 days
Inbred 1	154.33	133.47	363.4	234.3	83.4	73.7	25.42	23.93	48.02	30.14
Inbred 2	160.53	155.33	269.4	257.1	87.6	90.4	23.37	23.53	55.37	30.17
Inbred 3	173.6	157.47	339.2	253.1	110.4	100	25.87	26.05	59.3	26.93
Inbred 4	145.87	146.47	315.4	245.7	75	67.53	24.18	25.23	70.25	29.83
Inbred 5	168.8	159.43	330.6	298	98.33	95.8	21.94	25.35	58.35	30.63
Hybrid 1 (1X2)	188.2	176.7	315.5	231.2	105.2	93.5	24.07	21.05	49.64	56.38
Hybrid 2 (1X3)	204.2	155.9	262.1	254.25	121.5	94.2	22.76	23.39	31.13	48.82
Hybrid 3 (1X4)	203.1	182.9	330.8	271.75	113.5	105.8	23.5	23.66	38.17	50.59
Hybrid 4 (1X5)	198.6	182.4	369.9	292.6	115.2	102.6	24.12	20.94	38.33	52.57
Hybrid 5 (2X3)	192.5	187.8	412.3	193.2	114.8	117.2	25.1	23.99	42.93	47
Hybrid 6 (2X4)	179.3	182.2	383.6	266.05	95.9	100.8	23.93	25.29	41.86	57.79
Hybrid 7 (2X5)	203.6	194.5	369.4	285.65	130.2	114.4	26.77	25.04	43.88	44.71
Hybrid 8 (3X4)	199.9	190.3	427.6	303.25	115.2	106.7	26.1	21.42	68.35	63.76
Hybrid 9 (3X5)	200.8	199.5	432.3	305	112.8	121	26.54	21.5	58.24	74.38
Hybrid 10 (4X5)	197.7	191.9	441.4	294.75	127.9	118.6	21.55	23.2	58.85	69.54
L.S.D (0.05)	12.57		37.83		8.35		1.66		3.86	

Table 4
Yield characteristics of inbred lines and their half diallel hybrids under two irrigation intervals.

Genotypes	No of ear plant^{-1}		No of rows ear^{-1}		No of grains row^{-1}		500 grains weight (g)		Total yield (kg ha^{-1})	
	5 days	10 days	5 days	10 days	5 days	10 days	5 days	10 days	5 days	10 days
Inbred 1	1.13	1.13	21.03	18.93	34.2	26.27	98.54	83.89	9299.53	6713.95
Inbred 2	1.33	1.13	16.27	16.53	31.73	32.27	102.9	101	7223.45	6990.13
Inbred 3	1	1.07	17.67	16.87	37.87	30.4	120.47	97.76	9699.52	6609.19
Inbred 4	1.33	1.2	16.13	15.07	33.73	27.4	101.75	93.44	7880.56	5728.29
Inbred 5	1.2	1.27	18.2	15	33.67	29.67	115.21	95.06	8437.67	6637.76
Hybrid 1 (1X2)	1	1	20.6	18.1	39.3	30.6	86.98	68.54	9492.38	5756.86
Hybrid 2 (1X3)	1	1	15.4	13.4	31.3	22	127.24	81.31	7428.2	3756.96
Hybrid 3 (1X4)	1.2	1.1	17.8	17.4	32.6	32.6	105.4	101.9	8128.17	7742.47
Hybrid 4 (1X5)	1.1	1	17.8	16.2	35.4	28.4	110.12	97.4	9913.79	5306.88
Hybrid 5 (2X3)	1	1	16.2	15.4	41.7	25.6	118.44	73.09	10113.8	4256.93
Hybrid 6 (2X4)	1	1	19.4	16.6	41.8	31	111.84	91.41	11063.7	6556.82
Hybrid 7 (2X5)	1.6	1	20.2	18.3	35.1	29.5	94.38	77.52	9328.11	6592.53
Hybrid 8 (3X4)	1.3	1.1	15.4	15	41	36.2	111.6	99.12	11320.9	8356.72
Hybrid 9 (3X5)	1.1	1	16	15.3	38	35	116.56	98.18	9770.94	8356.73
Hybrid 10 (4X5)	1.4	1	17.8	15.5	38.6	30	115.75	100.2	12285.1	7613.91
L.S.D (0.05)	0.14		1.19		2.68		6.63		1274.48	

This gene fold number was calculated as a result of its expression under the two irrigation treatments (well-watered and drought treated). However, its expression was significantly higher in stressed plants (table 5) at various degrees ranging from 0.10 to 315.20 folds due to the genetic variation between genotypes under study and their pattern of response to water stress. It is, therefore evident that LOS5/ABA3 expression is induced as a response to water stress because it encodes MoCo sulfurase which in turn regulates AO required for the final step of ABA biosynthesis which then leads to enhance drought tolerance in plants. This gene also controls the stomatal closures which cause a reduction in water loss.[28]

Table 5
LOS5/ABA3 Expression (Actine1: Endogenous control) in inbred lines and their half diallel crosses of maize

Genotypes	Gene Expression (Number of Folds)
Inbred 1	0.6
Inbred 2	0.1
Inbred 3	0.6
Inbred 4	14.5
Inbred 5	2.8
Hybrid 1 (1X2)	0.8
Hybrid 2 (1X3)	0.8
Hybrid 3 (1X4)	0.1
Hybrid 4 (1X5)	315.2
Hybrid 5 (2X3)	7.9
Hybrid 6 (2X4)	60.1
Hybrid 7 (2X5)	14.8
Hybrid 8 (3X4)	32.9
Hybrid 9 (3X5)	0.9
Hybrid 10 (4X5)	16.4

It is also proved that LOS5/ABA3 led to an increase in the activity of some antioxidant enzymes such as CAT and SOD.[19,28] It has been noticed that highest expression of the aforementioned gene was 315.20 in the hybrid S1×S5 while the lowest expression was 0.10 in inbred line S2 and hybrid S3×S4, however, the expression was higher in hybrids in comparison with their parents. These results indicated that some of the genotypes expressed LOS5/ABA3 better than others which reflect their ability to adapt to water stress (irrigation each 10 days). Possibly, the tolerances of some genotypes belong to their ability to accumulate ABA in the pathway of LOS5/ABA3 which enhances drought tolerance accompanied with good yield.

Similar findings of Lu et al[20] indicated that LOS5/ABA3 expression led to ABA accumulation which contributed to plant adapting to water stress. On the other hand, some of genotypes expressed this gene at very low level (less than 1 fold) and this indicated that some of the genotypes were sensitive to water stress and these results are also in consistency with findings of Al-Sheikh[4] that maize genotypes varied in their response to irrigation intervals up to 14 days. It is clear from the table 5 that all half diallel hybrids showed better gene expression of LOS5/ABA3 except S1×S4 and S3×S5 as they showed lower expression while S1×S5 showed the highest expression over all genotypes (315.20 fold).

This result indicates that the aforementioned hybrid significantly responded at the molecular level in comparison with the rest of the genotypes understudy. Although hybrid vigor was under spotlight of researchers for more than a century but its molecular basis is still not completely understood and yet to be searched and discussed.[6] Recently, a progress had been made in this regard, thus Grozmann et al[17] indicated to the role of epigenetics in hybrid vigour while Shen et al[26] submitted valuable information on role of DNA methylation and the expression of small segments of RNA beside the pattern of gene expression in the inbred lines and their hybrids.

The activity of antioxidant enzymes is readily upregulated by abiotic stress such as drought. Figure 1 and 2 showed that SOD and CAT activities increased dramatically in maize plants when they were subjected to water stress. Results presented in the two figures 1 and 2 also clearly indicated that hybridization affected the level of antioxidant enzymes in maize. Thus, it has been noticed that most of the hybrids under study showed better defense to ROS and drought stress in comparison with their parents. This is evident that hybrid vigour also affected positively the level of antioxidant in plants.[14] Production of Reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (.OH) in plant is general reaction response to abiotic stress e.g. drought.[15]

In order to avoid the damaging effects of these toxic oxygen intermediates, plant cells and their organelles mainly chloroplast, peroxisomes and mitochondria employ antioxidant defense system. Superoxide dismutase (SOD) and Catalase (CAT) are considered among the major ROS scavengers in plants which can provide plants with highly efficient machinery for detoxifying ROS products such as O_2^- and H_2O_2. The results presented in this study confirmed the activity changes of SOD and CAT during water stress. ROS has an important role as signaling molecules[25] and may, therefore, regulates development and growth under drought conditions. Results here also indicated that drought-tolerant plants associated with higher activities of antioxidant enzymes.

Understanding the molecular base of drought tolerance in maize is fundamental for enabling plant breeders in developing new methods in order to enhance this trait. However, the first step of improving drought tolerance in maize is determining the morphological and physiological traits that contributes to crop adaptation and raise its production efficiency. Most of the current study indicated that half diallel hybrids were superior in most agronomical traits as well as their gene expression under water stress conditions over their parents.

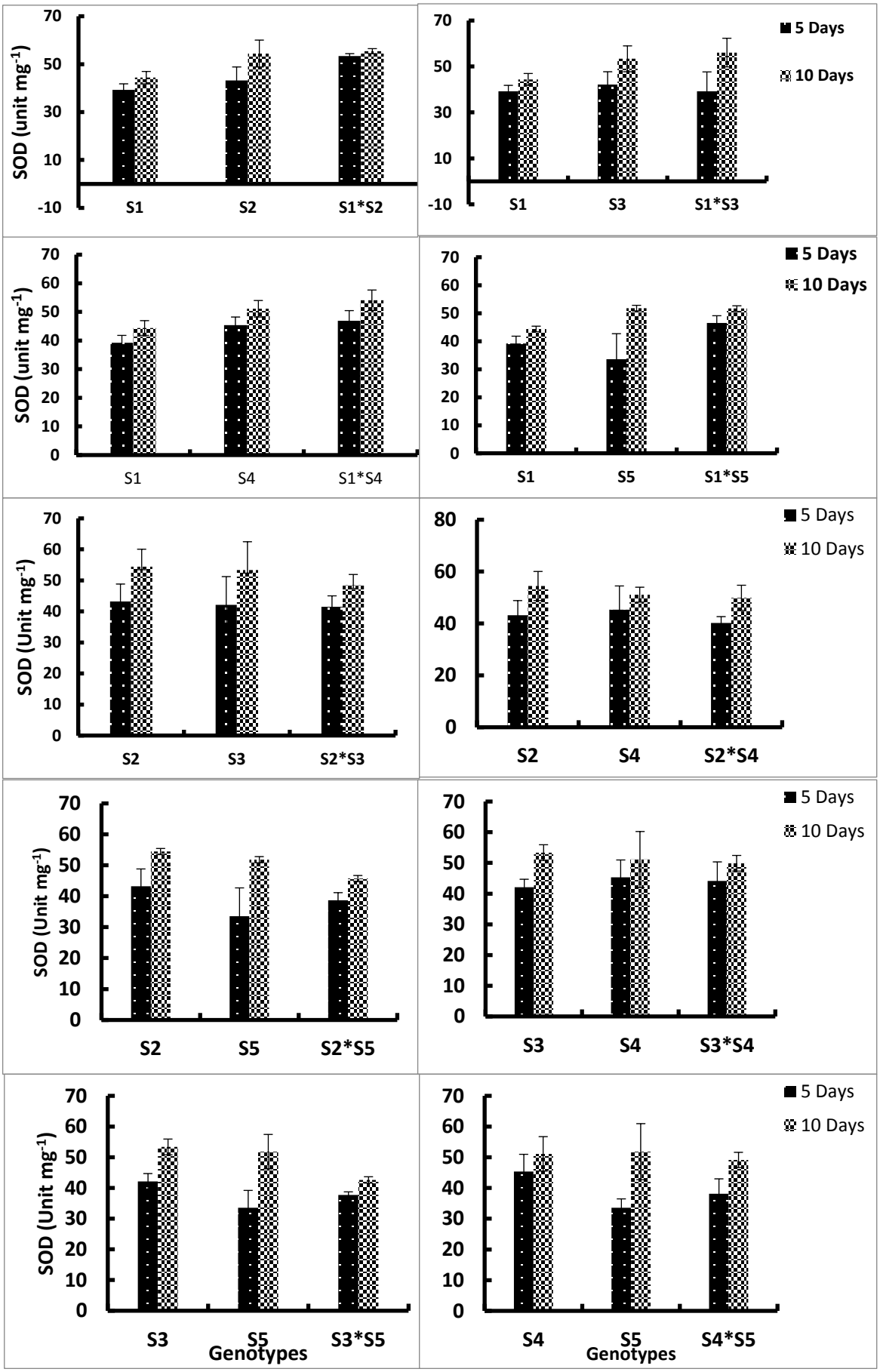

Figure 1: Activity of SOD enzyme in inbred lines and their half diallel crosses under two irrigation intervals (mean±SE)

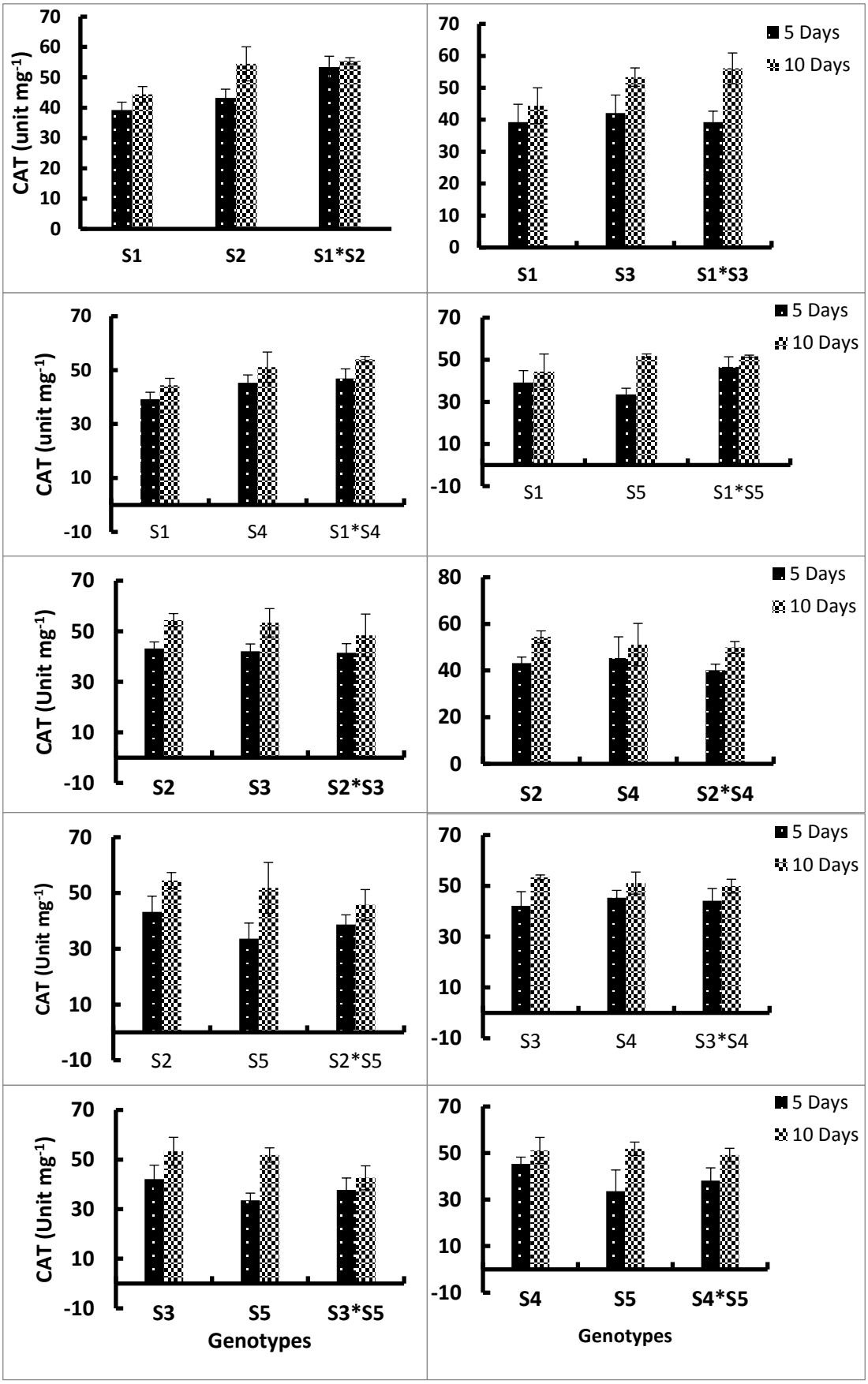

Figure 2: Activity of CAT enzyme in inbred lines and their half diallel crosses under two irrigation intervals (mean±SE)

Therefore, it is clear that S1×S5 that showed highest LOS5/ABA3 expression (315.20 folds) also showed significant 500 seed weight, plant height and plant dry matter. However, the hybrid S2×S4 that showed 60×10 fold of LOS5/ABA3 was also superior in number of seeds per ear while S3×S4 showed 32×90 fold of the gene and was superior in most of growth and yield traits such as plant yield, weight of 500 seeds and number of seeds per ear. All aforementioned traits are strongly related with the yield; therefore, it is worth mentioning that plants, which showed better response at molecular base had the tendency to show better growth and yield under water stress.

In sum, the genotypes used in this study (inbred lines and hybrids) significantly varied in their responses to irrigation intervals in terms of molecular and physiology. It can be concluded that LOS5/ABA3 had significant role in drought tolerance in maize because its expression had increased in all genotypes when water stress was applied. However, it was found that gene expression was correlated to some of growth and yield traits indicating its role in enhancing drought tolerance as well as giving better yield.[14]

Finally, it can be recommended that using more genetically divergent inbred lines, expanding irrigation intervals and applying them prior flowering stage in order to get more stable drought tolerant genotypes.

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