

Zinc and iron-mediated alleviation water deficiency of maize by modulating antioxidant metabolism

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Abstract

Microelements are inorganic compounds involved in the synthesis of enzymes and biologically active substances. To evaluate the physiological responses of maize to ZnSO₄ and FeSO₄ under drought stress, a field experiment was conducted on maize plants grown under different soil moistures and treated with foliar ZnSO₄ and FeSO₄ applications. Drought stress especially at early seed growth stage significantly reduced grain yield and *Fv/Fm* ratio; however, the activity of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione reductase (GR) was enhanced under drought stress. Foliar applied ZnSO₄ and FeSO₄ boosted the grain yield under non irrigation at vegetative growth stage and at early seed growth stage, respectively. Between grain yield and MDA concentration ($r = -0.73$), superoxide dismutase ($r = -0.57$), peroxidase ($r = -0.49$), H₂O₂ ($r = -0.67$) and catalase enzyme ($r = -0.42$) significant and negative correlation were observed. Combined application of ZnSO₄ and FeSO₄ resulted in alleviation of maize plant drought stress by Zn and Fe-mediated improvement in photosynthetic attributes. In addition, the foliar application of ZnSO₄ and FeSO₄ regulated physiological processes in maize plants and alleviated the adverse effects of water stress. According to the results, ZnSO₄ and FeSO₄ could be used for improving maize growth under drought stress.

Keywords: enzyme; maize; superoxide dismutase; water stress; zinc sulfate

Introduction

Maize (*Zea mays* L.) is the third most important crop in the world following wheat and rice, produced across almost 100 million hectares in developing countries (FAOSTAT, 2012). Water deficit is one of the major environmental factors limiting crop growth and productivity in many areas across the world (Falqueto *et al.*, 2017). In maize, water requirement is low at early growth stages, while being the most sensitive to drought stress during reproductive growth stages. Thus, pollination and fertilization period at the time of drought stress

will result in a significant yield loss (Ahmed-Amal and Mekki, 2005). Exposure of plants to environmental stresses leads to the generation of reactive oxygen species (ROS), such as H_2O_2 (Munne-Bosch and Penuelas, 2003). Production of ROS in plant cells during abiotic stress is mainly the outcome of enhanced photorespiration resulting in the production of H_2O_2 (Baishnab and Ralf, 2012; Kerchev *et al.*, 2016). These reactive species cause oxidative damage and impairs the normal functions of cells (Foyer and Fletcher, 2001). Several reports have shown the detrimental effects of ROS, whose production is stimulated under water stress (Yang *et al.*, 2014). Drought resistance in plants increases with changes in morphological, physiological, and biochemical responses such as changes in plant structure, growth rate, tissue osmotic potential, and antioxidant defense against water deficit (Anjum *et al.*, 2011; Ashraf *et al.*, 2015). Intervention of enzymatic and non-enzymatic antioxidant systems ensures resistance to environmental stress conditions such as drought (Sharma *et al.*, 2012). Zinc (Zn) and iron (Fe) are the most important micronutrients required for plant growth and have a significant effect on plant yield. They also play an important role in the synthesis of many enzymes. Chlorophyll biosynthesis and energy transfer also occur through iron (Gill and Tuteja, 2011). A research found that foliar application of Zn increased the seed yield of corn (Hussain *et al.*, 2012; Tabatabai *et al.*, 2015). Zinc plays an important role in reducing ROS generation and protects cells from the damaging effects of ROS (Cakmak, 2008). Zinc sulfate has been reported to play an important role in regulating the stomatal closure and maintaining ionic balance in the plant to reduce drought stress while also significantly enhancing SOD, POD, and CAT activities in response to drought stress (Tabatabai *et al.*, 2015; Yavas and Unay, 2016). Iron also plays an important role in reducing stress due to salinity, drought, and heavy metal stress by activating plant enzymatic antioxidants such as catalase (CAT), peroxidase, and superoxide dismutase (SOD), acting as a scavenger of reactive oxygen species (ROS) (Tripathi *et al.*, 2018). Drought has become a source of critical abiotic stress which impacts the maize growth and productivity. The benefits of Zn and Fe have been documented in studies with some stressful conditions, but minor research has focused on the combined effects of Zn and Fe on growth and physiological responses of maize to interrupted irrigation at different growth stages of maize, given that maize susceptibility to drought varies at different stages of growth. For this reason, we hypothesized that Zn and Fe would alleviate the negative effects of drought on yield components and physiological traits of maize plants.

Materials and Methods

Growth conditions and treatments

In order to evaluate the effect of iron and zinc on the yield and some antioxidant enzymes of maize under water deficiency conditions, an experiment was conducted during the summer of 2016-2017 and 2017-2018 (growing seasons) in an experimental field located in northwest of Ahvaz. The latitude of the test site was: 31° 20' N, longitude was 48° 40' E, and altitude was 22.5 m. Average meteorological parameters of the test site are shown in Table 1. The experiment was laid out as split-split plot in a randomized complete block design with three replicates. The main plot consisted of three levels of water deficit stress comprised of complete irrigation (control), no irrigation at vegetative growth stage (12-14 leaf), and no irrigation at early seed growth stage. water deficit stress treatment was specific to the above-mentioned stages, after which and until the end of the growth period, the water requirement of the plant was fulfilled. The sub-plot contained foliar solution of zinc sulfate at three concentrations (0, 5 and 10 g .L⁻¹) and sub-sub-plot included iron sulfate foliar solution at three concentrations (0, 3 and 6 g .L⁻¹). Prior to cultivation, soil samples were taken from 0-30 cm and 30-60 cm soil depth to determine the physical and chemical properties of the soil, as presented in Table 2. In the experiment, each plot consisted of 6 rows by 7 m long and spaced 75 cm apart. In this study, the seed of maize hybrid 704, which is one of the late hybrids, was used. Seed cultivation was done in a row via a row-crop machine in two crop years in August. The seeds were sown in a 3-5 cm depth. Extra seedlings were thinned at 2-4 leaf stage.

The first irrigation was done immediately after planting. Then, until the full establishment of seedlings (4 to 5 leaves), irrigation was done routinely providing 100% water requirements of the plant. Manual weeding and chemical control by nicosulfuron herbicide were performed during the weed control period. Foliar application of zinc and iron was carried out at the specified concentrations using a damp sprayer after calibration at the pressure of 1 atmosphere at two times in a six to eight-leaf stage and in a 12-leaf stage. The foliar solution of iron and zinc compounds consisted of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. Harvesting was carried out after physiological maturity and when grain moisture reached about 14%.

Table 1. Average biennial meteorological parameters of test site

Month	Rainfall (mm)	Average minimum temperature (° C)	Average maximum temperature (° C)	Average minimum relative humidity (%)	Average maximum relative humidity (%)
August	0	33.1	47.6	14.1	49.2
September	0	26.6	43.1	13.2	43.4
October	0	21.8	38.5	11.6	35.4
November	12.5	17.1	31.7	10.1	29.1
December	25	9	24	8.5	25.9

Table 2. Physical and chemical properties of the tested soil

2016-2017					
Soil depth (cm)	Soil texture	Organic carbon (%)	pH	EC (ds. m ⁻¹)	Total N (%)
0-30	Silty loam	0.76	7.4	2.5	0.05
30-60	Silty loam	0.52	7.7	2.1	0.04
Soil depth (cm)	Available P (mg. kg ⁻¹)	Available K (mg. kg ⁻¹)	Available Zn (mg. kg ⁻¹)	Available Fe (mg. kg ⁻¹)	
0-30	7.2	264	0.44	10.1	
30-60	6.4	217	0.3	9.8	
2017-2018					
Soil depth (cm)	Soil texture	Organic carbon (%)	pH	EC (ds. m ⁻¹)	Total N (%)
0-30	Silty loam	0.86	7.6	2.6	0.06
30-60	Silty loam	0.6	7.9	2.4	0.05
Soil depth (cm)	Available P (mg. kg ⁻¹)	Available K (mg. kg ⁻¹)	Available Zn (mg. kg ⁻¹)	Available Fe (mg. kg ⁻¹)	
0-30	8.8	265	0.48	10.5	
30-60	7.6	238	0.41	9.28	

Measurements

In this experiment, the following traits were measured: Leaf relative water content, Relative growth rate ($\text{g g}^{-1} \text{d}^{-1}$), Fv/Fm ratio, Malondialdehyde and Hydrogen peroxide concentrations, antioxidant enzymes assays (superoxide dismutase, peroxidase, catalase and Glutathione reductase), grain Protein, grain yield, biomass yield and harvest index.

Leaf relative water content (RWC)

Two leaves were collected from the young fully expanded leaves of two plants per replicate. Individual leaves first were detached from the stem and then weighed to determine fresh weight (FW). To determine turgid weight (TW), leaves were floated in distilled water inside a closed Petri dish. Leaf samples were weighed

periodically, after gently wiping the water from the leaf surface with tissue paper, until a steady state was achieved. At the end of the imbibition period, leaf samples were placed in a preheated oven at 80 °C for 48 h to determine dry weight (DW). Values of FW, TW, and DW were used to calculate LRWC using the following equation (Turan *et al.*, 2011).

$$\text{RWC (\%)} = [(FW - DW) / (TW - DW)] \times 100 \quad (1)$$

Relative growth rate (RGR)

The relative growth rate was calculated by following equation (Navabpour *et al.*, 2011):

$$\text{RGR (g g}^{-1} \text{ d}^{-1}) = (\text{Ln } W_2 - \text{Ln } W_1) / (T_2 - T_1) \quad (2)$$

W: weight of dry matter

T₂-T₁: The time interval between two consecutive sampling

Fv/Fm ratio

The maximum photochemical efficiency of photosystem II (Fv/Fm) was measured applying a fluorometer after 30 min of dark adaptation. The Fv/Fm ratio was calculated as: $F_v/F_m = (F_m - F_0) / F_m$; Where, F_m and F₀ represented the maximum and minimum yields of dark-adapted leaves, respectively.

MDA and H₂O₂ concentrations

The concentration of malondialdehyde (MDA) which is a product of lipid peroxidation was assessed by the thiobarbituric acid (TBA) according to Wang *et al.* (2009) and was calculated on a fresh weight basis, using the following formula:

$$(\text{nmol MDA g}^{-1} \text{ FW}) = 6.45 (\text{OD}_{532} - \text{OD}_{600}) - 0.56 (\text{OD}_{450}) \times 1000 \quad (3)$$

Hydrogen peroxide (H₂O₂) was assessed spectrophotometrically after the reaction with potassium iodide (KI), according to the method presented in Velikova and Loreto (2005). The content of H₂O₂ was calculated applying a standard curve prepared by identifying concentrations of hydrogen peroxide.

Enzyme (CAT, POD, SOD and GR) extraction and assay

Fresh foliar tissue (0.2 g) from fresh seedlings (uppermost leaves) was harvested, weighed, washed with distilled water, and then homogenized with a mortar and pestle with five ml chilled sodium phosphate buffer (50 mM, pH 7.8). The homogenates were centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was stored at 4 °C and used for CAT, POD, SOD, and GR assays. CAT activity was measured by the method of Blume and McClure (1980). The unit for CAT activity was micromoles of hydrogen peroxide oxidized per minute per milligram of protein. POD activity was determined spectrophotometrically, by measuring the oxidation of o-dianisidine (3, 3'- dimethoxy benzidine) at 460 nm as described by Ranieri *et al.* (2001) and expressed as units (μmol of dianisidine oxidized per minute) per mg of protein. SOD activity was estimated by recording the decline in absorbance of superoxide – nitro blue tetrazolium complex by the enzyme (Cavalcanti *et al.*, 2004). About 3 ml of the reaction mixture, containing 0.1 ml of 13 mM L- methionine, 0.1 ml of 75 μM p- nitro blue tetrazolium chloride (NBT), 0.1 ml of 100 μM EDTA, 0.1 ml riboflavin (2 μM) in a 1.5 ml of 50 mM potassium phosphate buffer pH 7.8, 50 μL of the enzymatic extract and distilled water to make the volume to three ml. The reaction was initiated under illumination of fluorescent lamp (30 W) at 25 °C and stopped five min later by turning it off. The blue formazan produced by NBT photo - reduction was measured as increase in absorbance at 560 nm. The control reaction mixture had no enzyme extract (with maximal colour formation). The blank solution had the same complete reaction mixture, but was kept in the dark. One SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photo - reduction in comparison with the tubes lacking the plant extract and expressed as a unit of enzyme activity per mg of protein. Glutathione reductase (GR) activity was identified by following the rate of NADPH oxidation at 340 nm according to Balabusta *et al.* (2016). The assay mixture contained 0.5 mM NADPH, 10 mM GSSG, 6.25 mM

MgCl₂ in 0.1 M phosphate buffer (pH 7.5), and 100 µl of the enzyme extract in the total volume of 400 µl. GR activity was expressed as micromoles of NADPH oxidized during 1 min per 1 mg of proteins (µ mol min⁻¹ mg protein⁻¹).

Grain protein

In order to measure grain protein percentage, initially, using Kjeldahl apparatus, the percentage of total grain nitrogen was measured and then whole-grain protein was calculated from the following formula (Walton 1989):

$$\text{Whole grain protein (\%)} = \text{Nitrogen percentage} \times 5.65 \quad (4)$$

Grain yield, biomass yield and harvest index

To determine the yield, 30 plants (ten plants from each replicate) were sampled randomly and harvested at maturity. The harvested plants were sun-dried (in an open place) and were shelled manually to record the grain yield (GY) per plant. The total plant dry biomass was weighed for each treatment and regarded as Biomass yield (BY). Harvest index was calculated by the following formula (Amanullah *et al.*, 2015):

$$\text{HI (\%)} = (\text{Grain yield} / \text{Biological yield}) \times 100 \quad (5)$$

Statistical analysis

Statistical analysis of data was performed using SAS (version 9.4; SAS Institute, Cary, NC, USA) after performing the Bartlett test and proving the homogeneity of the test variances in each year. Means were compared according to the Duncan's multiple range test at $P \leq 0.05$. Curves were drawn using Excel 2013 software.

Results

Relative water content (RWC)

Results Table 3 revealed that drought stress, ZnSO₄ and FeSO₄ at 1% probability levels had a significant impact on relative water content (RWC). Drought stress by stopping irrigation during different stages of maize growth (non-irrigation at vegetative and the early stage of seed growth) caused a significant decrease in RWC. The highest content of these parameters (RWC) was obtained in full irrigation conditions (79.1%) and the lowest RWC belonged to non-irrigation conditions at the early stage of seed growth treatments (73.3%). Foliar application of ZnSO₄ and FeSO₄ increased Relative water content of leaf and thereby reduced the negative effects of drought stress. The lowest reduction of this parameter was observed in 10 g L⁻¹ ZnSO₄ and 6 g L⁻¹ FeSO₄ treatments (Table 3).

Fv/Fm ratio

Drought stress caused a significant decrease in Fv/Fm ratio by stopping irrigation during different stages of maize growth (non-irrigation at vegetative and the early stage of seed growth). However, the smallest reduction of these parameters was observed with 10 g L⁻¹ ZnSO₄. No iron sulfate intake at a constant concentration of zinc sulfate (10 g L⁻¹) reduced the Fv/Fm ratio under non-irrigation at the early stage of seed growth. By increasing the amounts of ZnSO₄ and FeSO₄, the Fv/Fm ratio grew in the non-irrigation at the vegetative growth stage and in the non-irrigation at the early stage of seed growth treatment. This growth was more noticeable in non-irrigation at the early stage of seed growth treatment. The lowest amount of Fv/Fm ratio was obtained in non-use of zinc and iron treatments (with mean of 0.38) in the non-irrigation at the early stage of seed growth treatment (Figure 1).

Table 3. Simple effect of water deficit stress, zinc sulfate and iron sulfate on leaf relative water content and grain protein

Treatments	Relative water content (RWC) (%)	Grain protein (%)
Irrigation levels		
Full irrigation	79.1a	7.7b
Non-irrigation at vegetative growth stage	75.7b	7.9b
Non-irrigation at the early stage of seed growth	73.3b	8.7a
P≤0.01: **		
Zinc sulfate concentrations		
0	72.2c	7.7c
5 g. L ⁻¹	76.8b	8.2b
10 g. L ⁻¹	79a	8.5a
P≤0.01: **		
Iron sulfate concentrations		
0	72.8b	7.7c
3 g. L ⁻¹	76.6a	8.2b
6 g. L ⁻¹	78.7a	8.4a
P≤0.01: **		

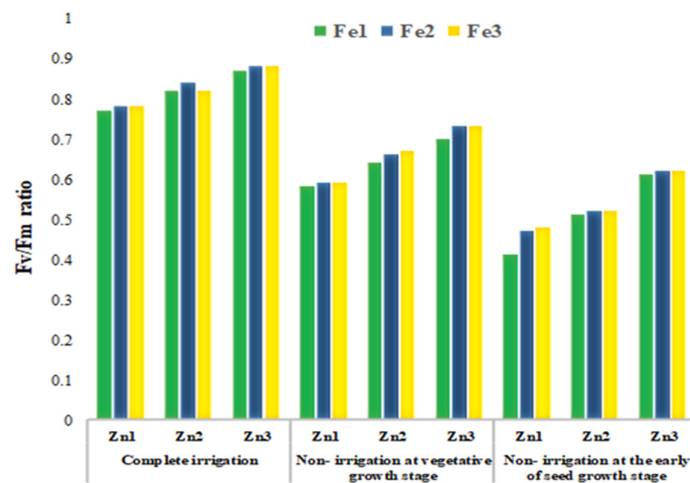


Figure1. Fv/Fm ratio means comparison against control, as affected by foliar application of ZnSO₄ and FeSO₄

S1: Zn1) Without zinc sulfate foliar application, Zn²) Foliar application with 5 g .L⁻¹ zinc sulfate concentration, Zn³) Foliar application with 10 g .L⁻¹ zinc sulfate concentration; Fe¹) Without iron sulfate foliar application, Fe²) Foliar application with 3 g .L⁻¹ iron sulfate concentration, Fe³) Foliar application with 6 g .L⁻¹ iron sulfate concentration

MDA and H₂O₂ concentration

Foliage-applied ZnSO₄ and FeSO₄ reduced the MDA content causing improved drought tolerance of maize.

The highest concentration of MDA content was obtained in the absence of application of zinc sulfate and iron sulfate in water deficit condition at the early stage of seed growth, which increased compared to the same treatment under full irrigation conditions. The results show that the non-irrigation at the early stage of seed growth, occurrence in the absence of Zn and Fe concentrations, the highest concentration of malondialdehyde is present. On the other hand, in other stages with the use of these elements, it is controlled to increase the concentration of malondialdehyde so that under complete irrigation conditions (control) which

is not interrupted. Upon increase in zinc sulfate and iron sulfate consumption, minimum concentration of malondialdehyde was obtained (Figure 2A). Foliage-applied $ZnSO_4$ and $FeSO_4$ reduced H_2O_2 content leading to improved drought tolerance of maize. Maximum H_2O_2 concentration was obtained in non-foliar application of zinc and iron. Also, the lowest values of this trait were obtained in full irrigation conditions. Between 3 and 6 $g \cdot L^{-1}$ concentrations of $FeSO_4$, there was no difference in water stress stages (Figure 2B).

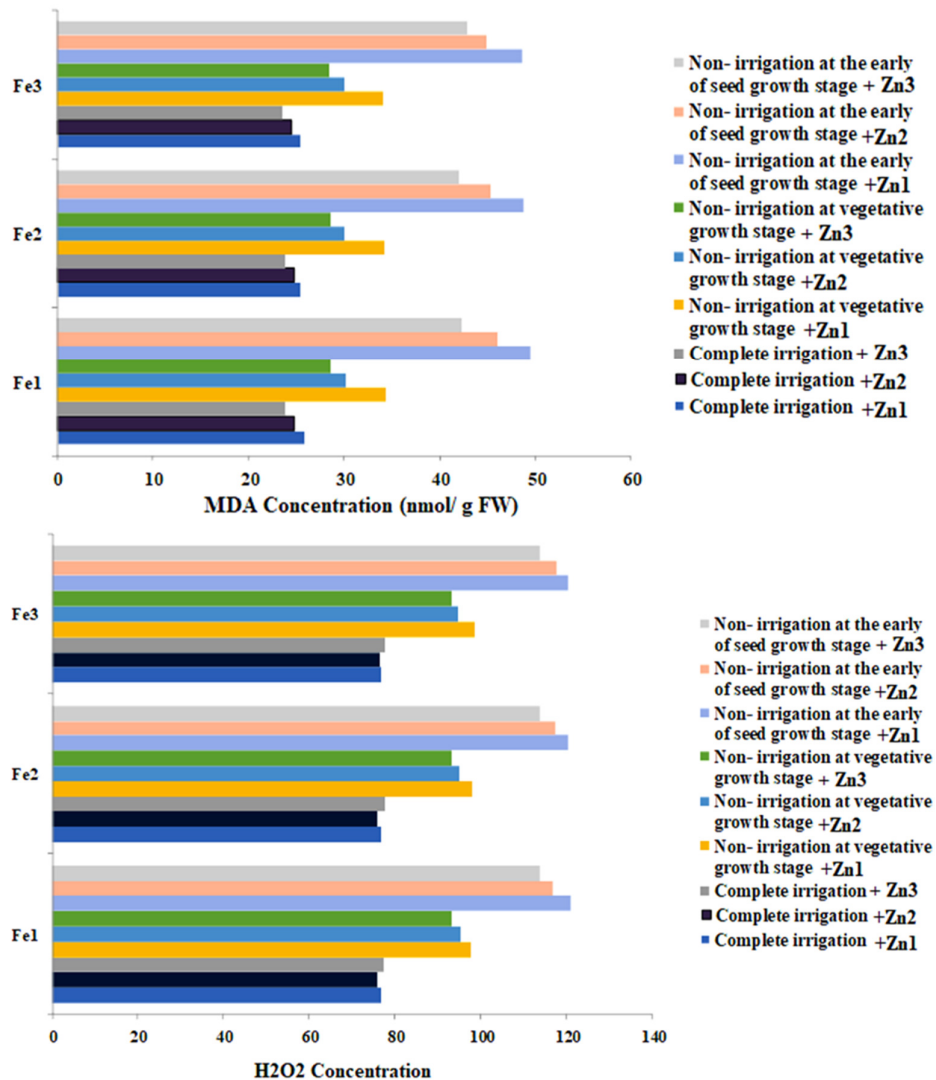


Figure 2. MDA and H_2O_2 concentration mean comparison against control, as affected by foliar application of $ZnSO_4$ and $FeSO_4$
 S1: Zn1) Without zinc sulfate foliar application, Zn2) Foliar application with 5 $g \cdot L^{-1}$ zinc sulfate concentration, Zn3) Foliar application with 10 $g \cdot L^{-1}$ zinc sulfate concentration; Fe1) Without iron sulfate foliar application, Fe2) Foliar application with 3 $g \cdot L^{-1}$ iron sulfate concentration, Fe3) Foliar application with 6 $g \cdot L^{-1}$ iron sulfate concentration

Relative growth rate (RGR)

Relative growth rate (RGR) curves (Figure 3) shows that between two water stress conditions highest RGR of maize obtained by foliar application of 10 $g \cdot L^{-1}$ $ZnSO_4$ and 3 $g \cdot L^{-1}$ $FeSO_4$ in non-irrigation at the early stage of seed growth ($0.07 g \cdot g^{-1}$). Lowest RGR by $0.047 g \cdot g^{-1} \cdot d^{-1}$ belonged to non-irrigation conditions at vegetative stage. In non-irrigation conditions at the vegetative stage and using 5 $g \cdot L^{-1}$ $ZnSO_4$ concentration, in

terms of this parameter was no difference between 3 and 6 g. L⁻¹ FeSO₄ concentration. However, foliar application of zinc and iron in water stress conditions, increased relative growth rate at the beginning of growing season and reduced it at the end of the growing season.

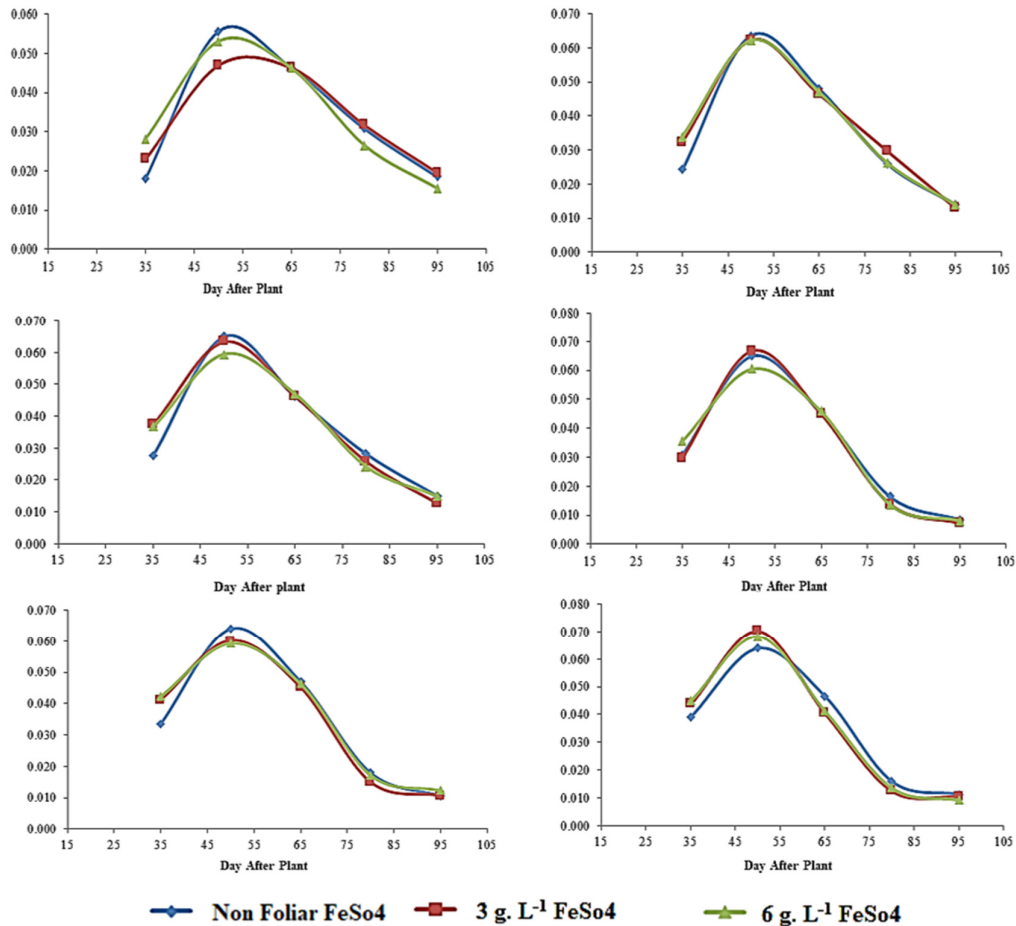


Figure 3. Trends of relative growth rate changes under influence of foliar application and water stress. A, B & C) Non-irrigation at vegetative growth stage (12-14 leaf); D, E & F) Non-irrigation at early seed growth stage. A & D) Without zinc sulfate foliar application; B & E) Zinc sulfate foliar application with 5 g. L⁻¹ concentration; C& F) Zinc sulfate foliar application with 10 g. L⁻¹ concentration

Superoxide dismutase (SOD) activity

The interactive effect of FeSO₄ × ZnSO₄ and water stress had a significant impact on superoxide dismutase enzyme. This enzyme in non-irrigation at the early stage of seed growth treatment has been more active than water deficit in the vegetative growth stage. According to our results, it was found that drought stress increased the activities of this enzyme. However, addition of ZnSO₄ and FeSO₄ in water-stressed plants caused a further rise in the activity of SOD enzyme. The highest superoxide dismutase enzyme levels were obtained when using 10 g. L⁻¹ zinc sulfate and 3 g. L⁻¹ iron sulfate in water deficit conditions at the early stage of seed growth and the lowest value of SOD enzyme was obtained under complete irrigation conditions (Figure 4A).

Peroxidase (POD) activity

Applying ZnSO₄ and FeSO₄ affected Peroxidase (POD) activity in maize in cut-off irrigation conditions. The highest POD was related to the highest level of ZnSO₄ (10 g L⁻¹) and foliar application with 3 g L⁻¹ iron sulfate concentration in water deficit condition at the early stage of seed growth, which increased compared to full irrigation treatment. Application of iron sulfate at a constant concentration of zinc sulfate did not show any significant differences in stopping irrigation at the vegetative growth stage. Under complete irrigation conditions, significant change was observed in the Peroxidase (POD) activity between two concentrations of 5 g L⁻¹ and 10 g L⁻¹ ZnSO₄. The lowest values of this trait obtained under complete irrigation conditions and no micronutrient intake (Figure 4B).

Catalase (CAT) activity

In terms of catalase activity, significant differences were seen in water stress conditions compared to full irrigation by zinc sulfate and iron sulfate foliar application. The highest catalase activity belonged to cut-off irrigation at the early stage of seed growth, using 10 g L⁻¹ zinc sulfate and 3 g L⁻¹ iron sulfate in this stage increased catalase activity. In water deficit at the vegetative growth stage, using 10 g L⁻¹ zinc sulfate and 6 g L⁻¹ iron sulfate, reduced the effects of water stress (Figure 4C). The activity of this enzyme, reduced the effect of water stress at different stages.

Glutathione reductase (GR) activity

Glutathione reductase activity under drought-stressed condition was more active than non-stressed condition. According to results of interactive effects (Figure 4D) was recording higher values of glutathione reductase enzyme in cut-off irrigation at the early stage of seed growth and using 10 g L⁻¹ zinc sulfate and 6 g L⁻¹ iron sulfate. The lowest values of this trait obtained under complete irrigation conditions. In cut-off irrigation at the vegetative growth stage and at constant concentration of zinc sulfate, no significant difference between applying 3 g L⁻¹ FeSO₄ and non- applying this element was observed. Also, under drought-stressed condition at the early stage of seed growth and in 5 g L⁻¹ concentration of zinc sulfate, no significant difference was recorded between applying 3 g L⁻¹ and non- applying FeSO₄.

Grain protein

According to results of Table 3, the comparison of the mean values showed that the highest grain protein content was observed in non-irrigation at the early stage of seed growth treatment (8.7%), which was interestingly higher than even the full irrigation treatments (7.7%). In our study non-irrigation at vegetative growth stage treatment shows 7.9 % average about this trait. However, foliar application of ZnSO₄ and FeSO₄ resulted in a significant increase in grain protein, as the highest grain protein was observed in the foliar application of zinc sulfate at the 10 g L⁻¹ concentration and significant difference was observed between the concentrations of 3 and 6 g L⁻¹ of FeSO₄. 6 g L⁻¹ FeSO₄ treatment with 8.4% average is the best treatment compared to other levels of iron sulfate foliar application.

Grain yield (GY), Biomass yield (BY) and Harvest index (HI)

Interactive effects of water stress × ZnSO₄ and FeSO₄ × ZnSO₄ had a significant impact on the grain yield ($P \leq 0.01$). The highest grain yield was obtained in full irrigation treatment and the lowest in the non-irrigation at the early stage of seed growth. In general, the lack of irrigation at the early stage of seed growth resulted in a significant decline in the grain yield compared to complete irrigation. Foliar application of zinc sulfate in non-irrigation at vegetative growth stage and the early stage of seed growth resulted in a significant increase in the grain yield. Specifically, 10 g L⁻¹ ZnSO₄ concentration significantly increased the grain yield compared to 5 g L⁻¹ concentration. There was significant difference between two concentrations of 3 and 6 g L⁻¹ FeSO₄ in terms of grain yield. Best concentration of this element was 6 g L⁻¹ foliar application (Table 4).

Statistical analysis of the data Table 4 showed that interaction effect of ZnSO₄ and FeSO₄ had significantly affected on biomass yield.

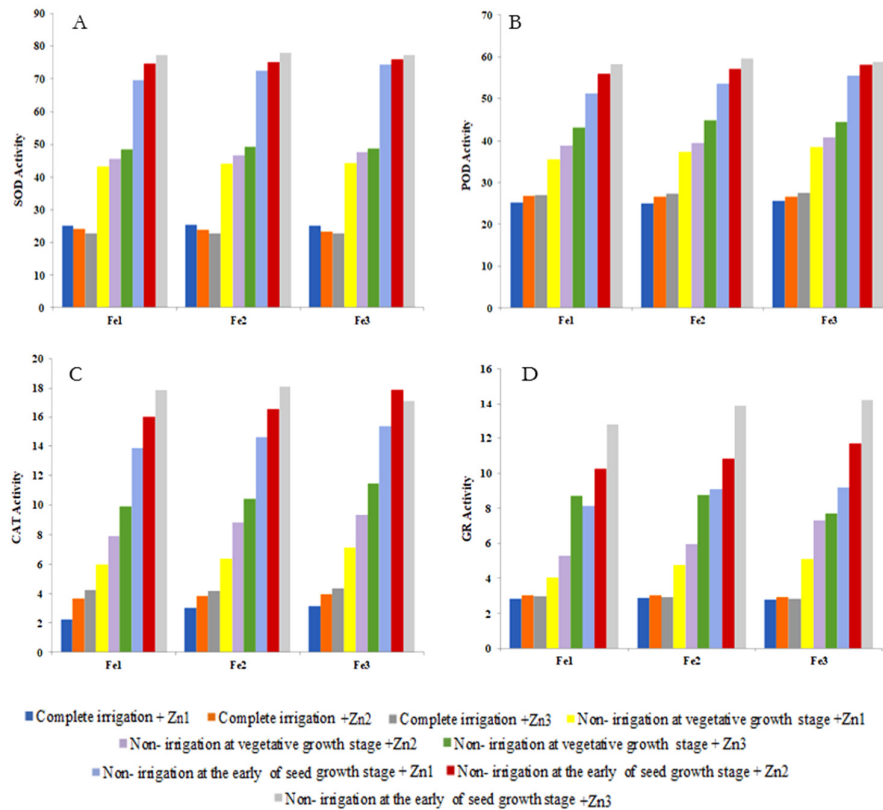


Figure 4. SOD, POD, CAT and GR enzyme means comparison against control, as affected by foliar application of ZnSO₄ and FeSO₄

Zn1) Without zinc sulfate foliar application, Zn2) Foliar application with 5 g L⁻¹ zinc sulfate concentration, Zn3) Foliar application with 10 g L⁻¹ zinc sulfate concentration; Fe1) Without iron sulfate foliar application, Fe2) Foliar application with 3 g L⁻¹ iron sulfate concentration, Fe3) Foliar application with 6 g L⁻¹ iron sulfate concentration

Table 4. Mean comparison effect of treatments on agronomic characteristics

	Water stress × ZnSO ₄		Water stress × FeSO ₄		ZnSO ₄ × FeSO ₄			
	GY	BY		GY	BY		GY	BY
	(Kg. ha ⁻¹)			(Kg. ha ⁻¹)			(Kg. ha ⁻¹)	
S1Z1	5328 e	ns	S1F1	5616 c	ns	Z1F1	3900 g	10466 d
S1Z2	6488 b		S1F2	6525 b		Z1F2	4385 f	10207 d
S1Z3	7062 a		S1F3	6735 a		Z1F3	4510 f	10656 d
S2Z1	3961 g		S2F1	4623 f		Z2F1	4853 e	12382 c
S2Z2	5668 d		S2F2	5405 d		Z2F2	5804 c	13566 ab
S2Z3	5980 c		S2F3	5580 cd		Z2F3	6230 b	13767 ab
S3Z1	3506 h		S3F1	4086 g		Z3F1	5573 d	13289 bc
S3Z2	4732 f		S3F2	4547 f		Z3F2	6291 ab	14375 a
S3Z3	5270 e		S3F3	4873 e		Z3F3	6449 a	13001 bc
P value	**			*		**	*	
CV(%)	GY:5.50				BY:7.20			
	S×Zn×Fe: ns				S×Zn×Fe×Y: ns			

ns = Not significant; * = P ≤ 0.05; ** = P ≤ 0.01. S1: Complete irrigation, S2: Non-irrigation at vegetative growth stage, S3: Non-irrigation at the early of seed growth stage; Zn1) Without zinc sulfate foliar application, Zn2) Foliar application with 5 g L⁻¹ zinc

sulfate concentration, Zn3) Foliar application with 10 g .L⁻¹ zinc sulfate concentration; Fe1) Without iron sulfate foliar application, Fe2) Foliar application with 3 g .L⁻¹ iron sulfate concentration, Fe3) Foliar application with 6 g .L⁻¹ iron sulfate concentration. Y: year

Foliar application at the rate of 10 g .L⁻¹ zinc sulfate and 3 g .L⁻¹ iron sulfate produced maximum biomass yield (14375 kg. ha⁻¹), whereas minimum biomass yield was observed in non-foliar application of zinc sulfate (10207 kg. ha⁻¹). Increase in Zn rate also increased biomass yield. In the case of Zn × Fe interaction, in 5 g. L⁻¹ zinc sulfate foliar application, greater biomass yield was produced by apply 6 g. L⁻¹ FeSO₄, however between two concentrations of FeSO₄ no significant difference was observed.

According to results presented in Table 5, harvest index was significantly affected by zinc, iron, water stress and their interaction, whereas interaction of Zn× Fe × S × Y was not significant for harvest index. Interaction of Zn× Fe × S revealed that increase in zinc sulfate and iron sulfate level in water stress conditions, increased harvest index. Most change of this parameter was observed in apply 10 g. L⁻¹ ZnSO₄ and 6 g. L⁻¹ FeSO₄. Water stress at vegetative growth stage and at the early stage of seed growth, decreased harvest index compared to full irrigation. Plots treated by 0 g. L⁻¹ micro elements with water stress at the early stage of seed growth achieved minimum harvest index (32.4%).

Table 5. Results of harvest index changes affected water stress and zinc and iron elements

Treatment		Complete irrigation	Harvest index (%)			
ZnSO ₄ (g. L ⁻¹)	FeSO ₄ (g. L ⁻¹)		Change percentage	At vegetative growth stage	Change percentage	At the early stage of seed growth
0	0	39.6 g-k	-4.3	37.9 i-l	-18.19	32.4 m
0	3	46.8 bcd	-10.69	41.8 e-i	-22.01	36.5 j-m
0	6	47.9 bc	-14.20	41.1 f-i	-25.89	35.5 klm
5	0	42.3 e-i	-1.9	41.5 e-i	-8.52	38.7 h-l
5	3	45.6 b-e	-2.64	44.4 c-f	-9.87	41.1 f-i
5	6	49.7 b	-3.22	48.1 bc	-11.88	43.8 c-g
10	0	42.8 d-h	-1.64	42.1 e-i	-9.35	38.8 h-l
10	3	45.7 b-e	-3.07	44.3 c-f	-12.48	40 f-j
10	6	57.1 a	-15.94	48 bc	-25.92	42.3 e-i
S; Zn; Fe: **		S×Zn×Fe×Y: ns			CV (%): 6.30	

The change percentage is calculated compared to the full irrigation conditions.
 ns = Not significant; * = P ≤ 0.05; ** = P ≤ 0.01. Y: year

Attributes correlation analysis

The correlation coefficients of grain yield and some traits such as antioxidant enzymes under different levels of zinc, iron and irrigation are presented in Table 6. Significant and positive correlations were observed between relative water content (r= 0.95; p≤0.001), Fv/Fm ratio (r = 0.84; p ≤ 0.001) and biomass yield (r = 0.84; p ≤ 0.001) with grain yield. Non-significant correlations were observed between glutathione reductase enzyme and grain protein with grain yield. Results showed that between grain yield and MDA concentration (r= -0.73; p≤ 0.001), superoxide dismutase (SOD) (r= -0.57; p≤ 0.001), peroxidase (POD) (r= -0.49; p≤ 0.001), H₂O₂ (r= -0.67; p≤ 0.001) and catalase (CAT) (r= -0.42; p≤ 0.05) were significant and negative correlation. It means by decreasing MDA and H₂O₂ concentration, superoxide dismutase, peroxidase and catalase enzyme, grain yield was increased. Relative water content and Fv/Fm ratio showed negatively and significantly correlated with MDA, H₂O₂ content, SOD and POD enzyme.

Table 6. The correlation coefficients of grain yield and some traits

Traits	RWC	Fv/Fm	MDA	H ₂ O ₂	SOD	POD	CAT	GR	GP	BY
RWC		0.74**	-0.62**	-0.56**	-0.47*	-0.38*	-0.32 ^{ns}	-0.25 ^{ns}	0.23 ^{ns}	0.76**
Fv/Fm	0.74**		-0.93**	-0.93**	-0.86**	-0.81**	-0.76**	-0.66**	-0.34 ^{ns}	0.62**
MDA	-0.62**	-0.93**		0.97**	0.93**	0.89**	0.87**	0.78**	0.54**	-0.39*
H ₂ O ₂	-0.56**	-0.93**	0.97**		0.97**	0.95**	0.93**	0.85**	0.61**	-0.35 ^{ns}
SOD	-0.47*	-0.86**	0.93**	0.97**		0.99**	0.98**	0.94**	0.69**	-0.21 ^{ns}
POD	-0.38*	-0.81**	0.89**	0.95**	0.99**		0.99**	0.96**	0.75**	-0.12 ^{ns}
CAT	-0.32 ^{ns}	-0.76**	0.87**	0.93**	0.98**	0.99**		0.97**	0.78**	-0.03 ^{ns}
GR	-0.25 ^{ns}	-0.66**	0.78**	0.85**	0.94**	0.96**	0.97**		0.81**	-0.06 ^{ns}
GP	0.23 ^{ns}	-0.34 ^{ns}	0.54**	0.61**	0.69**	0.75**	0.78**	0.81**		0.37 ^{ns}
BY	0.76**	0.62**	-0.39*	-0.35 ^{ns}	-0.21 ^{ns}	-0.12 ^{ns}	-0.03 ^{ns}	-0.06 ^{ns}	0.37 ^{ns}	
GY	0.95**	0.84**	-0.73**	-0.67**	-0.57**	-0.49**	-0.42*	-0.33 ^{ns}	0.09 ^{ns}	0.84**

Relative water content: RWC; H₂O₂: Hydrogen peroxide; Malondialdehyde: MDA; Superoxide dismutase: SOD; Peroxidase: POD; Catalase: CAT; Glutathione reductase: GR; Grain protein: GP; Grain yield: GY; Biomass yield: BY. * and **: Significant at the 5% and 1% probability levels, respectively. ns = Not significant

Discussion

The damage caused by drought in plants necessitates studies on the mechanisms of drought tolerance in plants to avoid a significant loss of yield under water stress conditions. Water deficiency at grain filling time can impair photosynthesis, causing diminished remobilization of assimilates to grain and reduced grain filling period, resulting in lower grain weight production (Farooq *et al.*, 2014). On the other hand, micronutrients such as zinc and iron are essential for plant growth and are involved in physiological processes such as photosynthesis, plant hormone production, and plant chlorophyll formation. Their deficiency can cause plant nutrient imbalances and ultimately reduce the quantity and quality of the product (Malakoti *et al.*, 2005). Chlorophyll fluorescence is very useful to study the effects of environmental stresses on photosynthesis in plants (Afrousheh, 2010). In the current research, foliar application of ZnSO₄ improved Fv/Fm ratio under water stress. Similar findings have also been reported in the other crops where the use of ZnSO₄ improved drought tolerance (Karim *et al.*, 2012). It was reported that Zn application under adverse conditions increased the chlorophyll content and photosynthesis rate, and improved the plant growth (Tavallali *et al.*, 2010). Also, Nogues and Baker (2000) believed that the reduction of fluorescence performance (Fv/ Fm) during drought tolerance in all species, as a physiological regulation of electron transfer, by energy-emitting processes, photosystem II receptors can be realized. The decrease in Fv/ Fm due to water stress is consistent with the results of Fathi's research (2010). Increasing the efficiency of photosensitivity II by zinc spraying is consistent with the results of Tavallali *et al.* (2010). The lack of microelements changed the physiological state of investigated maize plants, especially in drought stress conditions. It has been reported that in drought tolerance of maize, there is direct or indirect contribution of antioxidants (Farooq *et al.*, 2009). In response to drought stress, the activities of CAT, POD, and SOD have been reported to increase (Morteza-Salekjalali *et al.*, 2012; Yavas and Unay, 2016) which further support our results. The results of research by Amiri Nejad *et al.* (2015) showed that water stress and foliar application increased the activity of antioxidant enzymes, so that the highest activity of catalase, peroxidase and superoxide dismutase enzymes was obtained from the treatment of intense stress and foliar application of iron and zinc elements, which increased significantly compared to the control treatment. Kawakami *et al.* (2010) reported that the activity of superoxide dismutase increased significantly under water stress. The results of Akbari *et al.* (2013) showed an increase in peroxidase in the application of Zn + Fe under water stress conditions. In the current investigation, ZnSO₄ and FeSO₄ improved drought tolerance

of maize plants which was reflected in an enhanced activity of the above-mentioned enzymes. By absorbing oxygen or oxidative species from the lipid environment and biologically to remove free cell radicals, superoxide dismutase, catalase, and other antioxidant enzymes keep reactive oxygen groups at low concentrations, and catalyze the degradation of hydrogen peroxide in the cell membrane. They, they constitute an important biological defense mechanism against free radical damage (Mansouri *et al.*, 2018). Generally, the interaction of foliar application of micronutrients has an important role in increasing the amount of yield and antioxidant enzymes activity under water deficit stress conditions.

Conclusions

The results of this experiment showed that water deficit at the early stage of seed growth had the most destructive effects on the corn. Application of Zn + Fe nutrient had a better effect on grain yield, biomass yield, harvest index, leaf relative water content, relative growth rate and antioxidant enzymes compared to their single application and control. The highest grain yield of maize was recorded for non-drought stress treatment and was followed by foliar application of both ZnSO₄ and FeSO₄, while severe water stress and non-application of Zn and Fe resulted in the minimum grain yield. This study provides useful information for the underlying physiological and biochemical mechanisms involving ZnSO₄ and FeSO₄ foliar application and plant tolerance to drought stress. Furthermore, this study provides evidence for the use of ZnSO₄ and FeSO₄ in arid and semiarid environments to enhance the grain yield. Drought stress reduced maize agronomic traits and boosted the activity of antioxidant enzymes such as catalase, superoxide dismutase, and peroxidase, which increased the above enzymes under drought stresses. This suggests the effect of these enzymes in reducing oxidative stress damages and their important role in counteracting reactive oxygen species (ROS).

Authors' Contributions

Mojtaba Afshari conducted an experiment and collected the data. Ahmad Naderi and Mani Mojadam oversee how data is tested and analysed. Shahram Lack and Mojtaba Alavifazel have been involved in presenting and analysing the results. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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