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Effect of zinc and sulfur foliar applications on physiological characteristics of sunflower (*Helianthus annuus* L.) under water deficit stress

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Abstract

In order to study effect of zinc and sulfur foliar applications on quantitative and qualitative characteristics of sunflower under water deficit stress, an experiment was conducted at Varamin, Iran during 2013 growing season. The experimental design was laid out in a randomized complete block with a split plots arrangement of treatments in three replications. Main plots included three different levels of irrigation (complete irrigation, irrigation withholding at flowering stage and irrigation withholding at seed filling period stage) and sub plots were included four different zinc and sulfur application (untreated zinc and sulfur application, zinc foliar application, sulfur application and both zinc foliar application and sulfur application). The results showed that irrigation withholding at different growth stages significantly decreased seed number in cap, thousand seed weight, seed yield, biological yield, oil yield and total chlorophyll but by contrast increased proline content, membrane stability, super oxide dismutase enzymes activity. Both zinc foliar application and sulfur application treatment had positive effect on all attributes in this experiment. In general, the results of the present study indicate that usage of zinc foliar application reduces the harmful effects of water deficit stress and increases resistance to drought stress in sunflower plant.

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Introduction

In many regions of the world, including Iran, drought stress is one of the most important factors responsible for decreasing agricultural crop yield. Sunflower (*Helianthus anus* L.) is one of the most important oilseed plants in the world. Drought stress and high temperatures during the flowering and seed filling stages decrease crops yield. Researchers have shown that, in many flowering plants, the pollination and seed-filling stages are sensitive to drought stress (Liu *et al.* 2004). Under conditions of water stress and other types of environmental stress, reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, are generated (Zhu 2000). These free radicals can damage essential membrane lipids as well as proteins and nucleic acids (Noctor and Foyer, 1998). Plant cells contain an array of protection mechanisms and repair systems that can minimize the occurrence of oxidative damage caused by reactive oxygen species (ROS) (Abdel Latef, 2010). Mechanisms of active oxygen species detoxification exist in all the plants and include activation of enzymatic (superoxide dismuatase, catalase, ascorbat peroxidase, peroxidase, glutathione reductase (Meloni *et al.,* 2003). On the other hand, one of the most important effects of moisture shortage is that mobility of some elements such as zinc will reduce in the soil solution by decreasing soil moisture and the plant will be more encountered with deficiency of this element given the root growth restrictions (Kafi and Rostami, 2007). Among fertilizers, zinc sulfate plays a more important role in stomata regulation and ion balance in plant systems to reduce the tensions of draught. Thus, fertilizer consumption should be balanced and efficient during water shortage and the use of fertilizers such as zinc sulfate should be especially considered (Baybordi, 2006; Babaeian *et al.,* 2010). Kassab (2005) indicated that foliar application of Zn, Mg, Mn and Fe significantly increased growth parameters, yield and its components of mung bean plants. Given the above mentioned points, it seems necessary to achieve the zinc foliar application can reduce the effects of drought stress while meeting the plant needs; because the use of zinc increases the crop yield, promotes the quality of products and consequently, achieves the enrichment and improvement of the community health (Borrell *et al.,* 2008). Also, Sulfur has pronounced effects on plant growth. In cotton it diminished leaf size and stem elongation, protein and soluble sugars, and caused chlorosis. Chloroplasts contain proteins rich in S and chloroplast morphology is considerably affected by S deficiency (Repica *et al.*, 1971; Whatley, 1971). Pirson, (1955) concluded that S deficiency upsets photosynthesis in a profound way which, after radiation of external sulfate, can only be corrected slowly through the synthesis of new protein and Chl. The purpose of this study is to understand whether application of micronutrients, Zn and S, raises quantity and quality of sunflower under water deficiency.

Materials and methods

In order to study effect of zinc and sulfur foliar applications and limited irrigation on quantitative and qualitative characteristics of sunflower, an experiment was conducted at Varamin, Iran during 2013 growing season. Site of study was situated at 31° 51□ E and 20° 35□ N and 1050 m above sea level. Before beginning of experiment, soil samples were taken in order to determine the physical and chemical properties. A composite soil sample was collected at a depth of 0-30 cm. It was air dried, crushed, and tested for physical and chemical properties. The research field had a clay loam soil. Details of soil properties are shown in Table 1. After plow and disk, plots were prepared. The experimental design was carried out in a randomized complete block with a split plot arrangement of treatments in three replications. Main plots included three different levels of irrigation (complete irrigation, irrigation withholding at flowering stage and irrigation withholding at seed filling period stage) and zinc and sulfur application (untreated zinc and sulfur application, zinc foliar application, sulfur application and both zinc and sulfur foliar application) was allocated to subplots were. The plots were prepared with 5 m long and consisted of five rows, 0.60 m apart. Between all main plots, 2 m alley was kept to eliminate all influence of lateral water movement. Polyethylene pipeline was performed for control of irrigation as dropping irrigation. Sunflower seeds (*Helianthus annuus* L. Merr cv. Azargol) sowed in certain experimental plots with 30 cm apart each other Irrigation was performed immediately after seed sowing and after seedling establishment irrigation was done every week. At 4-5 leaf stage plants were thinned to appropriate density. Weeds were controlled manually at 5-leaf stage, stem elongation and flowering stage. In order to stress induction irrigation was stopped at stem elongation and seed filling stage.

At physiological maturity stage crop was completely harvested and seed number in cap, 1000 seed weight, seed yield, biological yield and oil yield were measured. Oil percentage was calculated using soxhlet methods. Oil yield was obtained by following formula:

Oil yield = Oil percentage × seed yield /100

Membrane stability assay

Leaf samples (0.5 g) were immersed into 10 ml of -2 bar mannitol solution (14.7 g mannitol per liter) and after 24 h electrical conductivity of the solution was measured.

Chlorophyll assay

Chlorophyll was extracted in 80 % acetone from the leaf samples according to the method of Arnon (1949). Extracts were filtrated and content of total chlorophyll was determined by spectrophotometry at 645 and 663 nm, respectively. The content of chlorophyll was expressed as mg g-1 fresh weight according to Equation 1 (Arnon, 1949).

Proline assay

Proline content of leaves was determined according to a modification of the method of Bates *et al.,* (1973). Samples of leaves (0.5 g) were homogenized in a mortar and pestle with 10 ml sulphosalicylic acid (3% w/v), and then centrifuged at 18 000 g for 15 min. Two millilitres of the supernatant was then added to a test tube, to which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. The test tubes were incubated in a water bath for 1 h at 100°C and then allowed to cool to room temperature. Four millilitres of toluene were then added to the tubes and then mixed on a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min, to allow separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube and its absorbance was measured at 520 nm in a spectrophotometer. The content of proline was calculated from a standard curve.

Antioxidant enzyme activity assay

Superoxide dismutase activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium according to the method of Giannopolitis and Ries (1977). The reaction mixture contained 100 µl l µm riboflavin, 100 µl 12 mM L-methionine, 100 µl 0.1 mM EDTA (pH 7.8), 100 µl 50 mM Na2CO3 (pH 10.2), 100 µl 75 µM nitroblue tetrazolium in 2300 nitroblue tetrazolium 25mM sodium phosphate buffer (pH 6.8) and 200 µl crude enzyme extract, in a final volume of 3 ml. Glass test tubes that contained the reaction mixture were illuminated with a fluorescent lamp (120 W), and identical tubes that were not illuminated served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. One unit of Superoxide dismutase activity was defined as the amount of enzyme which caused 50 % inhibition of photochemical reduction of nitroblue tetrazolium.

All data were analyzed from analysis of variance (ANOVA) using the GLM procedure in SAS (SAS Institute, 2002). The assumptions of variance analysis were tested by insuring that the residuals were random, homogenous, with a normal distribution about a mean of zero. Duncan's multiple range tests was used to measure statistical differences between treatment methods and controls.

Results and discussion

Analysis of variance showed that the effect of irrigation withholding in different growth stages was significant on all traits experiment. Also the effect of zinc and sulfur application was significant on all measured traits experiment except thousand seed weight (Table 2). Interaction of experimental factors (irrigation withholding in different growth stages × zinc and sulfur application was significant on total chlorophyll, membrane stability and super oxide dismutase enzyme activity. As can be seen from table 3, the highest seed number in cap was obtained from complete irrigation. Seed number in cap decreased as result of irrigation withholding at flowering stage and seed filling period stage at by 18.34 % and 7.58%, respectively with compared complete irrigation treatment conditions. Water deficit stress during flowering and pollination affect on metabolism, physiology and morphology of plants. It seems that decrease in seed number is due to lack of fertilization. In addition, water stress leads to reduction in nutrient uptake and photosynthesis rate and thus reproductive organs will damage. Also the result showed that the highest seed number in cap was obtained when zinc foliar application was applied along with sulfur application. The results of experiments by Agele *et al.* (2007) also indicate the positive effect of micronutrient fertilizers such as zinc sulfate and sulfur on the number of seeds regarding the sunflower. Moreover, some reports have been presented on the positive role of the use of zinc sulfate fertilizer on the number of seeds generated in the shrub of wheat, canola and sunflower. Interaction between irrigation withholding in different growth stages × zinc and sulfur application was not significant on seed number in cap (Table2).

Table 1. Soil properties of the experimental site.

Depth	EC ($ds.m^{-1}$)	pH	O.C (%)	T.N.V (%)	K (p.p.m)	P(p.p.m)	Total N (%)	Texture
0-30 cm	4.1	7.78	1.16	21.4	406.6	12.8	0.06	Clay loam

Table 2. Analysis of variance on sunflower attributes affected by water stress and zinc and sulfur applications.

S.O.V	d.f	Seed number in cap	1000 seed weight	Seed yield	Biological yield	Oil yield	Total chlorophyll	Membrane stability	Proline	Superoxide dismutase
Replication	2	316.19ns	0.095ns	2662.11ns	3197.44ns	626.96ns	0.005ns	36.94ns	0.000001ns	817.37ns
Water stress	2	240956.69**	347.11**	1202878.86**	953873.69**	594661.35**	265.72**	253932.72**	0.002**	687975.44**
Error (a)	4	342.52	0.707	29969.44	50410.86	8041.28	0.021	56.70	0.000002	663.03
Zinc and Sulfur applications	3	11505.81**	5.17ns	207023.58*	596562.85**	53840.90*	0.280*	6050.50**	0.000068**	4546.27*
Water stress × Zinc and Sulfur application	6	1725.06ns	0.22ns	10662.63ns	48821.76ns	1518.91ns	0.336**	1237.17**	0.0000071**	1617.73ns
Error (b)	18	1016.89	2.60	66048.37	113571.46	14969.009	0.069	198.71	0.000001	1108.92
C.V		2.27	2.17	7.24	3.47	7.21	1.93	1.76	4.63	4.06

*, ** and ns significant at 0.05, 0.01 probability level and no significant, respectively.

Also the result showed that the highest thousand seed weight was obtained from complete irrigation. By contrast, the lowest thousand seed weight related to irrigation withholding at seed filling period stage (Table 3). Water stress affect on available assimilates during seed filling stage and decreases sink capacity and leads to unfilled seeds and low seed weight (Li *et al.*, 2000). In addition, water deficit stresses affects on source and sink relations. These results are in agreement with those obtained by Unger, (1992) and Yegappan, *et al.*, (1982) they reported that the most important factor in increase of seed weight is soil water content during seed filling stage (Francois *et al.*, 1984). Seed yield decreased as result of irrigation withholding at flowering stage and seed filling period stage at by 16.07 % and 10.24%, respectively with compared complete irrigation treatment conditions. Decrease in seed yield due to decrease in yield components especially seed weight has been reported by other researchers previously (Unger, 1992 and Yegappan *et al.,* 1982). Decrease in length of seed filling stage due to water stress is the main factor to decrease seed weight (Cantagallo *et al.,* 1997). Furthermore, water deficit decreased seed yield via decrease in photosynthesis and seed number per ear. Similar results are accessible published by other researcher (Unger, 1992). Also the result showed that the highest seed yield was obtained when zinc foliar application was applied along with sulfur application. However, only significant difference was observed between this treatment and untreated treatment. Seed yield increased as result of zinc foliar application was applied along with sulfur application at by 9.04 %, when this treatment compared with untreated treatment (Table 3).

Table 3. Comparison of main means sunflower attributes affected by irrigation withholding in different growth stages and zinc and sulfur applications.

Treatments	Seed number in cap	1000 seed weight (g)	Seed yield (kg.ha^{-1})	Biological Yield (kg.ha^{-1})	Oil yield (kg.ha^{-1})	Total chlorophyll (mg.lit^{-1})	Membrane stability (µs cm-1)	Proline (mg.g-1 FW)	Superoxide dismutase (ΔA/mg pro.min^{-1})
Irrigation									
Complete Irrigation	1536.92a	78.85a	3889.8a	9992.6a	1951.28a	31.96a	639.37c	0.010c	555.32c
Irrigation withholding at flowering stage	1254.92c	75.83b	3264.4c	9432.0b	1578.20b	29.22b	838.78b	0.037a	1023.59a
Irrigation withholding at seed filling period stage	1420.33b	68.40c	3491.3b	9659.6b	1554.33b	22.79c	922.54a	0.029b	876.26b
Zinc and sulfur applications									
Untreated	1359.33c	73.58a	3379.9b	9432.1c	1606.73b	27.83b	825.89a	0.022c	806.95bc
Zinc foliar application	1409.44b	74.70a	3627.9ab	9815.6ab	1731.50ab	28.21a	789.96b	0.026b	829.85ab
Sulfur application	1401.00b	73.90a	3470.1ab	9536.2bc	1658.60ab	27.87b	816.34a	0.025b	793.34c
Zinc and sulfur foliar applications	1446.44a	75.25a	3716.2a	9995.0a	1781.59a	28.05ab	768.72c	0.029a	843.41a

Treatment means followed by the same letter within each common are not significantly different (*P* < 0.05) according to Duncan's Multiple Range test.

The obtained results are in full agreement with the findings of Basole *et al.,* (2003), Gupta *et al.,* (2003) and Kassab (2005). These results suggested that foliar application of nutrient solutions partially alleviates the adverse effects of water stress on photosynthesis and photosynthesis-related parameters, yield and yield components through mitigating the nutrient demands of water-stressed plants. In this concern, Ved *et al.*, (2002) stated that foliar applied zinc enhances photosynthesis, early growth of plants, improves nitrogen fixation, grain protein and yield.). Also, the researcher stated that sulfur effects on plant growth, leaf size and stem elongation, protein and soluble sugars and seed yield (Repica *et al.*, 1971; Whatley, 1971). As can be seen from table 3, the highest biological yield was obtained from complete irrigation. Biological yield decreased as result of irrigation withholding at flowering stage and seed filling period stage at by 5.61 % and 3.33%, respectively with compared complete irrigation treatment conditions. Anyia and Herzog (2004) indicated that water deficit caused between 11 and more than 40% reduction of biomass across the genotypes of cowpea (*Vigna unguiculata* L.) due to decline in leaf gas exchange and leaf area. Also the result showed that the highest biological yield was obtained when zinc foliar application was applied along with sulfur application. Biological yield increased as result of zinc foliar application was applied along with sulfur application at by 5.63 % when this treatment compared with untreated treatment (Table 3).

Table 4. Interaction between irrigation withholding in different growth stages and zinc and sulfur applications on some attributes of sunflower.

Irrigation	Zinc and sulfur applications	Seed number in cap	1000 seed weight (g)	Seed yield (kg.ha^{-1})	Biological Yield (kg.ha^{-1})	Oil yield (kg.ha^{-1})	Total chlorophyll (mg.lit^{-1})	Membrane stability (µs cm-1)	Proline (mg.g-1 FW)	Superoxide dismutase (ΔA/mg pro.min^{-1})
Complete Irrigation	Untreated	1516.33a	78.36ab	3771.3abcd	9827.7ab	1872.42ab	31.60b	645.13f	0.009g	545.43f
	Zinc foliar application	1542.33a	79.03a	3921.7ab	10003.3ab	1972.43a	31.91b	640.32f	0.010fg	560.53f
	Sulfur application	1528.67a	78.50ab	3854.3abc	9902.0ab	1934.63a	31.96b	638.07f	0.011fg	552.10f
	Zinc and sulfur foliar applications	1560.33a	79.50a	4012.0a	10237.3a	2025.65a	32.39a	633.96f	0.012f	563.20f
Irrigation withholding at flowering stage	Untreated	1182.33g	75.10c	3018.3f	9037.3d	1463.09c	29.09cd	867.11c	0.033c	984.43c
	Zinc foliar application	1245.67f	76.03bc	3395.3cdef	9674.7abc	1636.32c	29.50c	825.18d	0.038b	1046.55ab
	Sulfur application	1275.67ef	75.40c	3164.0ef	9160.3cd	1529.05c	28.87d	861.86c	0.036b	992.39bc
	Zinc and sulfur foliar applications	1316.00e	76.80abc	3480.0bcdef	9855.7ab	1684.33bc	29.42c	800.97e	0.043a	1070.97a
Irrigation withholding at seed filling period stage	Untreated	1379.33d	67.30d	3350.0def	9431.3bcd	1484.68c	22.81f	965.45a	0.026e	890.99de
	Zinc foliar application	1440.33bc	69.03d	3566.7abcde	9768.7ab	1585.73c	23.23e	904.38b	0.029d	882.47de
	Sulfur application	1398.67cd	67.80d	3392.0cdef	9546.3bcd	1512.11c	22.78f	949.12a	0.028d	835.54e
	Zinc and sulfur foliar applications	1463.00b	69.46d	3656.7abcd	9892.0ab	1634.80c	22.36g	871.22c	0.033c	896.05d

Treatment means followed by the same letter within each common are not significantly different ($P < 0.05$) according to Duncan's Multiple Range test.

However, no significant difference was observed between this treatment and zinc foliar application treatment. Modaihsh (1997) and Kaya and Higgs (2002) reported the foliar application of zinc can increase the production of biomass. It should be noticed that in crops, two key resources are carbon and nitrogen that are accumulated to grow plants and produce grain (Sinclair and Jamieson, 2008). Also the result showed that highest oil yield was obtained from complete irrigation. Oil yield decreased as result of irrigation withholding at different growth stages. Singh and Sinha (2005) reported the decrease in oil concentration may be due to oxidation of some polyunsaturated fatty acids. Water deficit stress effects on qualitative and quantitative characteristics of produced oil. Decease of oil percentage is due to decrease in seed weight. The oil percentage was decreased by drought stress, most likely because of reduction in photosynthesis and assimilates remobilization. In addition, drought stress reduces the seed filling period and oil content. Rudra naik *et al.* (2001) has reported that water deficit stress decreased seed weight and oil percentage of safflower plants. Since oil yield is obtained by multiplying seed yield by oil percentage so oil yield decreased on account of water deficit stress. Also the result showed that the highest oil yield was obtained when zinc foliar application was applied along with sulfur application. However, only significant difference was observed between this treatment and untreated treatment (Table 3). Oil percentage was affected by zinc foliar application. Zinc plays an important role in synthesis of chlorophyll and plant growth regulators. Zinc improves photosynthesis and assimilates material and finally increased oil percentage. Since oil yield is obtained by multiplying seed yield by oil percentage so oil yield increased on account of zinc foliar application. Also the result showed that the highest total chlorophyll belongs to normal irrigation. Irrigation withholding at different growth stages decreased total chlorophyll content (Table 3). It is consistent with the results of Yari *et al.* (2005), suggesting that moisture stress reduces leaf chlorophyll content. Also the result showed that the highest chlorophyll content was obtained of zinc foliar application treatment (Table 3). As can be seen from table 4, total chlorophyll increased as result of zinc foliar application under irrigation withholding at different growth stages. Chlorophyll maintenance and consequently photosynthesis durability in stressful conditions are among physiological indicators of stress resistance (Zhang *et al.,* 2006; Jiang and Huang, 2002). According to table 3 the highest electrolyte leakage was occurred when sunflower plants were treated with irrigation withholding at seed filling period stage. Electrolyte leakage decreased as result of zinc foliar application was applied along with sulfur application, when this treatment compared with untreated treatment (Table 3). According table 4, the lowest electrolyte leakage was obtained when sunflower plants treated with zinc foliar application was applied along with sulfur application irrigation withholding at different growth stages. Jabari *et al.* (2006) indicated that cell walls were destroyed under drought stress because stomata closure under drought conditions decreased carbon dioxide fixation, while photo reactions and electron transfer went on in their normal manner. Under such condition, NADP availability will be limited for electron acceptance. Therefore, oxygen can be an alternative electron acceptor which leads to the accumulation of poisonous oxygen species such as superoxide radicals (O2), peroxide hydrogen (H2O2) and hydroxyl radicals (OH-). The accumulation of active oxygen species, which are produced under stress, damages many cell compositions like fats, proteins, carbohydrates and nucleic acids (Jiang and Huang, 2001). As a result, fatty peroxides destroy cell membrane (Liang *et al.,* 2003). As can be seen from table 3, the highest proline content was obtained from irrigation withholding at flowering stage. Proline is a highly water-soluble amino acid. The accumulation of PRO is a common metabolic response of plants to adversity, and PRO is an indicator of adaptation to adversity and is involved in the succession resistant capability of plants (Bian *et al.* 1988). PRO protects membranes against the adverse effect of high concentrations of ions and may also function as a PROT-compatible hydrotrope and as a hydroxyl radical5scavenger (Kavi Kishor *et al.* 1995). In the current study, irrigation withholding at different growth stages and both zinc foliar application and sulfur application increased the leaf PRO content. Also the result showed that the highest superoxide dismutase enzyme activity was obtained from irrigation withholding at flowering stage (Table 3). It was proved that the drought stress increases the production of reactive oxygen species (ROS) (Mittler, 2002). To scavenge these ROS, plants either synthesize different antioxidant compounds or activate antioxidant enzymes. Plants can detoxify ROS by up-regulating antioxidant enzymes, such as SOD, CAT and POX as well as some non-enzymatic antioxidant compounds. It is evident that high levels of antioxidants are related to plant water deficit tolerance (Sankar *et al.,* 2007; Tahi *et al.,* 2008). Similar results were reported under drought stress in wheat (Shao *et al.,* 2005), *Phaseolus acutifolius* (Turkan *et al.,* 2005) and tomato plants (Sanchez-Rodrıguez *et al.,* 2010). The combined action of SOD and CAT converts the toxic O2.-, H2O2 to water and molecular oxygen, averting the cellular damage under unfavorable conditions such as drought stress (Reddy *et al.*, 2000; Chaitanya *et al.,* 2002). The highest superoxide dismutase enzyme activity was obtained increased as result of zinc foliar application treatments when these treatments compared with untreated this condition (Table 3). Zinc is an essential mineral nutrients and a cofactor of over 300 enzymes and proteins involved in cell (Marschner, 1986). Thus zinc foliar application due to increase antioxidant enzyme activity in drought stress condition.

Conclusions

Our results clearly demonstrate that he flowering stage was the most sensitive to water deficit. Drought during this period caused reduction in floret number per capitulum and in time to maturity; consequently, reduced sink capacity and shorter growing period lead to lower seed yield. Water deficit stress during seed filling stages resulted in similar seed yield. This suggested that it is possible to obtain high seed yield with less applied water when the irrigation stop happens at a tolerant phonological stage. The effects of applied Zn and S were more pronounced on seed than total biomass. Moreover, foliar Zn and S application increased total chlorophyll, proline content and super oxide dismutase enzyme activity whereas decreased membrane stability.

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