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Application of Zinc Fertilizer and Mycorrhizal Inoculation on Physio-Biochemical Parameters of Wheat Grown under Water-Stressed Environment

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Abstract: Drought stress and poor zinc (Zn) are major constraints for commercial agriculture. Their detrimental effects significantly decrease crop's growth and yield. Less water uptake disturbs the metabolic processes in plants. However, the deficiency of Zn leads to the inactivation of many enzymes. It is well documented that cereal crops, especially wheat, are susceptible to drought and Zn deficiency. Scientists suggest the supplementation of Zn along bio-fertilizers for the sustainable management of these issues. That is why the current experiment was conducted to explore the best combination of Zn and bio-fertilizer for wheat. There were two different recommended concentrations of Zn sulfate (Zinc level 1 (Zn1) = 20 and Zinc level 2 (Zn2) = 40 kg ha^{-1}) applied under normal irrigation (75% field capacity = FC) and severe drought stress (40% FC). Sole and combined inoculation of arbuscular mycorrhizal fungi (AM) with Zn1 and Zn2 was also performed. Osmotic stress (40% FC) significantly decreased the examined growth parameters. It also significantly enhanced antioxidant and oxidative indicators in wheat. A significant increase in root fresh weight, root dry weight, and shoot length while a significant decrease in EL, SOD, POD over the control validated the efficacious role of Zn2 + AM. It is concluded that Zn2 + AM can improve wheat root fresh weight and root length wheat under 40% FC. Under different climatic zones, wheat varieties, and soil types, more investigations are recommended to declare Zn2 + AM as the best amendment for improving wheat growth attributes under osmotic stress.

Keywords: mycorrhizal inoculation; antioxidants; wheat; zinc; osmotic stress

1. Introduction

Around the world, 1 billion hectares of land is hyperarid and 5.45 billion hectares are sub-humid, semiarid, and arid areas. About 70% of drylands worldwide (5.2 billion

hectares) are under cultivation with very limited production and where the whole yield relies on the mode of water availability [1]. Water availability is the most dominant factor limiting crop yield, especially in semi-arid and arid regions of irrigated agricultural lands. Drought stress induces several physiological and biochemical alterations in plants as defensive survival mechanisms [2–4].

Water scarcity directly affects the photosynthetic apparatus, particularly disrupting major components of the photosynthetic process, i.e., thylakoid electron transport, the stomatal conductance of CO_2 supply and carbon reduction cycle, and increased carbohydrate accumulation, lipid peroxidation, and disturbance in cellular water balance. Water stress imbalance between the generation of reactive oxygen species (ROS) and antioxidative defense mechanisms results in elevated ROS accumulation, which brings oxidative stress to proteins, lipid peroxidation, and disorganization of DNA fragments [5]. Among the various rhizosphere-inhabited microorganisms, some of them assist plant growth promotion and defense responses. These soil microorganisms act as bio-fertilizers such as rhizobacteria and fungi that colonize the rhizosphere and plant roots and confer advantageous effects to plants [6].

Mycorrhiza association is a symbiotic relationship among plant roots and fungi. Mycorrhiza association imparts various benefits to plants, including increased tolerance against various biotic and abiotic stresses, the production and accumulation of several secondary metabolites, improvement in nitrogen fixation, osmotic adjustments during drought stress, and an enhanced photosynthesis rate [7].

The mechanisms used by Mycorrhiza to improve the water status of the plant might occur by increased water absorption by external fungal hyphae, an increase in the activities of antioxidant enzymes, the regulation of the stomatal apparatus, and the absorption of mineral nutrients, particularly phosphorus [8]. Mycorrhizal inoculation to wheat under water stress enhanced enzymatic antioxidant activities such as peroxidase (POD) and catalase (CAT) [9]. Mycorrhizae can improve the host plant's physiological and water status by altering the rate of water movements by expanding their hyphae, resulting in an increased water absorption region [5,10].

Zinc (Zn) is an essential micronutrient for plant biological systems. It has a vital physiological role in protein and enzyme functions associated with essential biochemical pathways, photosynthetic processes, protein synthesis, and enzyme activation. Zinc application increases the photosynthetic rate and chlorophyll content [11–14] and enhances photochemical reactions in thylakoid membranes and electron transport in PSII [15].

Wheat (*Triticum aestivum* L.) is a major cereal crop and a staple food in most of the world's regions. It has 55% of carbohydrates and 8–12% of protein contents. It is also an important crop plant on account of its worldwide trade. Wheat cultivation in a limited water supply decreases its yield, while its global consumption increases at the rate of 1.6% per annum [16]. Although traditional breeding and water management practices are assumed to be quite effective in drought stress tolerance, high technicalities and difficult approaches intricated in these ways make them hard to adopt. Inoculating plants with growth promoting-microorganisms is an effective strategy that can be executed easily.

In recent literature, plant researchers focused on applying either mycorrhizal inoculation or Zn fertilizer to mitigate the drought stress on wheat. The novelty of this study is to investigate the combined effect of mycorrhizal inoculation and Zn fertilizer on wheat to alleviate drought effects. Considering the importance of wheat for humans, the present study was conducted to hypothesize that the co-application of mycorrhizal inoculation and Zn fertilizer could be more effective in alleviating drought stress effects.

2. Materials and Methods

2.1. Experiment Site

The experiment was carried out in the research area of the Department of Botany, University of Agriculture Faisalabad, Pakistan. The experiment was performed in 2016

following a completely randomized block design (CRD) with the factorial arrangement and was replicated three times. Faisalabad is located at 31.4504° N, 73.1350° E.

2.2. Seed Sterilization

Seeds of Wheat (*Triticum aestivum* L., cv. Lasani-2008) were collected from Ayub Agriculture Research Institute, Faisalabad. The seeds were disinfected with 95% ethanol and then washed with 70% sodium hypochlorite solution. Finally, the seeds were carotenoids three times with distilled water.

2.3. Experimental Design

The treatments consisted of (a) Control, (b) Zinc level 1 = Zn1 (20 kg ha^{-1}), (c) Zinc level 2 = Zn2 (40kg ha^{-1}), (d) Arbuscular mycorrhizal fungi = AM, (e) Zn1 + AM, (f) Zn2 + AM and arranged in two groups (well-watered and drought).

2.4. Seed Sowing and Application of Treatments

Zn fertilizer was supplemented at three levels of 0 (control), 20 (Zn1), and 40 (Zn2) kg ha^{-1} by using zinc sulfate. Zinc sulfate was given at seed sowing 5 cm underneath the soil. Before sowing, wheat seeds were inoculated with 250–300 active propagules of arbuscular mycorrhizal (AM) fungi (*Glumus intraradisis*). The seeds of wheat were first dispersed in sugar solution and then coated with powdered mycorrhizal propagules. Each pot was given the respective dose of Zn fertilizer. Eight wheat seeds were sown in plastic pots containing 7 kg soil (50% soil + 50% sand) mixed thoroughly and sieved. Five healthy seedlings were maintained at the end of the experiment by thinning. Well-watered conditions were maintained at 75% field capacity, and drought stress was maintained at 40% field capacity (FC).

2.5. Plant Harvesting and Growth Attributes

Plants were harvested after 50 days of seed sowing. Fresh weight was quantified instantly after harvest using a digital balance. Root and shoot lengths of plants were determined using the measuring tape. Two sample plants were oven-dried at 65 °C for 72 h to measure their dry weights. Plants were stored at −30 °C in a freezer for further fresh analysis.

2.6. Chlorophyll Contents

The carotenoids, chlorophyll (chl.) a, chl. b, and total chl. were estimated from fresh leaf samples following the Arnon method [17]. To determine chlorophyll, a 0.1 g sample was incubated in 95% acetone (8 mL at 4 °C) for 1 day in the dark. Light absorbance was recorded at 646, 663, and 450 nm using a spectrophotometer (UV-2550; Shimadzu, Kyoto, Japan).

2.7. Electrolyte Leakage

The method of Lutts et al. [18] was used for the estimation of leaf electrolyte leakage. To wipe out the impurities from the leaf surface, the leaves were rinsed with deionized water. One gram leaf samples were soaked in 20 mL distilled water in test tubes and incubated at 25 °C for one day. The initial Electrical Conductivity was recorded by using EC meter (pre-calibrated). The final Electrical Conductivity value was recorded after heating the samples at 120 °C for 20 min in the water bath. The extent of electrolyte leakage was estimated using the following equation:

$$\text{Electrolyte leakage (EL \%)} = \left(\frac{\text{Initial Electrical Conductivity}}{\text{Final Electrical Conductivity}}\right) \times 100 \quad (1)$$

2.8. Evaluation of Protein Contents

The protein determination was carried out by following the Bradford method [19]. To estimate leaf protein concentration, 10 µL extract was mixed with 5 µL Bradford solution and 290 µL of extraction buffer mixture. Finally, the color absorbance was recorded at 595 nm.

2.9. Estimation of Sugars, Proline, and Non-Enzymatic Antioxidants

To determine the osmolytes, i.e., soluble sugars, proline, and other non-enzymatic antioxidant constituents, the ethanol extracts of dried plant material were made by 50 mg samples blended in 10 mL of 80% ethanol. The obtained reaction mixture was then filtered, followed by a re-extraction in 80% ethanol. The final volume of 20 mL was maintained by mixing both samples. The obtained mixture was used to determine flavonoids [20], anthocyanin [21], phenolics [22], ascorbic acid [23], and total soluble sugars [24] constituents.

To determine proline contents, 0.1 g of fresh leaf samples were homogenized in 3% aqueous sulfosalicylic acid (5 mL) then centrifuged at 10,000× *g* for 15 min. After that, 1 mL of aliquot was taken in the test tube with 1 mL of acidic ninhydrin and 1 mL of the glacial acetic acid mixture. This mixture was boiled for 10 min at 100 °C and then immediately cooled down in an ice bath. Finally, the solution was vortexed for 20 s and again brought to room temperature. Absorbance was recorded at 520 nm by using a spectrophotometer [25]. Glycine betaine was determined by following Holmstrom et al. protocol [26].

2.10. Oxidative Stress Indicators

Malondialdehyde (MDA) in leaves was analyzed by using 0.1 g of fresh leaf samples. In 25 mL concentrated phosphate buffer (50 mM; pH 7.8) with polyethene pyrrole mixture (1%) leaves were grinded. After that, 10,000× *g* centrifuge for 15 min at 4 °C temperature was conducted. The reaction mixture was then boiled at 100 °C for 20 min and instantly cooled down in an ice bath. Finally, the supernatant was collected, and a spectrophotometer recorded absorbance at 450, 532, and 600 nm. Lipid peroxidation was assessed according to Heath and Packer [27]. Where Abs. = Absorbance

$$\text{MDA}\left(\mu\text{mol g}^{-1}\right) = 6.45\,(\text{Abs.532} - \text{Abs.600}) - 0.56 \times \text{Abs.450} \quad (2)$$

Estimation of H_2O_2 concentration of wheat plants was done by homogenous mixing of 3 mL sample extract with 1 mL solution of 0.1% titanium sulfate and 20% H_2SO_4 (v/v) as described by Jana and Choudhuri [28].

2.11. CAT, POD and SOD Assay

To estimate the enzymatic antioxidant activities, 0.2 g fresh leaf samples were disintegrated using liquid nitrogen. Then, 1 mL of 0.05 M Tris–HCl buffer (pH = 7.5) was added to the sample. The obtained solution was centrifuged at 13,000 rpm for 20 min at 4 °C. According to Kar and Mishra method, the supernatant was used to estimate CAT and POD enzyme activity [29]. Superoxide dismutase enzyme activity (SOD) was assayed by following Beauchamp and Fridovich [30].

2.12. Na, Ca, P, and K Concentration in Leaves

Phosphorus concentration was assessed by taking the absorbance at 420 nm on a spectrophotometer [31]. The K concentration in the shoot was measured by running the acid-digested samples on a flame photometer as directed by Nadeem et al. [32]. Calcium (Ca) and sodium (Na) ion concentrations were determined by following Yoshida et al.'s [33] method using flame-photometer Atomic Absorption Spectrum.

2.13. Statistical Analysis

Statistical analysis of data was carried out by two-factorial analysis of variance to calculate the significance of given treatments and drought stress. The treatments means

were compared by LSD test at $p < 0.05$. To compute associations among variables, Pearson's correlation analysis was computed by using OriginPro 2021 software.

3. Results

3.1. Growth Parameters and Chlorophyll Contents

Both main and interactive effects of treatments and irrigation (FC) were significantly different for root fresh weight (RFW) and root length (RL). The main effect of treatments was significant for shoot fresh weight (SFW), root dry weight (RDW), shoot dry weight (SDW), shoot length (SL), chlorophyll a (Chla), chlorophyll b (Chl b), and total chlorophyll (TChl) were significant. However, the main effect of FC was significant for SL, SDW, Chla, Chlb, and TChl contents. The results showed that Zn 2 (26.0 and 9.7%), AM (38.0 and 10.2%), Zn1+AM (34.4 and 16.6%), and Zn2+AM (43.6 and 22.4%) significantly increase RFW and RL over control at 75 and 40 FC. Treatments Zn1+AM and Zn2+AM differed significantly for improvement in RDW, SFW, SL, SDW, Chla, Chlb, and TChl over control. Treatment AM was significantly better than the control for the increase in SFW, Chla, Chlb, and TChl (Table 1).

Table 1. Mean comparisons for different growth parameters and photosynthetic pigments of wheat at different levels of experimental treatments in drought stress after 50 days of growth in pots.

Treatment (T)	RFW (g)			RL (cm)			RDW (g)		
	T × FC		Mean (T)	T × FC		Mean (T)	T × FC		Mean (T)
	75 FC	40 FC		75 FC	40 FC		75 FC	40 FC	
C	2.41 cd	2.05 e	2.18 C	0.51 fg	0.40 g	0.46 B	0.35	0.29	0.28 C
Zn 1	2.63 c	2.19 de	2.42 BC	0.73 ef	0.6 ef	0.67 B	0.43	0.41	0.43 BC
Zn 2	3.03 b	2.25 de	2.65 AB	1.43 bc	0.9 de	1.17 A	0.48	0.43	0.46 A-C
AM	3.33 ab	2.26 cd	2.80 AB	1.63 ab	0.83 ef	1.23 A	0.46	0.44	0.45 A-C
Zn1+AM	3.24 ab	2.39 cd	2.93 A	1.70 ab	0.9 de	1.30 A	0.52	0.54	0.53 AB
Zn2+AM	3.46 a	2.51 cd	2.88 A	1.8 a	1.16 cd	1.48 A	0.61	0.55	0.58 A
Mean (FC)	3.02 A	2.26 B		1.30 A	0.80 B		0.48	0.43	
p-value	Treatment	4.33124×10^{-5}		9.0904×10^{-9}			2.8426×10^{-4}		
	FC	4.63411×10^{-10}		5.75823×10^{-8}			0.2667 ns		
	Interaction	0.03579		0.0054			0.95431 ns		
Treatment (T)	**SDW (g)**			**SFW (g)**			**SL (cm)**		
C	0.51	0.30	0.41 C	0.64	0.46	0.51 C	2.16	1.77	1.97 B
Zn 1	0.59	0.43	0.52 BC	0.92	0.71	0.82 BC	2.36	1.73	2.05 AB
Zn 2	0.62	0.44	0.53 BC	1.32	0.94	1.14 A-C	2.43	2.03	2.23 AB
AM	0.55	0.39	0.47 C	1.36	1.14	1.25 AB	2.26	1.66	1.97 B
Zn1+AM	0.92	0.66	0.79 AB	1.52	1.34	1.43 AB	2.50	1.96	2.23 AB
Zn2+AM	1.06	0.86	0.97 A	1.57	1.51	1.54 A	2.66	2.19	2.43 A
Mean (FC)	0.71 A	0.52 B		1.23	1.00		2.40 A	1.89 B	
p-value	Treatment	9.45218×10^{-6}		3.72445×10^{-4}			0.01609		
	FC	9.90009×10^{-4}		0.0891 ns			1.99655×10^{-6}		
	Interaction	0.99351 ns		0.98412 ns			0.96762 ns		
Treatment (T)	**Chla (mg/g)**			**Chlb (mg/g)**			**TChl (mg/g)**		
C	0.50	0.29	0.39 C	0.33	0.11	0.22 D	0.82	0.40	0.61 D
Zn 1	0.84	0.68	0.76 B	0.69	0.55	0.62 C	1.52	1.23	1.38 C
Zn 2	1.66	1.25	1.46 A	0.83	0.63	0.73 C	2.49	1.88	2.19 B
AM	1.57	1.27	1.42 A	1.07	0.76	0.91 B	2.63	2.03	2.33 B

Table 1. *Cont.*

Treatment (T)	RFW (g)			RL (cm)			RDW (g)		
	T × FC		Mean (T)	T × FC		Mean (T)	T × FC		Mean (T)
	75 FC	40 FC		75 FC	40 FC		75 FC	40 FC	
Zn1+AM	1.59	1.45	1.52 A	1.22	0.99	1.10 A	2.81	2.43	2.62 A
Zn2+AM	1.63	1.53	1.58 A	1.23	1.01	1.12 A	2.87	2.54	2.70 A
Mean (FC)	1.30 A	1.08 B		0.89 A	0.67 B		2.19 A	1.75 B	
p-value	Treatment	6.93916×10^{-17}		6.31474×10^{-16}			3.13157×10^{-20}		
	FC	2.2081×10^{-6}		1.14979×10^{-8}			1.48905×10^{-10}		
	Interaction	0.06971 ns		0.59239 ns			0.0858 ns		

All values are the means of three replicates. Different labels (capital letters for main effects and small letters for interactive effect) showed significant differences using the LSD test. Non-significant interactive and main effects of T and FC did not have any lettering. Non-significant = ns. (75 FC = well-watered, 40 FC = drought stress; C = control, Zn1 = 20 kg ha^{-1}, Zn2 = 40 kg ha^{-1}, AM = arbuscular mycorrhizal fungi) (RFW = root fresh weight, SFW = shoot fresh weight, RDW = root dry weight, SDW = shoot dry weight, RL = root length, SL = shoot length, Chl. a = chlorophyll a, Chl. b = chlorophyll b, TChl = total chlorophyll).

3.2. Antioxidant Contents and Osmolytes

The main effects of the treatment were significant, but interaction FC × T was not significant for carotenoids (Car), ascorbic acid (Asa), anthocyanin shoot (AnthS), soluble sugars root (SSR), soluble sugars shoot (SSS), flavonoids root (FlavR), flavonoids shoot (FlavS), superoxide dismutase (SOD), and peroxide (POD). No treatment brings any significant change in Pro and phenolics (Phen) contents. For Asa, Zn2 + AM caused a significant decrease (26%) compared to control. All the treatments remained non-significant in AnthR from control. However, Zn2 + AM and Zn1 + AM caused a significant decrease compared to control in AnthS. The addition of Zn2, AM, Zn1 + AM, and Zn2 + AM remained significantly better for enhancement in SSS and SSR over the control. A significant decrease in FlavR (56%), FlavS (69%), POD (58%), and SOD (73%) showed the efficacious role of Zn2 + AM over control (Table 2).

Table 2. Mean comparisons for different osmolytes and antioxidant contents (on fresh weight basis) of wheat at different levels of experimental treatments in drought stress after 50 days of growth in pots.

Treatment (T)	Car (mg/g)			Pro (µmol/g)			Phen (mg/g)		
	T × FC		Mean (T)	T × FC		Mean (T)	T × FC		Mean (T)
	75 FC	40 FC		75 FC	40 FC		75 FC	40 FC	
C	0.06	0.05	0.06 B	2.96	2.84	2.90	2.18	4.92	3.55
Zn 1	0.07	0.06	0.07 AB	2.72	3.03	2.87	1.85	2.61	2.23
Zn 2	0.08	0.08	0.08 AB	2.56	2.85	2.71	1.68	2.37	2.02
AM	0.08	0.06	0.07 AB	3.04	3.35	3.19	1.93	2.71	2.32
Zn1+AM	0.10	0.07	0.08 AB	2.17	2.77	2.47	1.67	2.38	2.02
Zn2+AM	0.13	0.11	0.12 A	1.93	2.23	2.08	1.43	2.14	1.78
Mean (FC)	0.09	0.07		2.56	2.85		1.79 B	2.85 A	
p-value	Treatment	0.01486		0.14849 ns			0.12036 ns		
	FC	0.06848 ns		0.1351 ns			4.01679×10^{-4}		
	Interaction	0.97112 ns		0.98515 ns			0.98447 ns		

Table 2. *Cont.*

Treatment (T)	Car (mg/g)			Pro (μmol/g)			Phen (mg/g)		
	T × FC		Mean (T)	T × FC		Mean (T)	T × FC		Mean (T)
	75 FC	40 FC		75 FC	40 FC		75 FC	40 FC	
Treatment (T)	**Asa (mg/g)**			**AnthR (μg/g)**			**AnthS (μg/g)**		
C	23.67	31.00	27.33 A	0.81	1.21	1.01 ns	1.25	1.71	1.48 A
Zn 1	22.33	25.33	23.83 AB	0.64	1.41	1.02 ns	1.04	1.41	1.22 AB
Zn 2	19.67	23.00	21.33 AB	0.44	1.35	0.89 ns	0.69	1.20	0.94 A-C
AM	22.00	26.67	24.33 AB	0.33	1.42	0.88 ns	0.76	1.33	1.05 A-C
Zn1+AM	21.00	23.67	22.33 AB	0.51	1.11	0.81 ns	0.50	0.95	0.73 BC
Zn2+AM	18.30	22.33	20.32 B	0.74	0.95	0.85 ns	0.49	0.73	0.61 C
Mean (FC)	21.16B	25.33 A		0.58 B	1.24 A		0.79 B	1.22 A	
p-value	Treatment	0.02465		0.25072 ns			0.00101		
	FC	7.65328×10^{-4}		8.18068×10^{-9}			4.62156×10^{-4}		
	Interaction	0.97757 ns		0.07905 ns			0.96471 ns		
Treatment (T)	**SSR (mg/g)**			**SSS (mg/g)**			**FlavR (μg/g)**		
C	1.12	1.09	1.11 D	1.38	1.03	1.21 D	0.83	0.81	0.82 A
Zn 1	1.36	1.04	1.20 CD	1.65	1.31	1.48 CD	0.55	0.92	0.74 AB
Zn 2	1.47	1.20	1.33 A-C	2.00	1.48	1.74 A-C	0.49	0.73	0.61 AB
AM	1.39	1.12	1.26 BC	1.76	1.33	1.55 BC	0.65	0.87	0.76 AB
Zn1+AM	1.49	1.38	1.44 AB	2.13	1.79	1.96 AB	0.38	0.60	0.49 B
Zn2+AM	1.59	1.49	1.54 A	2.31	1.81	2.06 A	0.26	0.47	0.36 B
Mean (FC)	1.40 A	1.22 B		1.87 A	1.46 B		0.53 B	0.73 A	
p-value	Treatment	1.77298×10^{-6}		1.97823×10^{-4}			0.00377		
	FC	2.05111×10^{-5}		2.15272×10^{-4}			0.00375		
	Interaction	0.49373 ns		0.97856 ns			0.99365 ns		
Treatment (T)	**FlavS (μg/g)**			**SOD (U g^{-1})**			**POD (U g^{-1})**		
C	0.81	1.71	1.26 A	1.16	2.12	1.64 A	4.62	6.10	5.36 A
Zn 1	0.68	1.15	0.91 AB	0.96	1.77	1.36 A	3.54	4.23	3.88 B
Zn 2	0.56	0.96	0.76 A-C	0.71	1.62	1.17 AB	2.67	3.79	3.23 BC
AM	0.76	1.09	0.92 AB	0.83	1.76	1.30 AB	3.08	4.06	3.57 B
Zn1+AM	0.39	0.62	0.50 BD	0.43	1.28	0.86 AB	2.68	3.05	2.87 BC
Zn2+AM	0.23	0.55	0.39 C	0.15	0.73	0.44 B	1.97	2.47	2.22 C
Mean (FC)	0.57 B	1.01 A		0.71 B	1.55 A		3.09 B	3.95 A	
p-value	Treatment	0.00202		0.00725			6.69258×10^{-8}		
	FC	4.97548×10^{-4}		3.74663×10^{-5}			1.81577×10^{-4}		
	Interaction	0.92577 ns		0.99049 ns			0.5551		

All values are the means of three replicates. Different labels (capital letters for main effects and small letters for interactive effect) showed significant differences using LSD test. Non-significant interactive and main effects of T and FC did not have any lettering. Non-significant = ns. (75 FC = well-watered, 40 FC = drought stress; C = control, Zn1 = 20 kg ha^{-1}, Zn2 = 40 kg ha^{-1}, AM = arbuscular mycorrhizal fungi) (Caro = carotenoids, Pro = proline, Phen = phenolics, Asa = ascorbic acid, AnthoR = antho-

cyanin root, AnthoS = anthocyanin shoot, SSR = soluble sugars root, SSS = soluble sugars shoot, FlavR = flavonoids root, FlavS = flavonoids shoot, SOD = superoxide dismutase, POD = peroxide).

3.3. Oxidative Stress Indicators and Ionic Constituents

Treatments were non-significant over control for catalase (CAT) and glycine betaine (GB). The results showed that Zn2 + AM and Zn1 + AM were significantly different from control in increasing total soluble protein (TSP) and MDA. The addition of Zn2 + AM remained significantly different compared to the control for H_2O_2. However, Zn2 + AM (33%), Zn1 + AM (24%), and Zn2 (13%) showed a significant decrease in electrolyte leakage (EL) than the control. Treatments Zn2, AM, and Zn1 + AM caused a significant decrease in sodium (Na) compared to control. It was noted that Zn1 + AM, AM, and Zn2 + AM performed significantly best than the control for improvement in Ca. However, for an increase in K and P Zn2 + AM differed significantly better compared to control (Table 3).

Table 3. Mean comparisons for CAT, TSP, MDA, H2O2, EL, GB and nutrient concentration of wheat at different levels of experimental treatments in drought stress after 50 days of growth in pots.

Treatment (T)	CAT (U g^{-1} FW)			TSP (mg/g FW)			MDA (µmol/g)		
	T × FC		Mean (T)	T × FC		Mean (T)	T × FC		Mean (T)
	75 FC	40 FC		75 FC	40 FC		75 FC	40 FC	
C	1.10	1.20	1.15 ns	2.80	2.10	2.45 C	0.85	1.85	1.35 A
Zn 1	0.82	1.15	0.98 ns	3.33	2.69	3.01 BC	0.79	1.22	1.01 AB
Zn 2	0.74	1.07	0.90 ns	3.59	3.26	3.43 A-C	0.61	1.11	0.86 AB
AM	0.85	1.18	1.01 ns	3.28	2.53	2.91 BC	0.70	1.13	0.91 AB
Zn1+AM	0.52	0.85	0.68 ns	3.86	3.36	3.61 AB	0.55	1.09	0.82 B
Zn2+AM	0.45	0.79	0.62 ns	4.23	3.99	4.11 A	0.36	0.78	0.57 B
Mean (FC)	0.75 B	1.04 A		3.52 A	2.99 B		0.64 B	1.20 A	
p-value	Treatment	0.15084 ns		5.4797×10^{-4}			0.00301		
	FC	0.0268		0.01031			4.46462×10^{-6}		
	Interaction	0.99915 ns		0.96292 ns			0.45346 ns		
Treatment (T)	**H_2O_2 (µmol/g)**			**EL (%)**			**Na (µg/g)**		
C	2.47	3.00	2.74 A	22.67	30.00	26.33 A	14.83	18.00	16.42 A
Zn 1	2.32	3.09	2.70 AB	21.67	29.67	25.67 AB	13.33	20.83	17.08 A
Zn 2	2.19	2.68 ns	2.44 AB	19.00	27.00	23.00 BC	12.67	19.00	15.83 AB
AM	2.23	2.96	2.59 AB	20.00	28.33	24.17 A-C	13.33	19.67	16.50 A
Zn1+AM	2.14	2.53	2.34 AB	17.33	22.67	20.00 CD	11.00	14.00	12.50 BC
Zn2+AM	2.01	2.27	2.14 B	15.67	19.67	17.67 D	10.00	13.83	11.92 C
Mean (FC)	2.23B	2.75 A		19.39 B	26.22 A		12.53 B	17.56 A	
p-value	Treatment	0.04916		2.09285×10^{-6}			4.91172×10^{-5}		
	FC	2.31731×10^{-4}		3.42732×10^{-9}			2.12199×10^{-8}		
	Interaction	0.7903 ns		0.35393 ns			0.35391 ns		
Treatment (T)	**Ca (%)**			**K (%)**			**P (%)**		
C	1.57	1.20	1.38 C	2.83	2.00	2.42 C	0.12	0.04	0.08 B
Zn 1	2.20	1.87	2.03 BC	3.60	2.50	3.05 BC	0.13	0.12	0.12 AB
Zn 2	3.00	2.10	2.55 BC	4.67	3.67	4.17 AB	0.14	0.13	0.13 AB
AM	2.33	1.80	2.07 AB	3.50	2.57	3.03 BC	0.12	0.11	0.12 AB

Table 3. *Cont.*

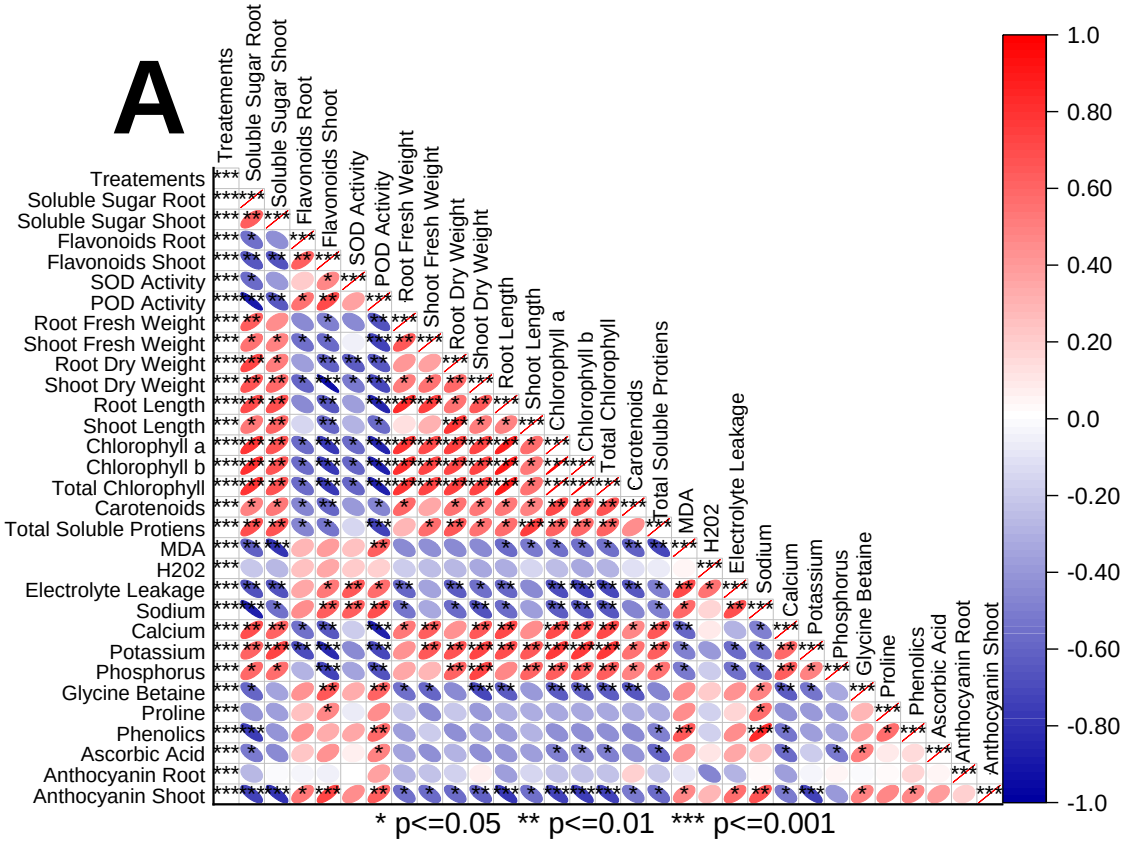
Treatment (T)	CAT (U g^{-1} FW)			TSP (mg/g FW)			MDA (µmol/g)		
	T × FC		Mean (T)	T × FC		Mean (T)	T × FC		Mean (T)
	75 FC	40 FC		75 FC	40 FC		75 FC	40 FC	
Zn1+AM	2.67	2.20	2.43 AB	5.00	3.83	4.42 A	0.14	0.14	0.14 AB
Zn2+AM	3.50	2.96	3.23 A	5.67	4.33	5.00 A	0.17	0.15	0.16 A
Mean (FC)	2.54 A	2.02 B		4.21 A	3.15 B		0.14 ns	0.11 ns	
p-value	Treatment	4.61249×10^{-5}		3.11022×10^{-6}			0.01961		
	FC	0.00407		7.41822×10^{-5}			0.14451 ns		
	Interaction	0.92374 ns		0.9964 ns			0.9518 ns		

Treatment (T)	GB (µmol/g)		
C	1.20	1.69	1.45
Zn 1	1.12	1.78	1.45
Zn 2	0.93	1.50	1.21
AM	0.99	1.83	1.41
Zn1+AM	0.69	1.22	0.95
Zn2+AM	0.33	1.10	0.71
Mean (FC)	0.88 B	1.52 A	
p-value	Treatment	0.0597 ns	
	FC	5.35532×10^{-4}	
	Interaction	0.99143 ns	

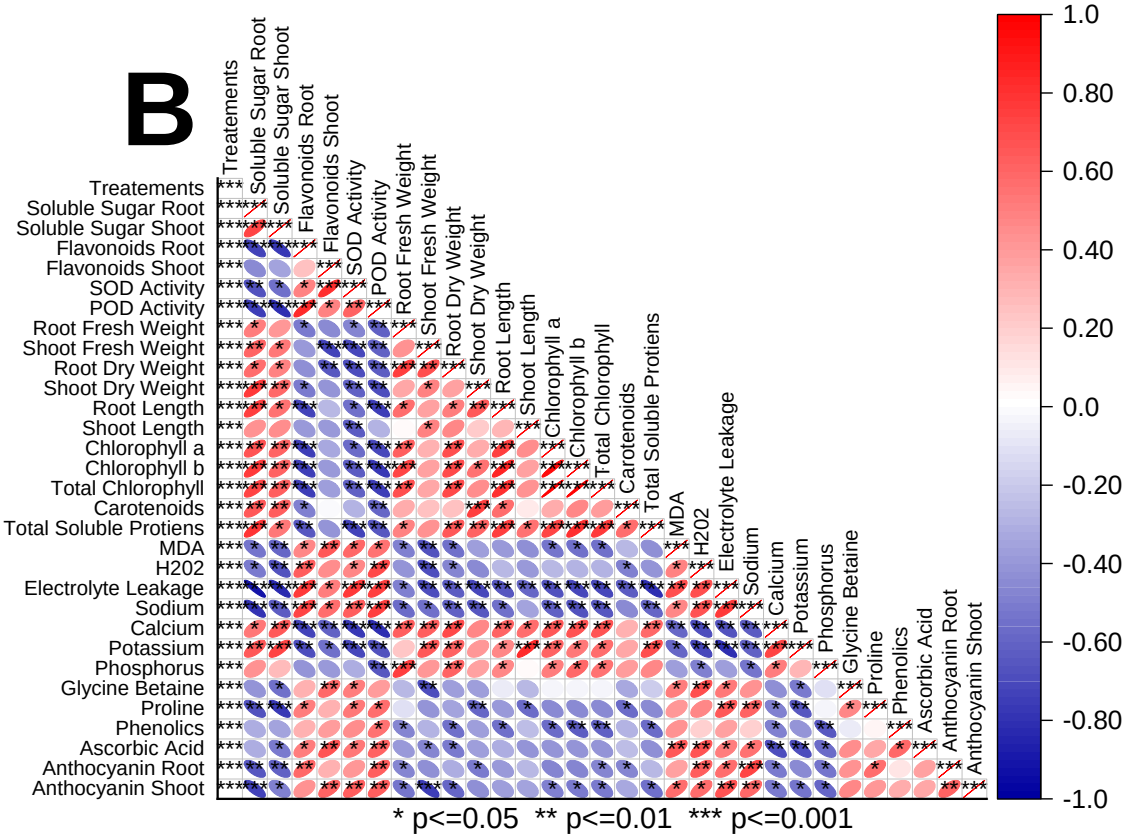
All values are the means of three replicates. Different labels (capital letters for main effects and small letters for interactive effect) showed significant differences using LSD test. Non-significant interactive and main effects of T and FC did not have any lettering. Non-significant = ns. (75 FC = well-watered, 40 FC = drought stress; C = control, Zn1 = 20 kg ha^{-1}, Zn2 = 40 kg ha^{-1}, AM = arbuscular mycorrhizal fungi) (CAT = catalase, TSP = total soluble proteins, MDA, malondialdehyde, H_2O_2 = hydrogen peroxide, EL = electrolyte leakage, GB = glycine betaine, Ca = calcium, P = phosphorus, K = potassium).

3.4. Pearson Correlation

The Pearson correlation showed that the effect of drought stress on shoot and root was significantly negative in correlation with the shoot and root fresh weight, dry weight, length, chlorophyll a, chlorophyll b, total chlorophyll, carotenoids, total soluble proteins, and total soluble sugar. Attributes (i.e., SOD, POD, catalase, flavonoids, phenolics, anthocyanin, ascorbic acid, electrolyte leakage, MDA, H_2O_2, and proline) were significantly positive in correlation with the drought effect (Figure 1a,b).



(**a**) Pearson correlation for studied attributes under normal irrigation (75% FC)



(**b**) Pearson correlation for studied attributes under drought (40% FC).

Figure 1. Pearson correlation of wheat attributes under normal (**a**) and drought stress (**b**) condition.

4. Discussion

The main objective of the present study was to analyze the role of Zinc (Zn) fertilizer and arbuscular mycorrhizal fungi (AM) for improving wheat growth during drought stress. Water deficiency badly affects wheat yield worldwide [34]. Bio-fertilizers are a consistent and cost-effective approach for sustainable agriculture and for minimizing environmental pollution [35]. Water stress interrupts cellular water relations and damages plant physiological and growth mechanisms. Plants adopt many defensive physiobiochemical mechanisms at both the cellular and organism levels, making tolerance to water stress an intricated and complex phenomenon [36]. Additionally, the supplementation of several other nutrient sources such as fertilizers reduces environmental stress and improves plant vigor during stress [35,37]. Therefore, in this study, wheat's potential adaptation and tolerance to osmotic stress through Zn fertilization, arbuscular mycorrhizal fungi, and their co-application were explored by considering variable physiological, biochemical, and ionic factors traits [38].

According to a scientific report published by Gargallo-Garriga et al. [39] osmotic stress hampered metabolic activities in plants. Lower metabolism rates help plants conserve more water and save energy for the transfer of assimilates within the plant body. The current study validates these facts. All studied growth attributes such as plant fresh weight, dry weight, and length were decreased during drought stress. Similar results were found in other studies. Extensive cell division and increased dry matter contents of root and shoot estimates enhanced plant growth. Water stress alters the osmotic potential of soil, leading to inadequate growth and cell division. Osmotic stress due to the variations in osmotic potential decreased the fresh weight of plants, while less cellular division resulted in a significant decrease in dry weight [40,41]. The present study followed these facts. The Zn2 + AM treatment effectively improved the fresh weight, dry weight, and plant length by sustaining more water contents in cells, thus ameliorating drought stress.

Mycorrhizal inoculated seedlings had enhanced water uptake efficiency compared to non-inoculated control ones in drought stress conditions. It is likely that arbuscular mycorrhizae (AM) hyphae expanded their surface area and moisture absorption regions of host plant roots and thus increased water absorption by roots [42]. In the current study, AM-inoculated plants performed better than control plants, and the effects were more profound with arbuscular mycorrhizae in combination with Zn fertilizer, i.e., in Zn2 + AM.

Zn improved chlorophyll synthesis, acting as a catalytic and structural protein component and co-factor of various enzymes. Zn has a protective and stabilizing effect on cell membranes, which causes improvement in the photosynthetic process [43]. Foliar application of zinc enhanced chlorophyll and photosynthetic enzyme (Carbonic anhydrase) activities in rice plants and thus, improved the photosynthetic process. Carbonic anhydrase is a Zn-containing enzyme that is part of plant photosynthetic machinery [44]. Similar findings were observed in the present study on wheat as supplementation with Zn increased all studied photosynthetic pigments.

Therefore, increasing the Zn concentration could enhance the plant potential of scavenging ROS molecules by positively inducing SOD gene expression, thus elevated SOD activities [43]. Therefore, Zn2 has more profound effects than Zn1 in the current study. Similarly, in another study, the Zn-treated tomato plants (*Solanum lycopersicum*), the superoxide dismutase (SOD), catalase CAT, and peroxidases activities were found to increase [45]. The current study validates these previous findings. Zinc fertilizer and increased dose rate increased plant vigor and physiological state during drought stress, with Zn2 + AM as the most effective treatment.

Drought stress (40) FC increased all antioxidant constituents (enzymatic and non-enzymatic) in wheat compared to control and well-watered plants. Zn1 + AM and Zn2 + AM were the most effective amendments in mitigating the lethal effects of drought stress. A study conducted on wheat under drought stress conditions concluded that mycorrhizal inoculation increased antioxidant enzyme activities such as catalase (CAT) and peroxidase (POD) in comparison with non-inoculated control plants [34].

Various metabolites and osmolytes, i.e., soluble sugars, soluble proteins, and proline, played vital roles in plant osmotic adjustments. Proline accumulation in stress is being documented in the literature [46]. We also observed similar findings. Zinc fertilizer and arbuscular mycorrhizal fungi efficiently increased wheat's proteins and soluble sugar contents in individual and combined addition under drought stress.

It is an unquestionable aspect that ROS regulate several physiological and developmental processes and are ubiquitously involved in cell signaling but only in small quantities. They accumulate in high concentrations during abiotic stress, and this interrupts normal plant metabolic functioning. Antioxidant compounds are well-known ROS scavengers [47]. The application of Zn increased the accumulation of total flavonoids and phenols in the berry plant [48]. This increase in antioxidant contents was mainly due to Zn's ability to improve the biosynthesis of antioxidants. Zn possibly modulates glutathione levels in plants by regulating its biosynthesis and protecting glutathione reactive cysteine residues through the efficient functioning of relevant enzyme groups [49]. In agreement with these results, wheat plants' non-enzymatic antioxidants such as phenolics, flavonoids, anthocyanin, and ascorbic acid contents were improved in drought stress. The application of Zn and AM co-application had significant positive impacts in decreasing their concentrations to resist ROS damage induced by drought stress.

Antioxidant enzymes decrease hydrogen peroxide (H_2O_2) levels and lipid peroxidation extent that causes membrane damage, thus maintaining normal cellular functions despite the higher ROS accumulation in plant cells under abiotic stress conditions. Our results are in line with this evidence. In the present research, MDA contents were higher under osmotic stress and H_2O_2 produced in low amounts in Zn- and AM-treated plants in drought stress and well-watered groups [34].

Mohamed et al. [50] reported increasing micro- and macronutrients with inoculation of arbuscular mycorrhizal fungi (AM) in onion (*A. cepa*). The uptake of Ca, P, K, and various other macronutrients was increased with Zn fertilizer and AM inoculation compared to non-treated plants. In another study, AM fungi improved the uptake of almost all essential plant nutrients except sodium (Na) and calcium (Cl) ions leading to growth stimulation [51]. The present study also follows these findings. Na ion concentration decreased when treated with Zn fertilizer and arbuscular mycorrhizal fungi [5].

5. Conclusions

In conclusion, applying Zn fertilizer and arbuscular mycorrhizal fungi positively affects wheat growth, antioxidants, and ionic attributes. Root fresh weight and root length were significantly enhanced with the supplementation of Zn2 + AM under 40% FC. Zn2 + AM had a more efficient main impacts for wheat over all other applied treatments. More investigation is needed at field levels and in other soil and climatic conditions to confirm the results of Zn2 + AM so that it can be declared as an effective amendment for improvement in root growth of wheat under osmotic stress.

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