Micronutrient and Redox Homeostasis Contribute to *Moringa Oleifera*-regulated Drought Tolerance in Wheat

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Abstract

Global food security is being severely affected by rapid increase in population and drastic climate change. With the changing climate, drought stress has become the most important limiting factor for sustainable production of several important agricultural crops, including wheat. Gradual rise in temperature and reduced precipitations are likely to cause frequent onset of droughts around the world. Therefore, alleviation of drought stress in crop plants has become an essential requirement to meet the increasing food demand. The present study explored the role of foliar application of *Moringa* leaf extract (MLE) in conferring drought tolerance in wheat during the anthesis stage. The results showed that MLE treatment improved the concentration of the macro- (K, Ca) and micronutrients (Cu, Fe, Na, Mn, Zn, Si) in flag-leaves of wheat under non-stressed conditions and also maintained their concentrations under drought stress. The micro-nutrients (Cu, Fe, Mn, Zn) being the co-factors of the enzymes also stimulated the antioxidant enzyme activities, eventually leading to significant reduction in the reactive oxygen species (ROS) and malondialdehyde (MDA) accumulations under drought stress. Furthermore, micronutrients played a crucial role in osmoregulation and sustainable plant growth under drought stress. Overall, the study provided insights into the functional role of micronutrients in improving drought tolerance and also indicated the potential to commercialize MLE as an effective bio-stimulant for sustainable agriculture in drought prone regions.

1. Introduction

Global food security is being severely affected by the rapid increase in population and drastic changes in the climate. With the changing climate, drought stress has become the most important limiting factor to sustainable agricultural productivity in greatest part of the world (Daryanto et al. 2017). Drought stress regularly affects major wheat cultivating regions throughout the world (Zhang et al. 2018). Most of the wheat genotypes cultivated in the Indo-Gangetic Plains (IGP) are developed for the irrigated cropping systems are therefore, extremely susceptible to drought (Zhang et al. 2017). High temperature-induced severe increase in soil moisture evaporation is the primary reason for drought stress in this region. Continuous increase in average atmospheric temperature and asymmetric precipitation is predicted to intensify the incidence of drought around the world causing about 9–12% increase in yield loss of wheat by the end of 21st century (IPCC 2014; Leng and Hall 2019). Furthermore, rapid depletion of groundwater table in larger part of the IGP may cause frequent onset of droughts (Nath et al. 2017). Therefore, amelioration of drought stress has become an essential requirement to encounter the global food security.

Drought can occur at any stages of the plant life cycle but anthesis is considered as the most vulnerable period related to the production of wheat (Barnabas et al. 2007). Therefore, it is the serious requirement to increase the drought tolerance during the reproductive development in wheat (Senapati et al. 2019). Drought has significant negative influences on morpho-physiological (Stallmann et al. 2018) and biochemical responses (Hameed et al. 2011) of plants ultimately impeding the crop productivity. Water stress also disrupts plant-water relationships in wheat affecting plant growth (Yadav et al. 2019).

Chlorophyll biosynthesis process is also inhibited under drought stress that accelerates flag-leaf senescence in plants (Kumar et al. 2020). Drought stress in plants is associated with the excess production of reactive oxygen species (ROS) that causes severe oxidative damages (Hameed et al. 2011). Accumulation of superoxide anion ($^{\bullet}O_2^-$), singlet oxygen (O_2^*), hydrogen peroxide (H_2O_2), hydroxide ion (OH^-) induced by water stress triggers membrane lipid peroxidation and cell death (Basu et al. 2021a).

Antioxidant defence system comprising non-enzymatic and enzymatic antioxidants contributes drought tolerance in plants through detoxification of ROS (Basu et al. 2021b). Non-enzymatic antioxidants, including ascorbate, glutathione and primary antioxidant enzymes, such as, superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (POX; EC1.11.1.7) and ascorbate peroxidase (APX; EC1.11.1.11) are involved in ROS scavenging (Dwivedi et al. 2019; Basu et al. 2020). Growth enhancers like proline, soluble sugars also minimize the effect of stress through osmotic adjustment (Loutfy et al. 2012).

Mineral nutrients comprising the macro and micronutrients also play a major role in drought tolerance in plants (Egilla et al. 2001). The role of microelements in the regulation of the plant growth has been widely studied. However, the role of the mineral nutrients under the abiotic stresses is so far less explored. Reduced soil moisture under drought stress impairs the nutrient uptake and aerial translocation in plants (Silva et al. 2011). Drought-induced stomatal closure also restricts the root-to-shoot transport of the mineral nutrients by reducing the transpiration rate. Major inorganic solutes, including potassium (K), calcium (Ca) play significant role in osmoregulation and plant growth under different abiotic stresses (Wu et al. 2019; Basu et al. 2021c). The major elements may directly act as osmotic solutes or indirectly as regulators in biosynthesis of organic solutes intended for water conservation in plants. On the other hand, micronutrients such as iron (Fe), copper (Cu), zinc (Zn), manganese (Mn) being the major co-factors of the antioxidant enzymes protect the plants from oxidative damage by effective ROS scavenging (Ahanger et al. 2016). Silicon (Si) also prevents the oxidative damage in plants by activating the antioxidant defence system (Basu and Kumar 2021).

Moringa oleifera leaf is a natural bio-stimulant that enhances the growth, physiological performance and yield attributes of different crop plants (Kumar et al. 2021). Previous studies have also established foliar sprays to be the effective way for the application of bio-stimulants as the chemicals provided are readily available to plants (Basu and Kumar 2021). Several studies on bio-stimulants have focused on the application of different biomolecules, organic fertilizers or seaweed extracts in ameliorating abiotic stress in plants (Wang et al. 2019; Sharma et al. 2019). However, application of *Moringa* leaf extract (MLE) in conferring drought tolerance by enhancing the micronutrient levels during the anthesis stage of wheat has not been extensively studied. Therefore, there is a significant gap in the knowledge about the mechanism of action for MLE required to be explored. Comprehensive understanding of the role of MLE in improving the macro- and micro-nutrient contents and antioxidant defence mechanism may contribute in enhancing drought tolerance in wheat. The present study explored the role of MLE in conferring drought tolerance in wheat during the anthesis stage. The study also evaluated the association of non-

enzymatic and enzymatic antioxidant-mediated enhanced ROS detoxification in the flag leaves with the improved drought tolerance in wheat.

2. Material And Methods

2.1. Plant material and experimental design

Present study was accomplished with wheat genotype of IGP (*Triticum aestivum* L. cv. HI1544). Seeds of the IGP wheat genotype were surface sterilized with 0.5% sodium hypochlorite (10 min), followed by thorough washing with de-ionized water. Then the seeds were soaked for overnight and equal numbers of seeds were germinated for 3 d under dark conditions in pots (diameter 25 cm, height 19 cm) filled with equal amount of soil. After germination of the seeds the pots were transferred to greenhouse conditions (22 ± 2°C; 16 h light/8 h dark cycle; relative humidity 75–80%). Crop management was optimized with normal irrigation.

2.2. Preparation of *Moringa* leaf extract (MLE)

Fresh and matured leaves of *Moringa oleifera* were dried and ground into fine powder. The leaf powder was homogenized with ethanol in a ratio of 10:1 (v/w). The extract was filtered through Whatman No. 1 filter paper and the filtrate was subjected to evaporator for evaporation of ethanol (Kumar et al. 2021). Prior to use as a foliar spray, *Moringa* leaf extract (MLE) was diluted in a ratio of 1:30 (v/v) and mixed with 0.1% (v/v) surfactant for optimal penetration.

2.3. Stress imposition and application of MLE

During the anthesis of the spikes (anthesis halfway), the pots were divided into four groups; plants of the first group grown under normal conditions (Control), plants of the second group was foliar sprayed with MLE (MLE), plants of the third group was exposed to the drought stress by withholding water for the next 10 d (Drought), plants of the fourth group was also exposed to drought stress (similar to the third group) but simultaneously foliar sprayed with MLE for 10 d (Drought + MLE) (Fig. S1A).

2.4. Sampling time/stage

Crop phenology was monitored according to Zadoks decimal code (Zadoks et al. 1974). All the studies were performed at anthesis of the spikes (Z65, i.e., anthesis halfway).

2.5. Growth parameters

Height of the wheat plants exposed to different experimental conditions was measured at their respective anthesis stages with measuring tape. In this experiment, the untreated plants grown under normal conditions were considered as control (C). In contrast, the plants exposed to drought stress were considered as stressed (S). Relative growth rate for each genotype was calculated according to Kumar et al. (2009) using the following formula.

$$\text{Relative growth rate} = (C - S) \times 100 / C$$

2.6. Estimation of relative water content and total chlorophyll content

Relative water content (RWC) was determined according to Weatherley (1950). Fully expanded topmost flag leaf was collected and weighed to record the fresh weight. The samples were hydrated to full turgidity by floating on de-ionized water and immediately weighed to obtain fully turgid weight. The dry weight of the samples was taken after proper drying in a hot air oven at 80 ºC for 24 h. RWC was calculated for each sample following the formula.

$$\text{RWC}\,(\%) = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \times 100$$

Total chlorophyll was extracted from the flag-leaf samples with 80% chilled acetone. Absorbance was recorded at 645 and 663 nm on a UV-VIS spectrophotometer (Motras Scientific, India). Total chlorophyll content was calculated following the Arnon's Eq. (1949); where, V = volume of solvent and W = weight of leaf (g).

$$\text{Total chlorophyll} = \frac{(20.2 \times \text{O.D645} + 8.02 \times \text{O.D663}) \times \text{V}}{1000} \times \text{W}$$

2.7. Determination of electrolyte leakage and membrane stability index

Freshly chopped flag-leaves were dipped in de-ionized water and incubated at 32 ^{o}C. The electrical conductivity of the solution (E1) was measured with the conductivity meter (Hanna Instruments, India). Total conductivity (E2) was determined after heating the solution at 121 ^{o}C. Relative electrical conductivity or electrolyte leakage (EL) was calculated by the formula described by Kumar et al. (2009).

$$\text{EL}\,(\%) = \frac{\text{E1}}{\text{E2}} \times 100$$

Membrane stability index (MSI) was calculated following the equation proposed by Basu et al. (2021a).

$$\text{MSI}\,(\%) = 1 - \frac{\text{C1}}{\text{C2}} \times 100$$

2.8. Quantification of proline and total soluble sugar content

Proline was estimated according to Bates et al (1973). Flag leaves were homogenized in 3% salicylic acid and filtered. The filtrate was mixed with 0.2% ninhydrin, glacial acetic acid and incubated at 100^{o}C for 1

h. Then the samples were cooled on ice. Proline was extracted from the mixture with toluene and absorbance was recorded at 520 nm. Proline content was calculated from the standard.

Total soluble sugar (TSS) from flag leaves were extracted with 80% hot ethanol (Yemm and Willis, 1954). Total sugar hydrolysed to glucose and was measured at 620 nm with anthrone reagent and was quantified from the glucose standard.

2.9. Estimation of macro and micro-nutrients

Endogenous K, Na and Ca contents were determined according to Kumar et al. (2009). Flag-leaf tissue was digested with 0.1% HNO_3. Ions were extracted in distilled water by boiling it twice for 30 min each. The filtrate was used to measure specifications with a flame photometer (Systronics, India). Ionic concentrations were determined from their respective standard curves.

The micronutrient contents were determined according to Kumar et al. (2012). Plant samples were dried at 105°C in an oven, crushed to fine powder using a mortar-pestle grinder. Dry plant powder thus obtained was pressed by using 15-ton pressure and tablets of 100.0 mg were made. EDXRF measurements were performed on the EDXRF spectrometer with a Ge solid state detector. The source of X-ray was 100 keV Gadolinium tube which allows fluorescence efficiency for K-lines higher than for L-lines of elements. All samples were measured for a period of 2000 seconds on the sample holders made of Titanium rings. Relative quantitative analysis of element was performed using Epsilon software.

2.10. Assay of reactive oxygen species

Hydrogen peroxide (H_2O_2) was determined according to Sergiev et al. (1997) with some modifications. H_2O_2 was extracted from 0.5 g flag-leaf tissue with 0.1% (w/v) TCA. After centrifuged at 12,000 g for 15 min, the supernatant was incubated with 0.1 mM potassium phosphate buffer (pH 7.0) and 1 M potassium iodide solution and absorbance was measured at 390 nm. H_2O_2 content was obtained from a standard curve.

Superoxide anion ($^{\bullet}O_2^-$) was estimated as described by Chaitanya and Naithani (1994) with minor modifications. Fag-leaf samples were homogenized in ice cold 0.2 M phosphate buffer (pH 7.2) containing 10^{-3} M diethyldithiocarbamate to inhibit SOD activity. The homogenate was centrifuged at 10,000 g for 10 min. The $^{\bullet}O_2^-$ was measured in the supernatant by its capacity to reduce NBT (2.5×10^{-4} M).Change in absorbance due to formation of $^{\bullet}O_2^-$ was measured at 540 nm for 1 min.

2.11. Measurement of lipid peroxidation

Lipid peroxidation was measured in terms of malondealdehyde (MDA) content. The MDA content was determined by thiobarbituric acid (TBA) test according to Heath and Packer (1968). After extracting MDA from flag-leaf tissue with 0.5% TBA in 20% trichloroacetic acid (TCA) absorbance was measured at 532

nm. Values of non-specific absorption recorded at 600nm were subtracted from the values recorded at 532 nm. MDA content was calculated according to its extinction coefficient ε = 155mM^{-1} cm^{-1}.

2.12. Estimation of total ascorbate and glutathione content

Total ascorbate (AsA + DHA) was quantified according to Law et al. (1983). Ascorbate was extracted from flag-leaf tissue with 6 % TCA and estimated with 2% dinitrophenyl hydrazine (DNPH) and 10% Thiourea. Absorbance recorded at 530 nm and concentration of ascorbate was calculated from the standard curve prepared with pure ascorbate.

Total glutathione (GSH + GSSG) was estimated according to Griffith (1980). Glutathione was extracted from flag-leaf tissue with 5% sulphosalicylic acid and estimated with 5,5'-dithiobis-(2-nitrobenzoic acid) and 3 units of glutathione reductase. Change in absorbance was measured at 412 nm. Total glutathione content was calculated from a standard curve with GSH.

2.13. Determination of antioxidant enzyme activities

Lyophilized plant tissue was homogenized with ice-cold phosphate buffer (pH 7.0) (Basu et al. 2017). Total protein was quantified in the plant tissue according to Bradford (1976). The absorbance of Coomassie Brilliant Blue after binding with the protein was recorded at 595 nm and was calculated from the BSA standard curve. The extracted protein was used to detect different antioxidant enzyme activities.

Superoxide dismutase (SOD) (EC1.15.1.1) activity was determined at 560 nm by its ability to reduce the formation of blue coloured formazone by NBT and $^{\bullet}O_2^-$ radical (Dhindhsa et al. 1981).

Catalase (CAT) (EC 1.11.1.6) activity estimation was performed according to Aebi (1983). Change in absorbance with addition of H_2O_2 was recorded at 240 nm for 1 min. Enzyme activity was expressed as unit min^{-1} mg^{-1} protein and a change in absorbance by 0.01 corresponded to 1 unit of enzyme activity.

Peroxidase (POX) (EC 1.11.1.7) activity was determined at 436 nm by its ability to convert guaiacol to tetraguaiacol (Polle et al. 1994). The increase in absorbance was recorded by the addition of H_2O_2 at 436 nm for 1 min.

Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was estimated following the method of Nakano and Asada (1981). Change in absorbance with was recorded at 290 nm for 1 min after addition of ascorbate and H_2O_2.

2.14. Native-PAGE and activity staining for antioxidant enzymes

Plant extracts containing equal amounts of protein were subjected to discontinuous polyacrylamide gel electrophoresis (PAGE) under non-denaturing and non-reducing conditions (Laemmli 1970).

SOD activity was detected following the method of Weisiger and Fridovich (1973) with some minor modifications. The gels were incubated in the dark in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2.45 mM NBT, 28 mM TEMED and 3 µM riboflavin. The gels were illuminated until colourless SOD bands appeared against a purple background.

CAT activity was determined by incubating the gels in 3.5 mM H_2O_2 followed by washing in de-ionized water. The gels were stained with 1% potassium ferricyanide and 1% ferric chloride (Scandalios 1968).

POX activity was detected using the method of Graham et al. (1964). Gels were incubated in darkness, till the POX activity-containing band visualized carefully.

Enzyme activities were estimated by measuring the relative intensities of bands with the Adobe Photoshop version 7.

2.15. Western blotting

Plant extracts containing equal amounts of proteins were electrophoresed on the SDS-Polyacrylamide gel (Laemmli 1970). A pre-stained marker was run along with to verify the transfer. The proteins were electro-blotted to the nitrocellulose membrane using a semi-dry trans-blot cell (Bio-Rad, India). The blotted membrane was blocked with 5% skimmed milk and was subsequently incubated with the anti-catalase primary antibody (Agrisera, Sweden) of 1:1500 dilutions. This was followed by 1 h incubation with 1:1000 diluted goat anti-rabbit IgG-Horse radish peroxidase conjugated secondary antibody (Agrisera, Sweden). Bands were observed by using DAB substrate. Similarly, primary antibody of anti-Cu/Zn superoxide dismutase (Agrisera, Sweden) of 1:1500 dilution was used. Rabbit anti-chicken IgY-conjugated with Horse radish peroxidase (Agrisera, Sweden) of 1:1000 dilution was used as secondary antibody for visualizing anti-Cu/Zn SOD.

2.16. Statistical analyses

All treatments were given three replications (n = 3) with proper randomization to eliminate bias, whatsoever. Each value is presented in the form of mean ± standard error (± SE) and the lowest standard deviations of mean with a reading considering at least three samples per experiment for each genotype and each condition. The data were statistically analysed for analysis of variance (ANOVA) as a 2×2 factorial completely randomized block design using SAS 9.3 software by SAS Institute Inc., USA. Influence of main and interaction effects of the treatments were carefully evaluated. The standard error of mean and differences between the treatments was compared pair wise by critical difference (CD) at 5% level of significance.

3. Results

3.1. Micronutrients sustained plant growth, physiological activities and improved osmotic adjustment under drought stress

Application of MLE as the foliar spray sustained the plant growth of the wheat genotype HI-1544 during the anthesis stage (Fig. S1B and 1A). In the present study, MLE treated plants exhibited considerably enhanced growth than that of the control (18.9% increase). Drought markedly reduced the plant growth than the control (18.4% decrease), whereas, Drought + MLE rejuvenated the plant growth and showed increased growth as compared to that of the control (3.4% increase). Similarly, the flag-leaf RWC under MLE treatment was higher than the control (85.6 and 80.9%, respectively) (Fig. 1B). Conversely, the plants had reduced RWC under drought condition (39.4%). However, application of MLE under drought stress (Drought + MLE) sustained the RWC almost comparable to that of the control condition (56.9%). Water stress also resulted in increased EL in the flag leaves (Fig. 1C). The results showed MLE plants had the minimum EL than the control (6.1 and 9.5%, respectively). Imposition of drought stress caused increase in the EL (80.3%); whereas, the Drought + MLE plants maintained considerably higher EL (32.3%). Likewise, the Drought + MLE plants maintained higher MSI (67.8%), while, drought stressed plants had the lowest MSI (19.7%; Fig. S2). Flag-leaf chlorophyll content was also significantly reduced under drought stress (Fig. 1D). The MLE plants exhibited higher chlorophyll content (1.16 mg g^{-1} FW) as compared to that of the control plants (0.97 mg g^{-1} FW). Water stress caused significant decrease in chlorophyll content (43.6% decrease), whereas, Drought + MLE exhibited the lowest decrease in flag leaf chlorophyll content (9.9% decrease).

Drought stress also caused accumulation of osmolytes in the wheat genotype HI-1544. Control and MLE plants had almost equivalent proline content (Fig. 1E; 0.27 ± 0.3 mg g^{-1} DW). Incidence of drought stress during anthesis caused significant increase in proline content (1.7-fold increase); whereas, the highest increase in proline content was observed in Drought + MLE plants (3.1-fold increase). Similar trend was observed in TSS content (Fig. 1F). Control and MLE plants had the lowest TSS content, ranging between 0.3–0.4 mg g^{-1} DW. Drought stress caused marked increase in TSS content (2.5-fold increase); whereas, the maximum increase in TSS content was observed in Drought + MLE plants (4.0-fold increase).

3.2. MLE improved macro- and micro-nutrient contents under drought stress

The MLE treatment increased the macro- and micro-nutrient concentrations in both drought-stressed as well as non-stressed plants (Fig. 2). Application of MLE improved the K content in the non-stressed plants of the wheat genotype (9.8% increase than control; Fig. 2A). Drought stress caused significant decrease in K content (30.7% decrease than control). However, MLE application maintained the K concentration in the Drought + MLE plants almost equivalent to that of the control conditions (18.4% decrease than control).

Likewise, MLE treatment also increased the Ca content in the non-stressed plants (10.9% increase than control; Fig. 2B). Incidence of drought stress during anthesis caused severe decrease in Ca content (43.0% decrease than control), which was revived in the Drought + MLE plants (30.2% decrease than control). The micronutrients showed the similar trend as that of the macronutrient contents. Drought stress led to significant decrease (51.8% decrease than control; Fig. 2C) in boron content; whereas, MLE treatment uphold boron concentration under drought stress (34.2% decrease than control). Similarly, the Drought + MLE plants exhibited higher Cu content (37.2% decrease than control; Fig. 2D) contrasting with that of the drought-stressed plants (21.0% decrease than control). The Fe concentration was also observed to be significantly decreased under drought stress (57.8% decrease than control; Fig. 2E) while maintained in the Drought + MLE plants (27.0% decrease than control). Drought stress also severely impeded the Mn (52.6% decrease than control; Fig. 2F), Zn (49.4% decrease than control; Fig. 2G) and Si (55.4% decrease than control; Fig. 2H) contents in wheat. However, MLE treatment sustained the Mn (32.1% decrease than control), Zn (26.5% decrease than control) and Si (28.5% decrease than control) concentrations in the Drought + MLE plants.

3.3. Micronutrients decreased oxidative stress under drought stress

Drought stress resulted in significant ROS accumulation in the flag leaves of the wheat genotype studied (Fig. 3). Enhanced ROS accumulation was determined by measuring endogenous H_2O_2 content and $^{\bullet}O_2^-$ production rate. In the present study, MLE plants showed considerably lower H_2O_2 content in flag leaves than the control plants (Fig. 3A; 11.1 and 15.4 μg g^{-1} FW). Following drought stress during anthesis, the H_2O_2 content in flag leaves was significantly increased ($P \leq 0.5$) in the wheat genotype studied (2.1-fold increase), whereas, Drought + MLE plants had the minimal increase in H_2O_2 (1.7-fold increase). Similar trend was observed in $^{\bullet}O_2^-$ production rate (Fig. 3B). The smallest $^{\bullet}O_2^-$ production rate was noted in the control and MLE plants that ranged between 24.5–28.2 nmol min^{-1} mg^{-1} protein. Drought stress caused marked increase in $^{\bullet}O_2^-$ production rate (1.8-fold increase), contrasting with the Drought + MLE plants (1.4-fold increase). Oxidative damage in the drought stressed wheat genotype was measured from the lipid peroxidation, estimated in terms of MDA content. The MLE and control plants had the lowest MDA content ranging between 2.4–2.6 nmol g^{-1} FW (Fig. 3C). After perceiving drought stress during anthesis, the wheat plants exhibited significant increase in MDA content (12.0 nmol g^{-1} FW), whereas, the Drought + MLE plants maintained considerably lower MDA content (7.0 nmol g^{-1} FW). Drought-induced ROS overproduction caused remarkable increase in relative cell death in the wheat genotype studied (Fig. 3D). The lowest cell death was observed in the control and MLE plants (0.95-1.0). Drought stress resulted in marked increase in cell death (1.6-fold increase) as compared to the control. However, Drought + MLE plants maintained considerably lower cell death (1.2-fold increase).

3.3. Micronutrients stimulated antioxidant defence system under drought stress

Redox homeostasis mediated by several key non-enzymatic and enzymatic antioxidants was measured in the flag leaf of the wheat genotype studied under different experimental conditions (Fig. 4). Non-enzymatic antioxidants were estimated in terms of total ascorbate and glutathione content. Following the present study, total ascorbate content under the control and MLE ranged between 4.0-4.9 µmol g^{-1} FW (Fig. 4A). Drought stress caused significant increase ($P \leq 0.05$) in total ascorbate content (39.9 % increase), whereas, the maximum increase in total ascorbate content was noticed in the Drought + MLE plants (51.8 % increase). Likewise, total glutathione content was also significantly increased under drought stress (Fig. 4B; 54.7 % increase). However, the Drought + MLE plants had the maximum increase in total glutathione content (62.5 % increase).

Study of antioxidant enzymes included SOD, CAT, POX and APX. Under control and MLE conditions the constitutive level of SOD activity in the wheat genotype ranged between 3.4–7.5 Unit min^{-1} mg^{-1} protein (Fig. 4C). However, SOD activity was significantly increased under drought stress condition (78.5% increase). The maximum increase in SOD activity was observed in the Drought + MLE plants (89.4% increase). Similarly, CAT activity was also lower in the control and MLE plants ranging between 6.1–8.7 Unit min^{-1} mg^{-1} protein (Fig. 4D). Drought stress during anthesis enhanced CAT activity by 37.6% in the wheat plants; whereas, the highest increase in the activity was observed in Drought + MLE plants (60.5% increase). POX activity of the wheat genotype under different experimental conditions also exhibited an increasing trend by water stress (Fig. 4E). The lowest POX activity was observed in the plants grown under the control and MLE conditions, ranging between 4.7–6.8 Unit min^{-1} mg^{-1} protein. Drought stress caused 59.9% increase in POX activity. However, the maximum increase in POX activity was noted in Drought + MLE plants (70.7% increase). APX activity was also significantly increased by drought stress (Fig. 4F). The minimum APX activity was exhibited by the plants grown under control and MLE conditions that ranged between 2.2–5.1 Unit min^{-1} mg^{-1} protein. Following drought stress, APX activity was increased by 78.9%, whereas, the highest increase in APX activity was observed in Drought + MLE plants (84.8% increase).

The *in gel* activities of antioxidant enzymes were well coordinated with the data obtained in the kinetic measurements (Fig. 5A-D). Native-PAGE revealed a single isozyme band with POX activity in flag-leaves of wheat under different experimental conditions (Fig. 5A). However, the band intensity was considerably higher in plants under drought stress as compared to the control plants (2.7-fold higher than control). Drought + MLE plants showed the maximum band intensity (3.1-fold higher than control). CAT activity also showed only one isozyme band in native gels under different experimental conditions (Fig. 5B). Similar to the CAT activity, drought stressed plants showed higher intensity bands as compared to the control conditions (6.5-fold higher than control). The highest band intensity was observed in Drought + MLE plants (7.6-fold higher than control). Regarding SOD activity, three isozymes bands for MnSOD, FeSOD and Cu/ZnSOD were observed in native gels under different experimental conditions which followed similar trend (Fig. 5C). Three isozymes exhibited a modest increase in activities represented by higher intensities of bands in the plants under drought stress compared to the control (2.1, 6.9 and 2.2-fold higher than control in MnSOD, FeSOD and Cu/ZnSOD, respectively), whereas, the highest band

intensity was observed in the Drought + MLE plants (2.5, 7.9 and 2.7-fold higher than control in MnSOD, FeSOD and Cu/ZnSOD, respectively).

Western blot analysis also exhibited harmony with the spectrophotometric and *ingel* analyses of CAT and Cu/ZnSOD enzyme activities (Fig. 5E and F). CAT proteins showed differential expression in western blot (Fig. 5E). Control and MLE plants showed the minimum CAT expression. However, drought stress significantly up-regulated the CAT expression level. However, the maximum level of expression was observed in the Drought + MLE plants. In contrast, control and MLE plants had the minimum increase in CAT expression. The Cu/Zn SOD expression showed similar trend as that of the CAT (Fig. 5F). Control and MLE plants showed the minimum Cu/Zn SOD expression contrasting with the drought stressed condition showing marked increase in the expression. The maximum Cu/Zn SOD expression was observed in the Drought + MLE plants.

4. Discussion

Drought stress severely affects the anthesis stage of wheat leading to dramatic yield loss. Gradual increase in average atmospheric temperature and irregular rainfall is predicted to exacerbate the conditions (Leng and Hall 2019). Therefore, enhancing drought tolerance in wheat, particularly during the reproductive development has become an essential requirement to support the global food security (Senapati et al. 2019). Several studies have revealed the potential role of different bio-stimulants in improving abiotic stress tolerance in plants (Sharma et al. 2019). However, the role of micronutrients in MLE-mediated drought tolerance in wheat has not been studied so far. The present study explored the potential role of the micronutrients in improving drought tolerance in Indian bread wheat during the anthesis stage grounded on the morpho-physiological and biochemical evaluation. The study also critically analysed the association of micronutrient-induced drought tolerance with redox homeostasis.

Drought stress induces the ROS accumulation in plants leading to oxidative stress. Following the present study the wheat plants showed increased H_2O_2 content and $^{\cdot}O_2^-$ production under drought stress (Fig. 3A-B). The excessive ROS burst resulted in the increased MDA accumulation, loss of membrane integrity and cell death, consequently impeding the plant growth (Hameed et al. 2011; Dwivedi et al. 2018). The increased ROS accumulation and loss of membrane integrity might be attributed to the drought-induced boron and Zn deficiency in plants (Hajiboland and Farhanghi 2011). The Fe deficiency under drought stress also led to H_2O_2 accumulation in plants by inhibiting the activities of the haem-containing enzymes POX and CAT (Rotaru 2011). Likewise, ROS accumulation was also induced by the loss of activities of the Cu, Zn, and Mn containing antioxidant enzymes Cu/ZnSOD and MnSOD due to their deficiencies under drought stress (Ahanger et al., 2016).

Drought tolerance in wheat is associated with the conservation of the micronutrient homeostasis with higher level of micronutrients in the plant cells. Following the study, MLE application improved the concentrations of B, Cu, Fe, Mn, Zn and Si in both the non-stressed wheat plants and also upheld their level under drought stress (Fig. 2C-H). The micronutrients (Fe, Mn, Cu, Zn and Si) induced the activities of

the antioxidant enzymes, including SOD, POX, CAT and APX in the Drought + MLE plants and protected them from the ROS-induced oxidative damages (Ahanger et al. 2016). Additionally, B, Cu, and Fe maintained the flag leaf chlorophyll content in the Drought + MLE plants. Increased accumulation of micronutrients in the Drought + MLE plants was also responsible for the osmoregulation and rejuvenation of the plant growth during drought stress adaption (Figs. 1 and 2; Wu et al. 2019). MLE-induced conservation of boron concentrations helped in sustaining the water uptake and cell turgidity by directly influencing the uptake of K^+ and solutes into cells. On the other hand, Zn and Si also increased the water retention of cells, thereby maintaining the cellular membrane integrity and chlorophyll content under drought stress (Basu and Kumar 2021). Micronutrients also stimulated the accumulation of osmo-protectants consequently improving drought tolerance in wheat (Loutfy et al. 2012). The present study showed significant increase in proline and TSS content in the Drought + MLE plants, which conserved the flag leaf RWC by acting as potential osmoprotectant and ROS scavenger (Fig. 1E and F; Yadav et al. 2019).

Micronutrients also stimulates the antioxidant enzyme activities that played an important role in ROS detoxification in wheat under drought stress. Correspondingly, Drought + MLE plants had enhanced antioxidant enzyme activities in the flag leaves that facilitated in eliminating oxidative damage through successful scavenging of ROS (Figs. 4 and 5; Wu et al. 2019). Furthermore, the present study illustrated the increased accumulation of non-enzymatic antioxidants, including ascorbate and glutathione in the Drought + MLE plants, which played a significant role in ROS detoxification (Fig. 4A and B; Basu et al. 2021b). Effective ROS detoxification in the flag leaves significantly decreased the MDA content and cell death in Drought + MLE plants, thereby sustaining the plant growth (Fig. 3C and D; Stallmann et al. 2018). In addition, micronutrient-induced ROS detoxification reduced the damage of photosynthetic pigments and maintained higher chlorophyll content in the MLE-treated plants under drought stress (Fig. 1D; Basu et al. 2017).

Following the study, MLE treated plants exhibited micronutrient-mediated potential drought tolerance through enhanced ROS detoxification and osmoregulaton. Micronutrients facilitated the sustainable plant growth by protecting the cells from ROS-induced oxidative damage. Micronutrient-mediated exceptional osmotic adjustment in Drought + MLE plants also helped in conserving the plant water status in the flag leaf cells. Considering the present study, MLE might be considered as the potential bio-stimulant for enhancing drought tolerance in Indo-Gangetic wheat genotype during the anthesis stage.

5. Conclusions

The present study explored the role of in micronutrients conferring MLE-mediated drought tolerance in wheat during the anthesis stage. Drought stress-induced excessive ROS accumulation caused cellular membrane damage ultimately leading to cell death. Application of MLE increased the concentrations of the micronutrients in plants leading to the activation of the antioxidant defence system that played a significant role in ROS detoxification and amelioration of oxidative stress. The increased micronutrient contents rejuvenated the plant growth and physiological activities of wheat under drought stress. The

micronutrients also increased proline and TSS contents that contributed in osmotic adjustment and plant growth under drought stress. As a whole, the study provided insights into the functional role of MLE in improving drought tolerance and indicated the potential to commercialize MLE as an effective bio-stimulant for sustainable agriculture in drought prone regions of IGP.

Abbreviations

DW– Dry weight, FW – Fresh weight, DNPH – dinitrophenyl hydrazine, DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid), EL – Electrolyte leakage, MDA – Malondialdehyde, MSI – Membrane stability index, ROS – Reactive oxygen species, RWC – Relative water content, TBA – Thiobarbituric acid, TCA – Trichloroacetic acid

Declarations

Conflicts of Interest

The authors declare no conflicts of interest.

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Figures

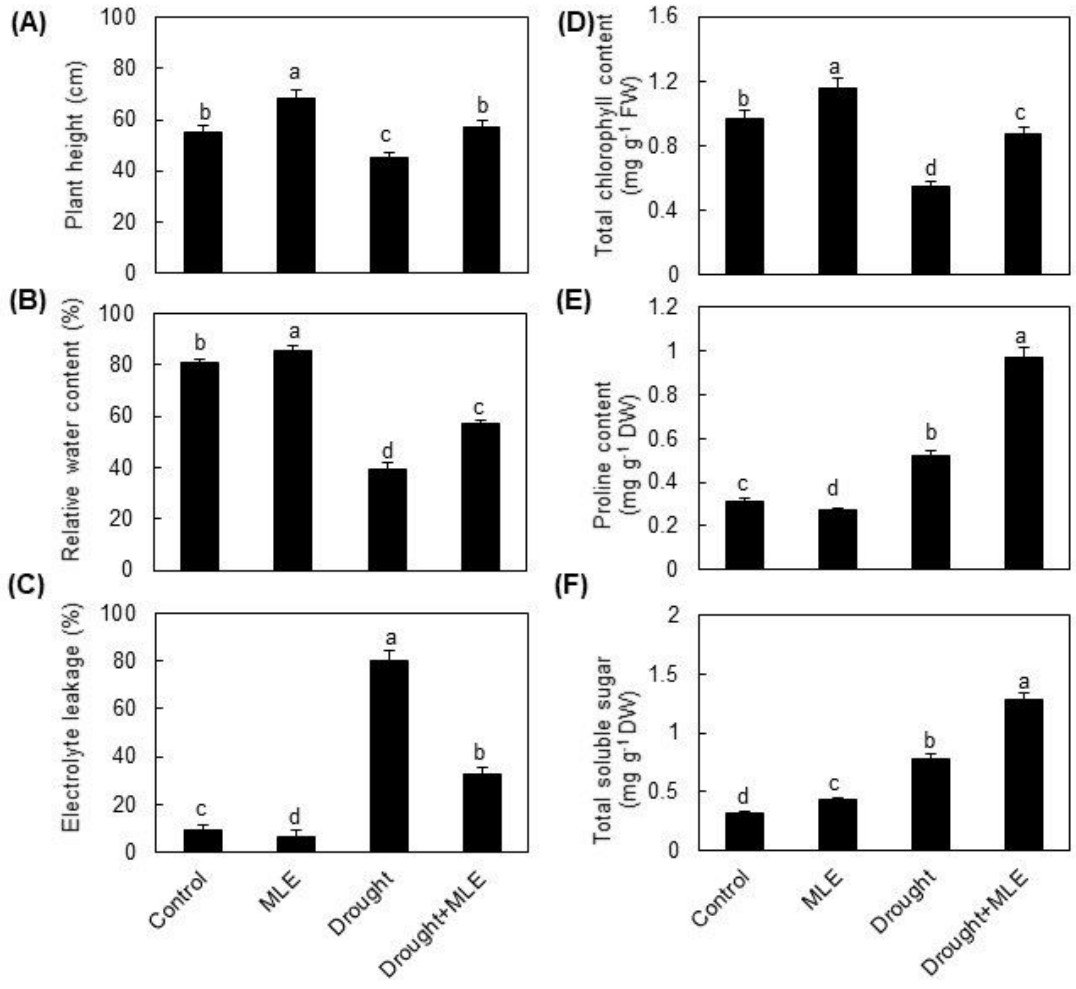

Figure 1

Effect of micronutrients on MLE-regulated drought tolerance in wheat based on morpho-physiological performances during the anthesis stage. Effect of MLE treatment on (A) plant height, (B) relative water content, (C) electrolyte leakage, (D) total chlorophyll content, (E) proline content and (F) total soluble sugar content of the wheat genotype HI-1544 under drought stress. Data represent means ± standard error (n = 3). Values for different letters indicate significant differences at $P \leq 0.05$.

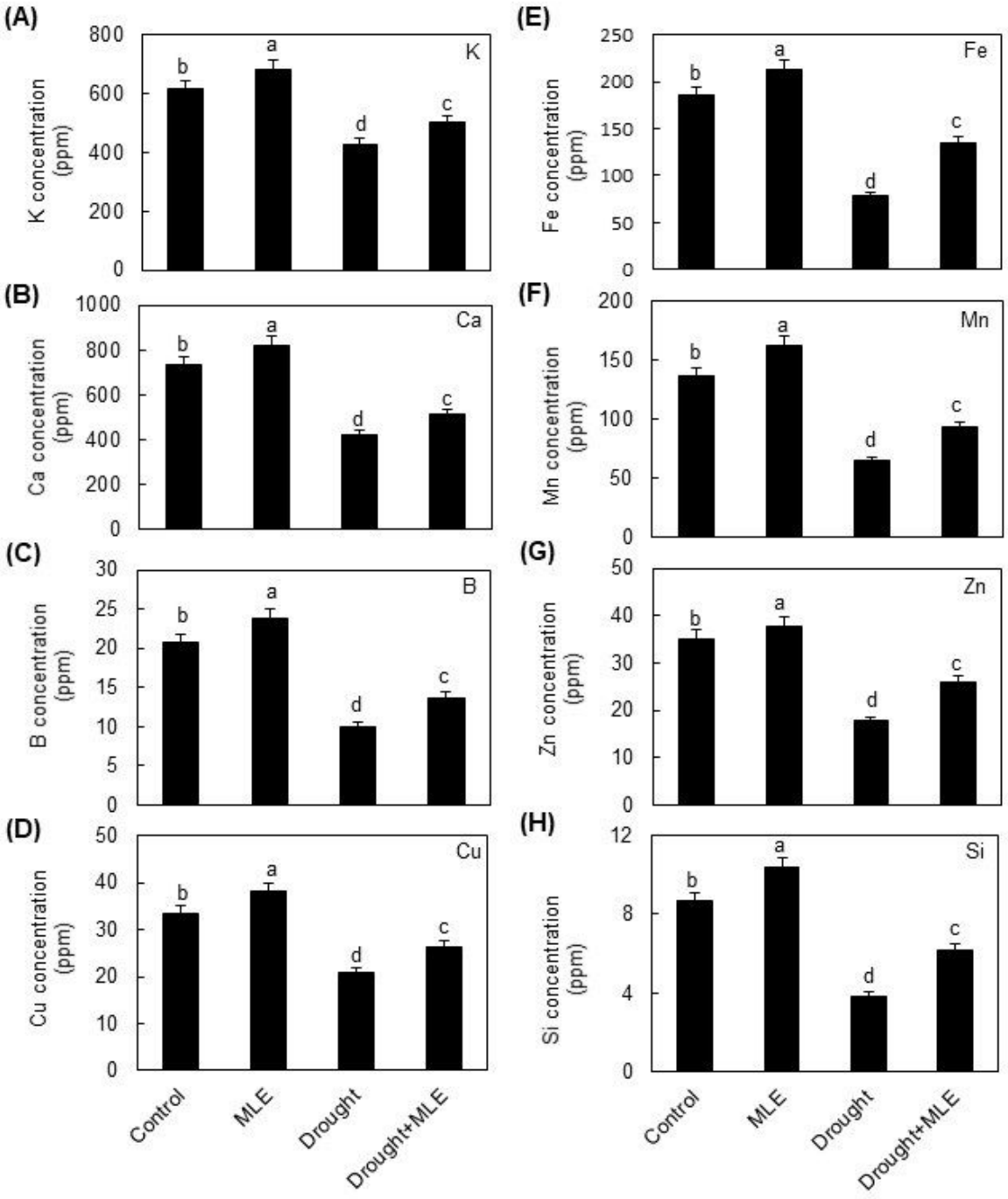

Figure 2

Effect of MLE on macro- and micro-nutrient contents in wheat during the anthesis stage under drought stress. Effect of MLE treatment on (A) potassium (K), (B) calcium (Ca), (C) boron (B), (D) copper (Cu), (E) iron (Fe), (F) manganese (Mn), (G) zinc (Zn) and (H) silicon (Si) concentrations of the wheat genotype HI-1544 under drought stress. Data represent means ± standard error (n = 3). Values for different letters indicate significant differences at $P \leq 0.05$.

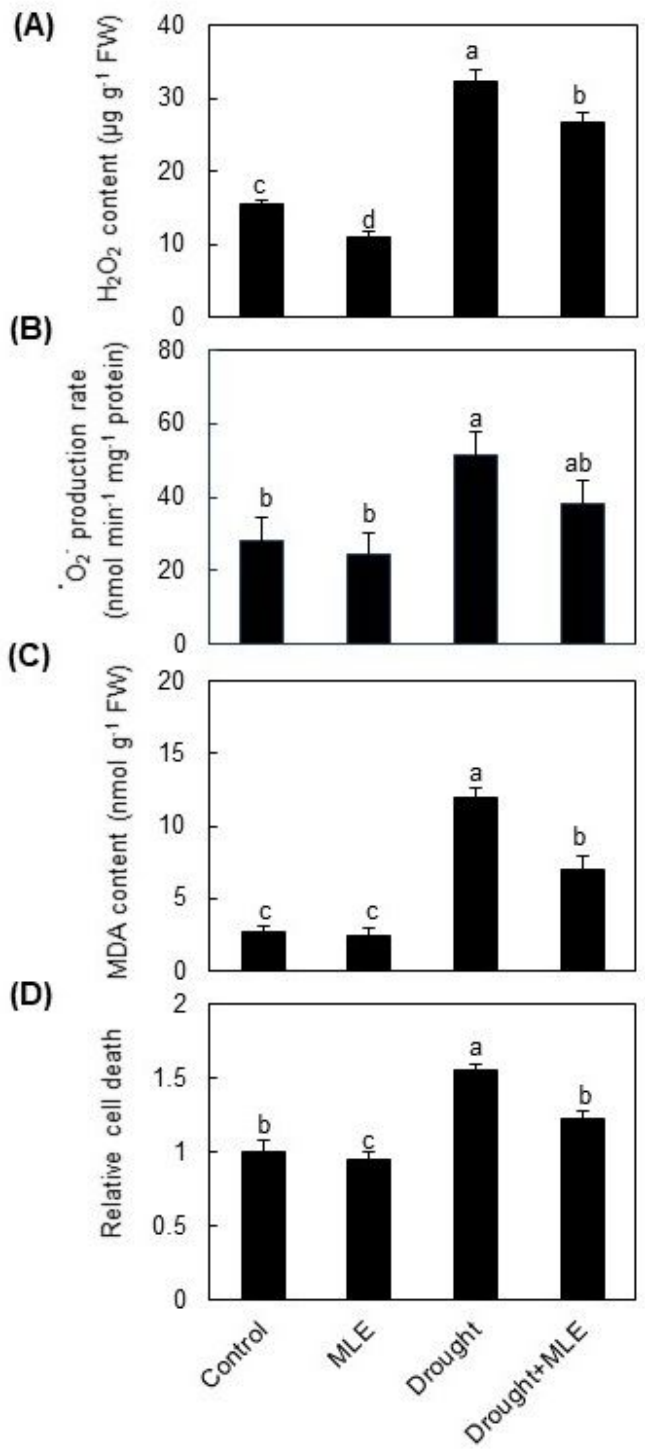

Figure 3

Effect of micronutrients on ROS accumulation and oxidative damage in wheat during the anthesis stage under drought stress. Effect of MLE treatment on (A) H2O2 content, (B) •O2-production rate, (C) MDA content and (D) relative cell death of the wheat genotype HI-1544 under drought stress. Data represent means ± standard error (n = 3). Values for different letters indicate significant differences at $P \leq 0.05$.

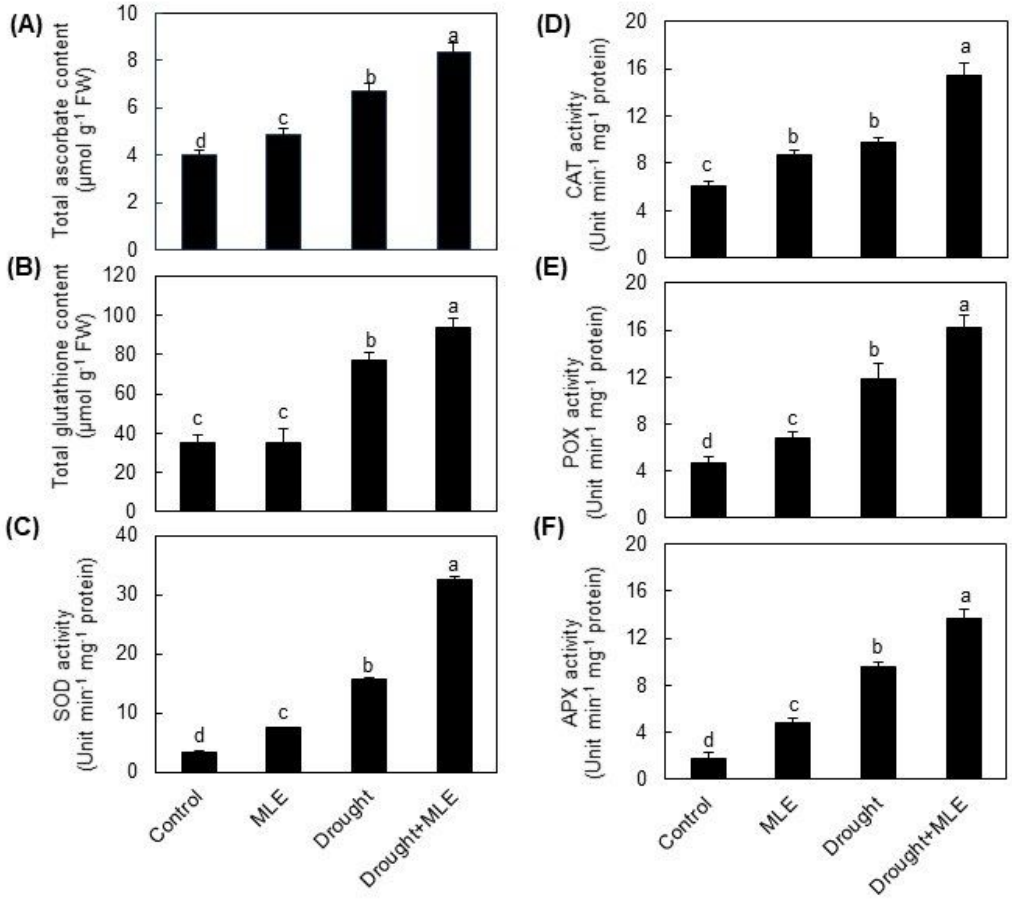

Figure 4

Effect of micronutrients on redox homeostasis in drought stressed wheat genotype the anthesis stage. Effect of MLE treatment on (A) total ascorbate content, (B) total glutathione content, (C) SOD activity, (D) CAT activity, (E) POX activity and (F) APX activity of the wheat genotype HI-1544 under drought stress. Data represent means ± standard error (n = 3). Values for different letters indicate significant differences at $P \leq 0.05$.

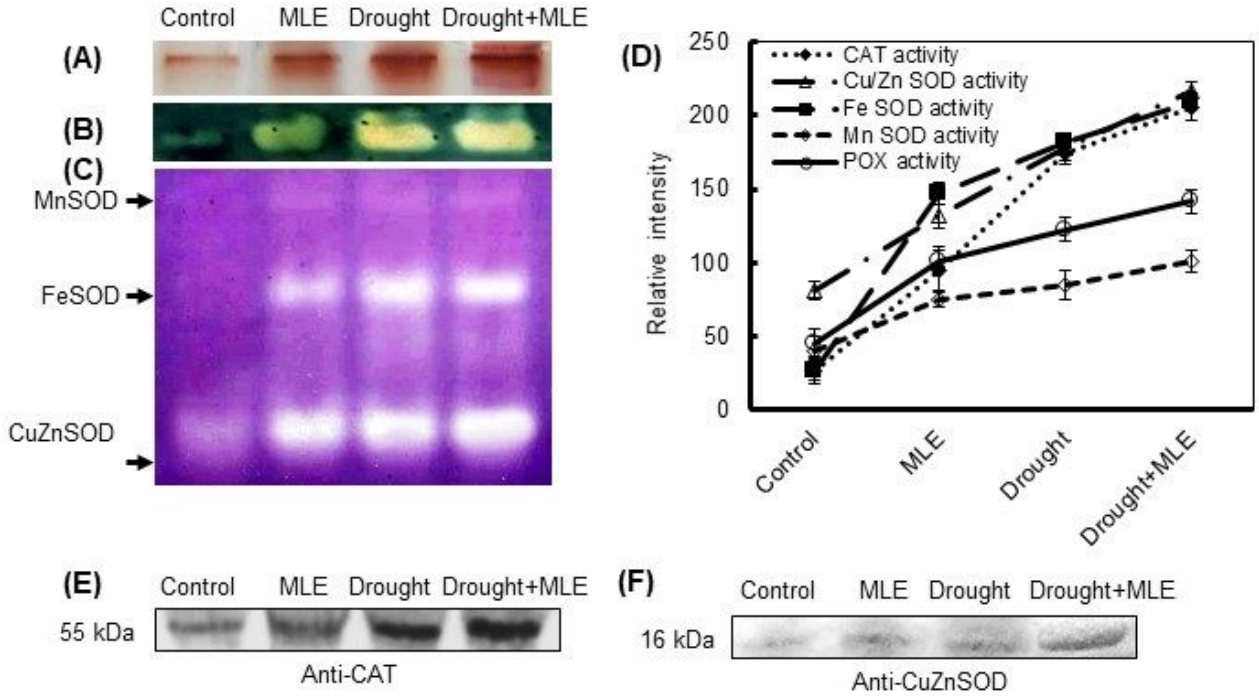

Figure 5

Effect of micronutrients on activity of POX (A), CAT (B) and SOD isozymes (C) in wheat genotype HI-1544 during the anthesis stage under drought stress. (D) Relative intensity of bands in the respective gels. Data represent means ± standard error (n = 3). Western blot analyses using antibodies against (E) CAT and (F) Cu/Zn-SOD.

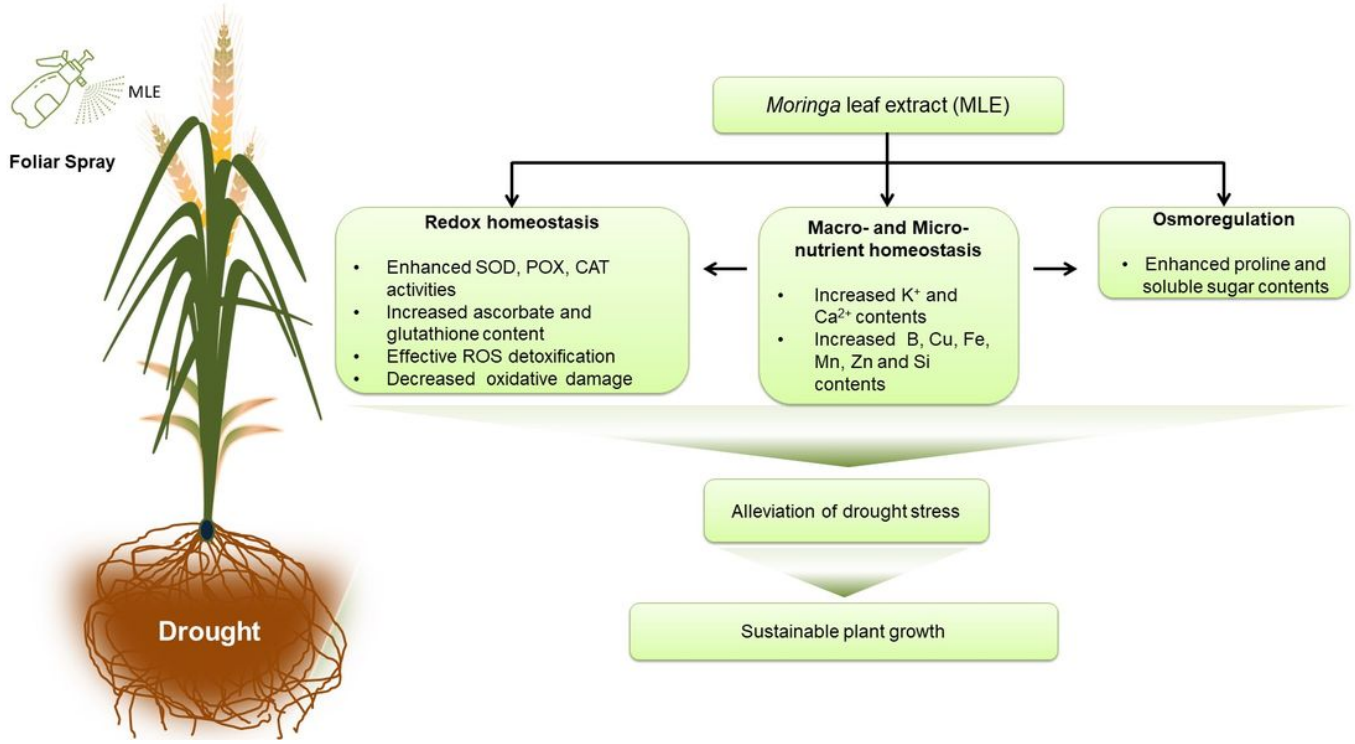

Figure 6

Mechanisms underlying micronutrients on MLE-regulated drought tolerance in wheat. Micronutrients in drought-exposed wheat plants led to effective ROS scavenging through enhanced non-enzymatic (ascorbate, glutathione) and enzymatic (SOD, POX, CAT) antioxidants, consequently minimizing oxidative damages. Micronutrients also stimulated endogenous macro- and micro-nutrient contents, thereby conserving the ion homeostasis. Micronutrients also improved the proline and total soluble sugar contents, thereby maintaining the osmotic balance under drought stress. Thus micronutrient mediated conservation of redox homeostasis and osmoregulation promoted plant growth in wheat under drought stress.

Supplementary Files

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