Phytology

Molybdenum improves *Sorghum bicolor* tolerance to salt stress by regulating the antioxidant system and the heat shock protein 70 expression

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Abstract
Salinity is the most severe abiotic stress affecting crop growth and yield worldwide. Molybdenum (Mo), a micronutrient required in small quantities by plants, has the potential to alleviate effects of stress in plants. This study aimed to determine the mechanism of Molybdenum-induce salt tolerance in *Sorghum bicolor* using the lowest Mo concentration. Sorghum plants grown on potting soil stressed with NaCl (0 mM - 200 mM NaCl) were treated with 0.5 and 1 µM Molybdenum [$(NH_4)_6Mo_7O_{24}.4H_2O$]. NaCl reduced shoot growth and caused severe deformation in the epidermis and xylem layers of sorghum shoots, and these growth attributes were restored by Mo. While chlorophyll content was also reduced by NaCl, proline content increased by 11-fold, and these effects were also reversed by Mo. NaCl-induced oxidative damage was also reversed by Mo, resulting in significantly low levels of reactive oxygen species (ROS) in 0.5 µM Mo treated plants. NaCl also increased superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities, suggesting a high antioxidant scavenging capacity in sorghum. Mo further increased SOD activity in the roots of control and NaCl-treated plants. APX activity increased in control plants, while a decrease occurred in NaCl-treated plants upon Mo application. The HSP70 expression, which was highly induced by NaCl, was slightly reduced by 0.5 µM Mo and completely reduced by 1 µM Mo. The study concludes that low (0.5 µM) Mo concentrations effectively reduced NaCl-induced oxidative damage by regulating ROS detoxification and the expression of HSP70 thereby restoring membrane structure and improving growth.

Keywords: antioxidant, molybdenum, oxidative, reactive oxygen species, sorghum, salt stress, heat shock protein

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important cultivated cereal crops, ranked the 5th in the world and the 2nd in Africa. Globally about 70 million tons of sorghum grains are produced annually, which serve as staple food for millions of people living in semi-arid regions mostly in African and Asian countries [1, 2]. Despite being moderately drought and salt tolerant [3, 4], sorghum growth and yield can be negatively affected by prolong exposure to stress conditions [5]. Salinity is one of the major abiotic stresses, which hinders plant growth and crop yield worldwide [6]. Estimates have indicated that at least 20% of irrigated land is already salinized [7]. Salinity affects plant growth by limiting the absorption of water from the soil due to osmotic stress and prevents the uptake of essential nutrients due to ionic stress, resulting from high concentrations of toxic Na^+ within plant cells [8-10]. The exposure of plants to these stresses affects the physiological mechanism of plants, which can have deleterious effects on plant growth and development [11].

Plant survival under stressful conditions, depend on the integration of physiological, biochemical and molecular adaptive responses, including the accumulation of low molecular mass scavengers, activation of the antioxidant defence system [12], and expression of stress responsive proteins like the heat shock proteins [13, 14]. The antioxidant system includes enzymes [mainly superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR)] and metabolites [ascorbic acid (AsA), flavonoids, phenols, carotenoids, glutathione (GSH) and osmolytes], which are crucial for the detoxification of ROS [superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$)] thereby protecting cells from oxidative damage [15, 13]. Thus, it is important to improve sorghum's tolerance to salt stress, increase its yield and thereby increase its usefulness as a food source, especially in dry and more saline regions of the world. This can be partly achieved through the exogenous application of bio-stimulants to alleviate the effects of salt stress.

Molybdenum [Mo; $[(NH_4)_6Mo7O_{24}.4H_2O]$ is a micronutrient required in small quantities by plants for their growth and metabolic activities. While Mo itself is biologically inactive, it plays both structural and catalytic roles in several enzymes, including nitrate reductase, sulphite oxidase, xanthine dehydrogenase, aldehyde oxidase and the mitochondrial

amidoxime reductase [16, 17, 18]. Its deficiency in soils is a widespread agricultural problem that result in low crop yields and hence crop loss [19]. Conversely, its overuse can be associated with the risk of environmental toxicity, especially when used in high concentrations. Several studies reported on the effective use of Mo to alleviate the effects of abiotic and biotic stresses on crops [20, 21, 18, 22], but using high Mo concentrations, in the millimolar range. The objective of this study was to investigate the mechanisms that are associated with the alleviation of salt stress effects on sorghum plants using low concentrations of exogenously applied Mo in the micromolar range. This study is the first to describe the role of Mo in mediating antioxidant activities and HSP70 expression-induced salt stress tolerance in crops.

Material and methods

Plant growth and treatments

Red sorghum [*Sorghum bicolor* (L.) Moench] seeds were purchased from Agricol, Brackenfell, Western Cape, South Africa. Seeds were surface decontaminated as described previously [24], followed by imbibing overnight in doubled distilled water (ddH_2O) at room temperature with shaking. After air-drying under the laminar flow, seeds were sown on sterilized petri dishes containing Whatman filter paper wetted with autoclaved ddH_2O. Petri dishes were placed in the dark and seeds were allowed to germinate for 7 days in a growth chamber set at 25 ˚C. After 7 days plants were individually transplanted in pots containing a mixture of potting soil and vermiculite (2:1). Plants were allowed to grow in the green house under controlled conditions [25 ˚C/22 ˚C day/night and 16 hrs light/8 hrs dark regimes] for 14 days. Watering was done every 2nd day, whereas plant treatment began after 14 days of growth and continued for 7 days. Treatment solutions included NaCl (0 mM and 200 mM) and Mo (0 µM, and 0.5 µM and 1 µM). After stress treatments, plant roots and shoots were separated, flash frozen in liquid nitrogen and stored at -80 ˚C until further use.

Growth attributes

Growth of sorghum was assayed based on the morphology of the plants in correlation with fresh weight (FW) and dry weight (DW) of the plants. The FW was obtained from fresh samples, which were measured on a Mettle Toledo AE50 analytical balance (Marshall Scientific, Hampton US). The DW was determined after oven-drying the plant shoots at 55 °C until constant weight was attained.

Microscopic analysis

Growth was also assayed based on the anatomical analysis of the epidermis and xylem surface layers as described elsewhere [25]. All Spectra were analysed using the built in Oxford Inca software suite. Samples were then imaged and collected using a Tescan MIRA field emission gun scanning electron microscope, operated at an accelerating voltage of 5 kV using an in-lens secondary electron detector.

Physiological analysis

All spectrophotometric measurements in this study were done using a Helios® Epsilon visible 8 nm bandwidth spectrophotometer (Thermo scientific Waltham, Massachusettes U.S) unless otherwise stated.

Total chlorophyll, chlorophyll a and b contents were measured as described previously [26]. whereas free proline content was determined according to the method described previously [27]. Oxidative damage was estimated based on accumulation of Reactive Oxygen Species (ROS). Histochemical detection of ROS markers was done as described previously [28]. with slight modification. To detect $O_2^{\bullet-}$, leaves were excised and immersed in the reaction mixture [50 mM phosphate buffer (pH 7.5); 0.2% nitroblue tetrazolium (NBT)] and incubated for 4 hrs at room temperature. For localization of H_2O_2, excised leaves were immersed in 1mg/mg of 3',3'-diaminobezidine (DAB; pH 3.8) solution overnight at room temperature. From all samples, chlorophyll was removed by boiling the leaves for 15 min in 80% (v/v) ethanol. $O_2^{\bullet-}$ was detected based on the appearance of dark blue spots resulting from its reaction with NBT, whereas H_2O_2 occurs as brown spots resulting from its reaction with DAB. H_2O_2 content was determined as described elsewhere [29].

Biochemical and molecular analysis

Total soluble proteins were extracted following the method of Niu et al. [30] and quantified using the Bradford assay [31]. The clear supernatant was used for enzyme activity and immunoblot assays. Superoxide dismutase (SOD, EC: 1.15.1.1) and Ascorbate peroxidase (APX, EC: 1.11.1.11) activities were measured as previously described [32].

Immunoblotting assays were done using a dot blot, following a western blot method described previously [33] with slight modifications. Briefly, 5 µL of extracted proteins were directly spotted onto the Polyvinylidene Fluoride (PVDF) membrane that was previously soaked in ethanol. The membrane was incubated for 1 hr in 1% casein made up with 1x Phosphate Buffered Saline and 0.1% Tween (PBS-T), while shaking. The membrane was washed briefly with 1x PBS-T. The mouse monoclonal anti-Hsp70 primary antibody (1:500) (Product# ab2787, Abcam, Cambridge, England) was added to the membrane and allowed to shake overnight on a rotary shaker. The next day the membrane was washed with 1x PBS-T while shaking for 10 min; this was then followed by two 5 min washes. Following the washes, the membrane was incubated with the StarBright Blue 520 goat anti-mouse IgG (1:1000) secondary antibody (Cat# 12005867; Bio-Rad Laboratories, Inc, Hercules, CA) for 1 hr, while shaking. This was followed by washing thrice for 5 min intervals. The membrane was then incubated with ECL detection solution (Bio-Rad Laboratories, Inc, Hercules, CA) and viewed using a ChemiDoC TM imaging system (Bio-rad, Hercules, US).

Statistical analysis

All experiments were repeated at least three times and data were statistically analyzed by the one-way analysis of variance (ANOVA) using GraphPad Prism 9.2.0 (https://www.graphpad.com). Data in figures and tables represent the mean ± standard deviation. Statistical

significance between control and treated plants were determined by the Bonferroni's multiple comparison test and represented as *** = $p \leq 0.001$, ** = $p \leq 0.01$, and * = $p \leq 0.05$. The data represents three independent experiments (supplementary data).

Results

Plant growth and membrane structure

NaCl stress considerably affected the growth by reducing shoot lengths (Figure 1), fresh and dry weights (Table S1) of sorghum plants, however Mo did not show any significant growth improvement. Application of 0.5 µM Mo on control plants did not show any significant effects on shoot growth, whereas 1 µM Mo slightly decreased growth (Figure 1a). NaCl stress affected the growth of sorghum plants, and the growth reduction was revered by the application of 0.5 µM and 1 µM Mo (Figure 1b).

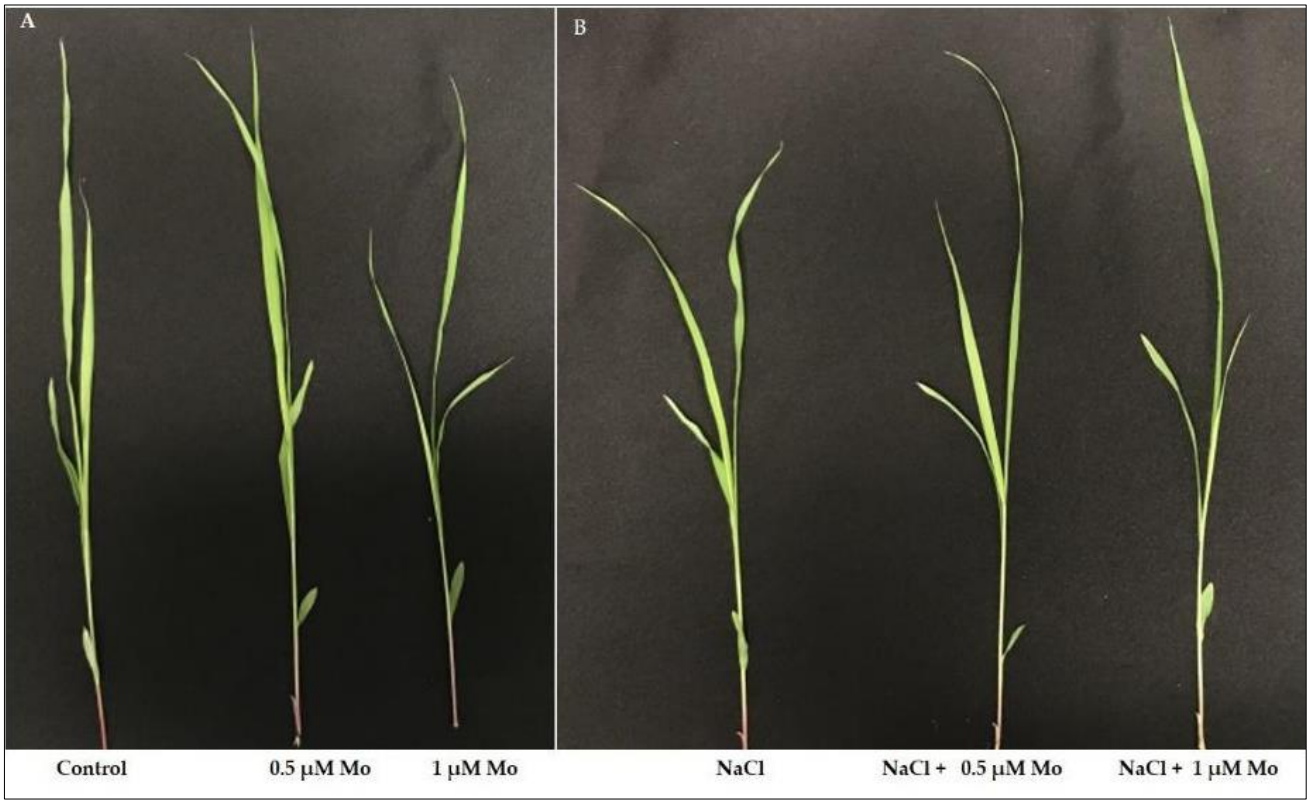

Fig 1: Effect of Mo on the growth of *Sorghum bicolor* plants treated with NaCl. Sorghum plants treated with Mo only (a), sorghum plants treated with a combination of NaCl and Mo (b)

Analysis of the anatomical structure on the effects of Mo on sorghum shoots treated with NaCl was done using Scanning Electron Microscopy (Figure 2). SEM images from control plants revealed smooth epidermis and xylem layers (Figure 2a), but upon treatment with NaCl, the epidermis and xylem layers revealed severe damage and showed signs of shrinking (Figure 2d). Application of Mo to control plants did not show significant changes (Figure 2b), except for treatment with 1 µM Mo, which caused some shrinkage on the sorghum shoot anatomical structure (Figure 2c). The epidermis and xylem layers of NaCl-treated plants concurrently treated with Mo showed less deformation and shrinkage as observed by improved surface structure (Figure e-f).

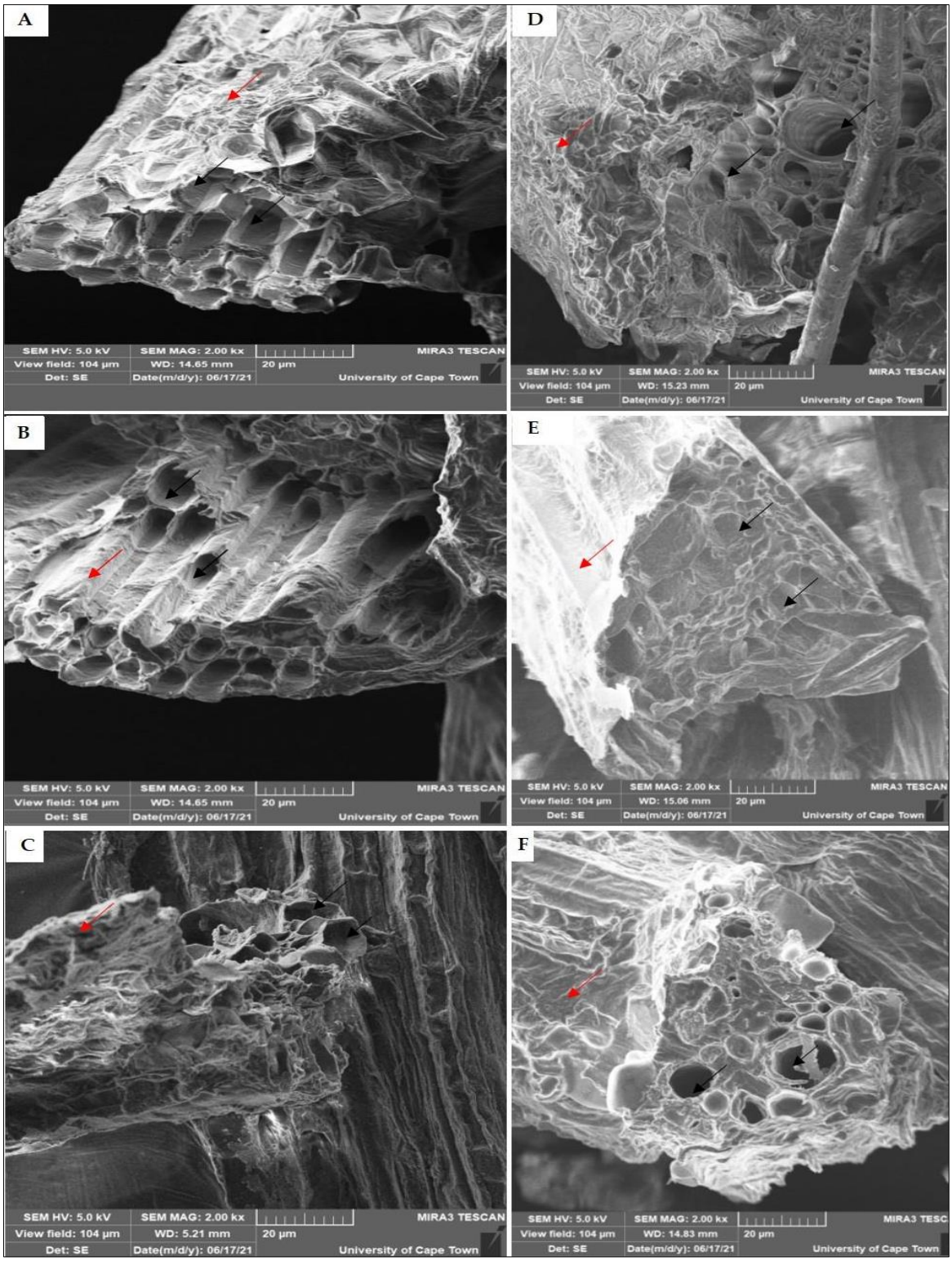

Fig 2: Scanning Electron Microscopy images showing the effect of Mo on the epidermis and xylem layers of *Sorghum bicolor* plants treated with NaCl. Epidermis and xylem layers of control plants only (a), plants treated with 0.5 µM Mo (b), and 1 µM Mo (c). Epidermis and xylem layers of sorghum shoots treated with NaCl only (d), NaCl + 0.5 µM Mo (e) and NaCl + 1 µM Mo (f). The red and black arrows indicate the surface area of the epidermis and xylem respectively.

Chlorophyll and proline content

Sorghum plants treated with NaCl resulted in decreased chlorophyll a content (1.6-fold) as compared to the control (Figure 3a). Application of 1 µM Mo to control plants, decreased total chlorophyll content (Figure 3b) whereas in NaCl-treated plants both total chlorophyll and chlorophyll a content were significantly ($p \leq 0.01$) improved (Figure 3c). No significant changes were observed for chlorophyll b content in sorghum shoots treated with 1 µM Mo (Figure 3c).

NaCl significantly ($p \leq 0.01$) increased proline content in the roots and shoots of sorghum plants by 11-fold as compared to the control (Figure 3d). Exogenous application of Mo significantly ($p \leq 0.01$) decreased proline content in the roots by ~2-fold and shoots by 4-fold of sorghum plants treated with a combination of NaCl and 0.5 µM Mo, whereas 1 µM Mo led to slight decreases by ~1-fold (Figure 3f).

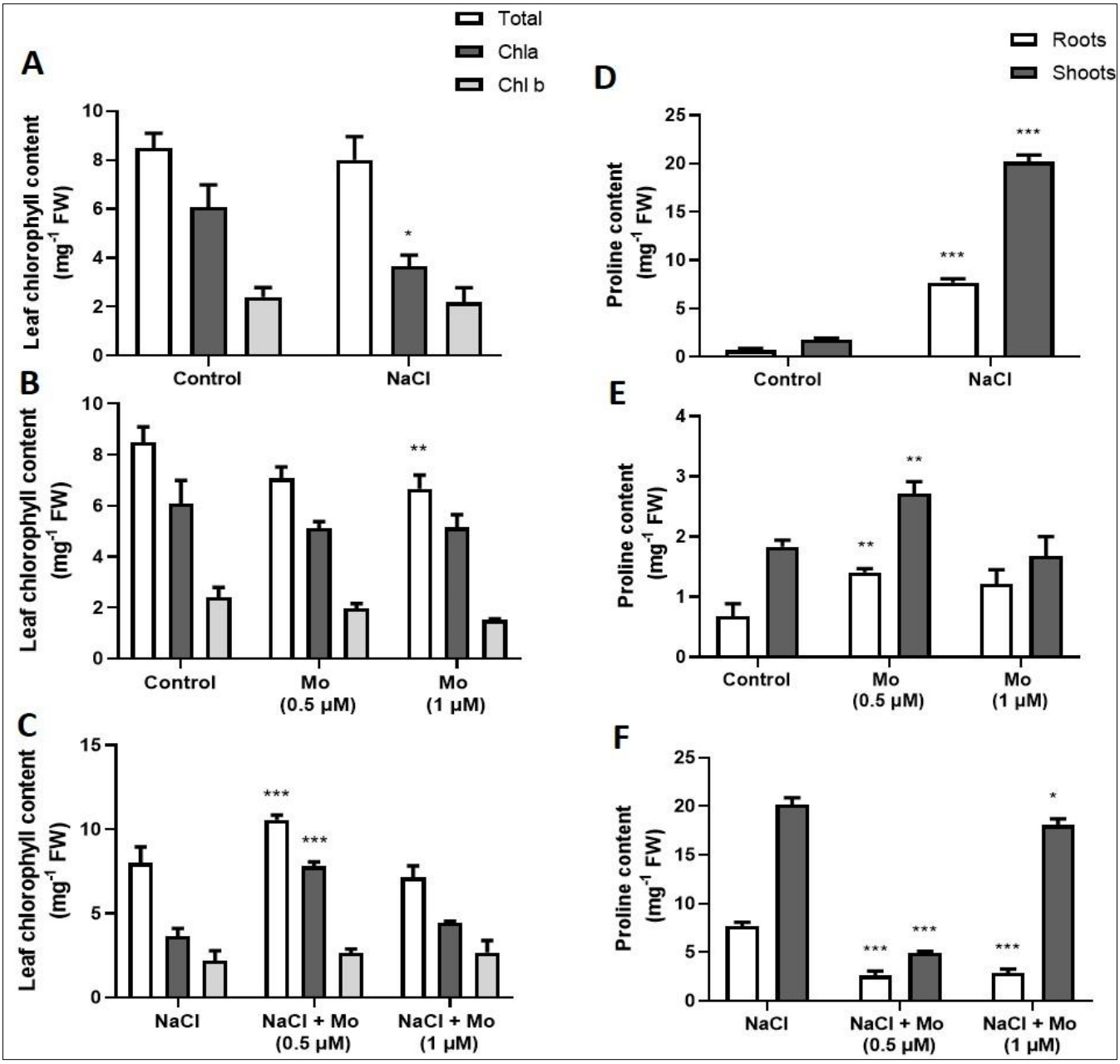

Fig 3: Effect of Mo on chlorophyll (a-c) and proline (d-f) content of sorghum plants under NaCl stress. Chlorophyll and proline contents for plants treated with 200 mM NaCl (a & d), 0.5 µM and 1 µM Mo (b & e), a combination of NaCl and Mo (c & f). Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way and two-way ANOVA as conducted on GraphPad Prism and indicated as *** = p≤0.001, ** = p≤0.01, * p≤0.05 according to the Bonferroni's multiple comparison test.

Markers of oxidative stress

Oxidative damage due to NaCl stress was determined based on ROS formation (Figure 4). Overproduction of ROS was confirmed by histochemical staining based on the presence of dark blue spots representing $O_2^{\bullet-}$ (Figure 4a) and brown spots representing H_2O_2 (Figure 4b) accumulation. Application of Mo resulted in ROS production in control plants, whereas ROS spots diminished in the leaves of plants treated with a combination of NaCl and Mo as compared to plants treated with NaCl only. H_2O_2 content significantly (p≤0.01) increased in the roots by 1-fold and shoots by 4-fold of sorghum plants treated with NaCl (Figure 4c). Application of Mo in control plants increased H_2O_2 content (Figure 4d), whereas in NaCl-treated plants (Figure 4e), H_2O_2 content was significantly reduced in the roots by 2.8-fold and shoots by 1.78-fold, whereas 1 µM Mo did not show any significant effect in the shoots.

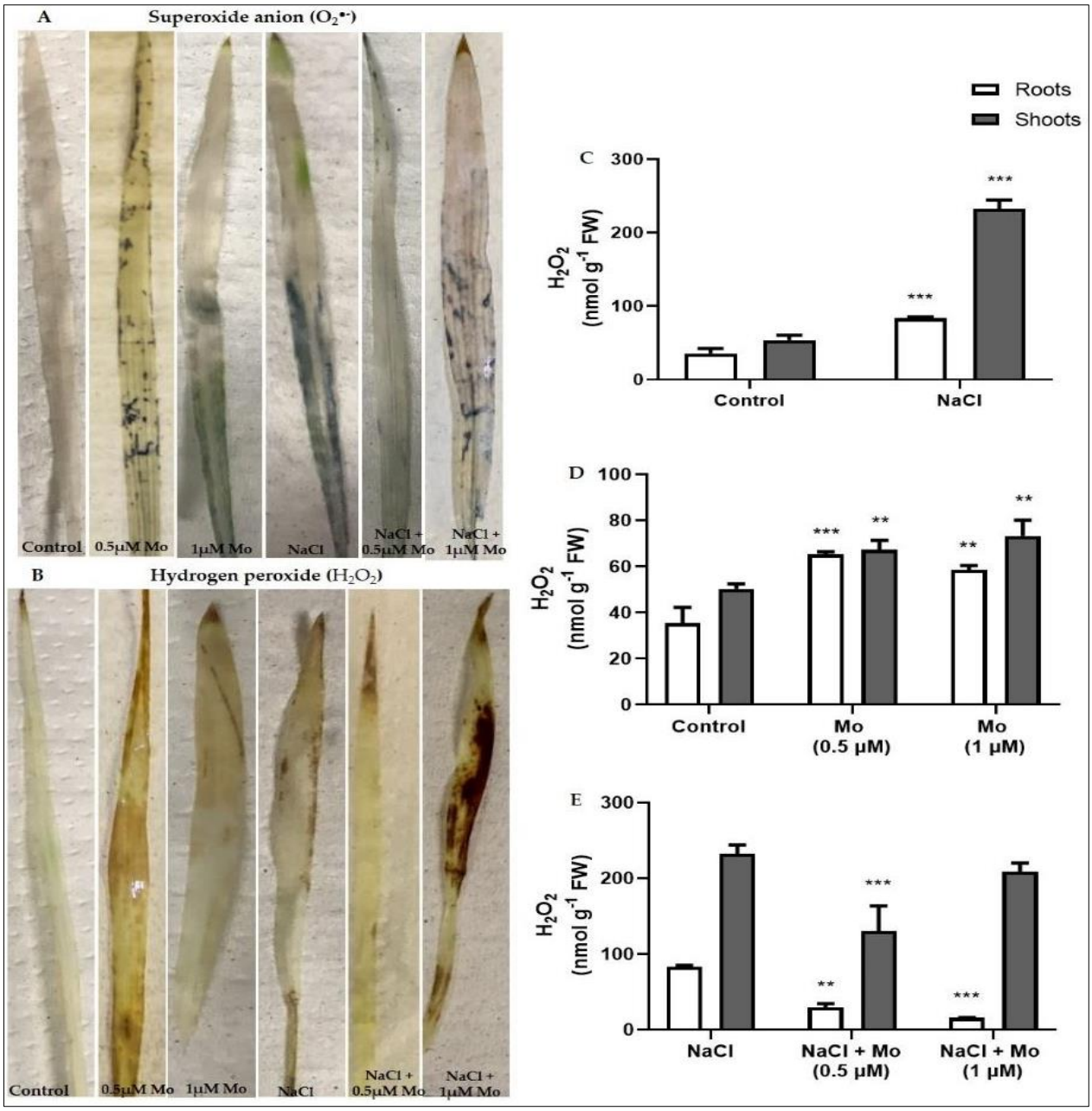

Fig 4: Effect of Mo on oxidative stress markers under NaCl stress. Histochemical detection of $O_2^{\bullet-}$ (a) and H_2O_2 (b) in sorghum shoots. Measurement of H_2O_2 content in sorghum tissues treated with 200 mM NaCl (c), Mo [0.5 and 1 µM] (d), a combination of NaCl and Mo (e). Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way and two-way ANOVA as conducted on GraphPad Prism and indicated as *** = p≤0.001, ** = p≤0.01, * = p≤0.05 according to the Bonferroni's multiple comparison test.

Antioxidant enzyme activities

To determine the effect of Mo on the antioxidant scavenging capacity of *Sorghum bicolor* plants, the activities of ROS scavenging enzymes (SOD and APX) were determined (Figure 5). NaCl significantly (p≤0.001 & p≤0.01) increased SOD activity by more than 2-fold in both the roots and shoots (Figure 5a). Application of Mo also significantly (p≤0.001) increased SOD activity in the roots of control plants by ~5-fold (Figure 5b) and NaCl-treated plants by ~5-fold (Figure 5c). However, no significant changes were observed in the shoots. APX activity increased in the roots by 3.9-fold and shoots by 3.75-fold of sorghum plants treated with NaCl only (Figure 5d). Application of Mo in control plants only increased APX activity in both tissues by more than 2-fold (0.5 µM) and 1-fold (1 µM) as shown in Figure 5e. Mo application in NaCl-treated sorghum plants significantly (p≤0.001) reduced APX activity in the shoots by ~2-fold, whereas the reduction in the roots was not significant (Figure 5f).

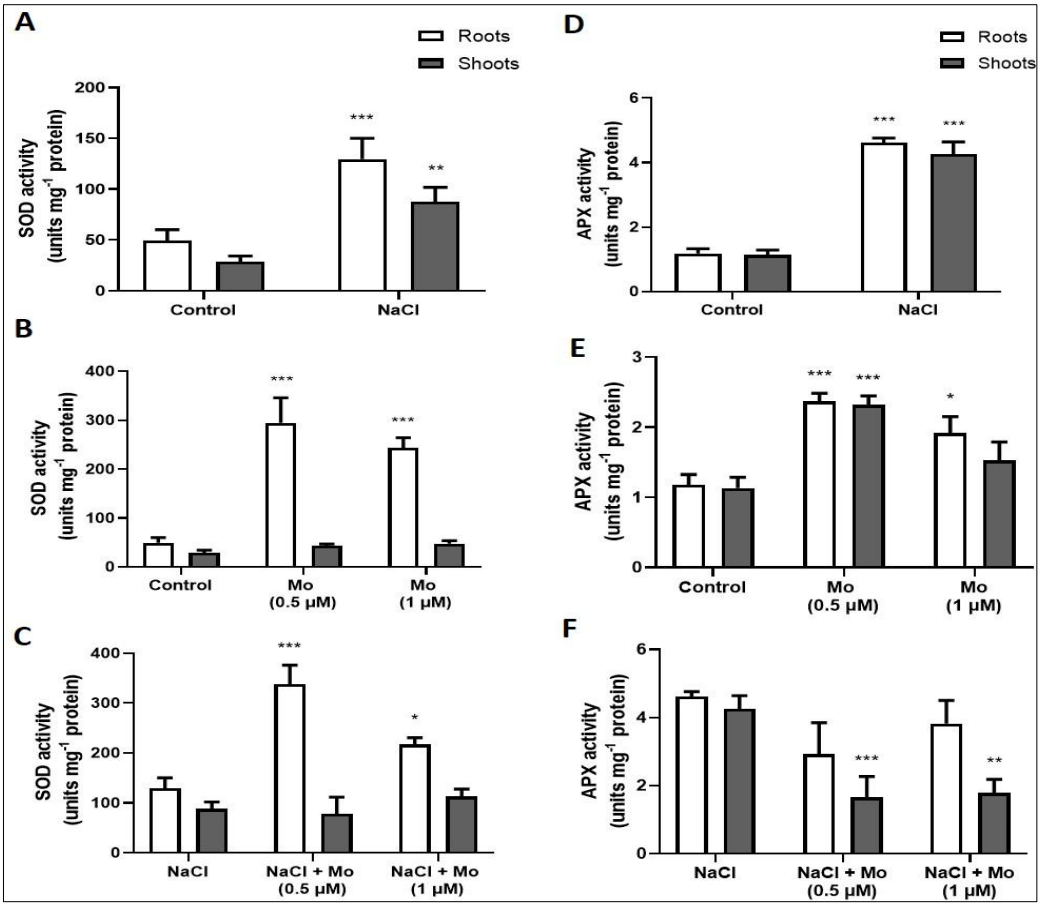

Fig 5: Effect of Mo on the antioxidant enzyme activities of SOD (a-c) and APX (d-f) in sorghum tissues under NaCl treatment. SOD and APX activities for plants treated with NaCl [200 mM] (a & d), Mo [0.5 and 1 µM] (b & e), a combination of NaCl and Mo (c & f). Error bars represent the SD calculated from three biological replicates. Statistical significance between the control and treated plants was determined using one-way and two-way ANOVA as conducted on GraphPad Prism and indicated as *** = p≤0.001, ** = p≤0.01 according to the Bonferroni's multiple comparison test.

Heat Shock Protein 70 expression

The molecular response of sorghum to NaCl treatment and the effect of exogenous Mo was also investigated by determining the expression level of the Heat Shock Protein 70 using a dot blot (Figure 6). HSP70 bands were not detectable in the shoots of control plants (Figure 6a), however HSP70 protein expression was highly induced in sorghum shoots treated with NaCl (Figure 6d). Application of low concentration of Mo (Figure 6b) induced HSP70 protein expression in control plants, as compared to 1 µM Mo (Figure 6c) and the control. HSP70 expression in sorghum shoots under a NaCl treatment, was reduced to a lesser extent in plants treated with 0.5 µM Mo (Figure 6e) and completely in 1 µM Mo treated plants (Figure 6f). The expression level of HSP70 was evidently higher in NaCl-treated plants than in plants treated with Mo only and those treated with a combination of NaCl and Mo.

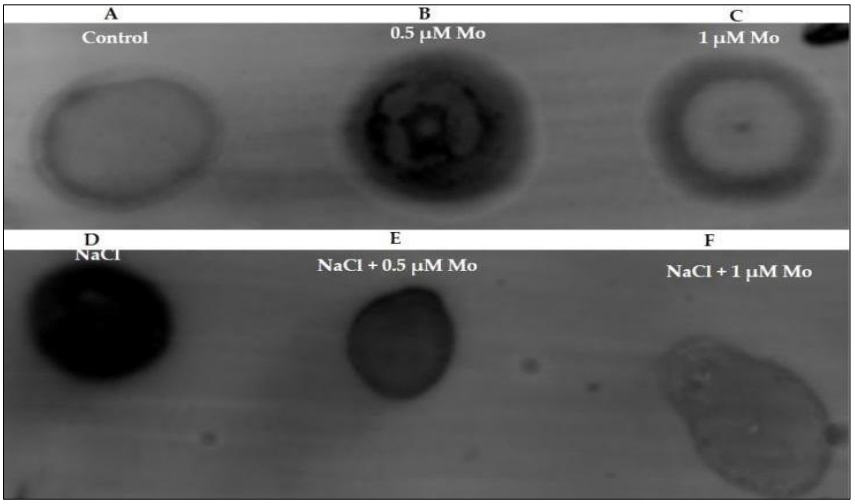

Fig 6: Dot blot analysis of the effect of Mo on the expression of the Heat shock protein 70 (HSP70) in *Sorghum bicolor* shoots treated with NaCl. HSP70 expression in (a) control, (b) 0.5 µM Mo, (c) 1 µM Mo, (d) NaCl, (e) NaCl + 0.5 µM Mo, (f) NaCl + 1 µM Mo

Discussion

Sorghum is considered a moderately stress tolerant crop, thus its growth and yield can be affected by high salt stress [3, 34, 35, 25]. In this study, 200 mM NaCl was chosen as the moderate salt treatment and to demonstrate the effects of exogenously applied Mo on the physiological, biochemical and molecular responses of sorghum. Reduction in biomass under salt stress is indicative of growth limitations in plants [36]. In this study, a reduction was observed on sorghum plants (Table S1; Figure 1). This reduction is attributed to the influence of osmotic stress, which interferes with metabolic processes, reducing turgo and the energy required for maintaining plant growth [37]. Exogenous application of Mo improved sorghum growth as compared to plants treated with NaCl only. Consistently, previous studies reported the ability of Mo to promote growth under different abiotic stresses, including salinity stress, in *Brassica campestris* L. ssp. Pekinensis [21], *Agropyron cristatum* [18] Fabaceae spp [22, 23], *Hordeum vulgare* [20. 38], temperature stress in *Triticum aestivum* [39], drought stress in *Triticum aestivum* [40] and cadmium stress in *Brassica napus* L [41] and *Oryza sativa* L [42].

Exogenous application of Mo also reversed the NaCl-induced reduction in the sheath thickness of the epidermis and xylem layers as compared to the control (Figure 2). Salt stress had the same effects in forage grass [43]. Since leaf epidermis contribute to the regulation of growth [44, 45], this might explain the improved growth seen on sorghum, possibly since the structure and thickness of the epidermis and xylem layers prevent moisture loss as reported for *Suaeda maritima* [46], *Phaseolus vulgaris* [47], and *Imperata cylindrica* (L.), Raeuschel [43], under salt stress.

A significant decrease in chlorophyll a content in NaCl-treated sorghum plants was observed (Figure 2). Similarly, several studies also reported a decrease in chlorophyll content in plants under salinity stress [48, 21, 49]. The decrease in chlorophyll content due to salt stress can be attributed to the increased activity of chlorophyllase [43, 50] and ROS generation [51-53]. Exogenous application of Mo reduced chlorophyll contents in control plants, whereas in NaCl-stressed plants, 0.5 µM increased chlorophyll contents indicating the importance of Mo (especially low concentrations) in alleviating the negative effects of salt stress on plant growth. The increased chlorophyll under salt stress in this study might be due to the ROS scavenging effect mediated by Mo as previously described in cabbage and *Phaseolus vulgaris* L [21, 54].

We further investigated the Mo-regulated mechanism of salinity stress tolerance associated with osmoregulation, antioxidant detoxification and expression of the Heat shock Protein 70. The uptake of high amounts of salts by the plant leads to increased osmotic pressure in the cytosol and under these conditions cell homeostasis is maintained by osmotic adjustment, which is mediated by the synthesis of organic osmolytes [55]. Proline increased significantly in NaCl-treated sorghum tissues as compared to the control (Figure 2b). The increased synthesis of proline might be due to high activities of enzymes involved in proline biosynthesis including pyrroline-5-carboxlyate synthetase, glutamine dehydrogenase and proline dehydrogenase [56]. In this study it is most likely that proline accumulation is associated with tolerance, since osmolytes are produced to protect cells against the adverse effects of stress [57, 58, 59]. Exogenous Mo effectively reduced the effects of salt stress in sorghum as seen by very low proline content in NaCl-treated plants. This result support the fact that sorghum is moderately tolerant to salinity and hence the high proline accumulation. The accumulation of ROS is some of the responses to salinity stress as observed in other plants [60]. NaCl led to the increased accumulation of $O_2^{\bullet-}$ and H_2O_2, as observed in sorghum histograms (Figure 4a-b). Consequently, increased H_2O_2 content in the shoots and roots was quantified in NaCl-treated sorghum plants as compared to the control (Figure 4c-e). The application of Mo played an effective role in reducing ROS accumulation, which further improved membrane stability. We observed a negative correlation between oxidative stress and growth reduction in sorghum plants exposed to NaCl stress. This is a clear indication that the growth reduction was caused by oxidative stress induced at a cellular level and that Mo was able to mediate a reduction of ROS levels in the cells. Overaccumulation of ROS leads to cell toxicity, membrane malfunction and hence cell death [61]. However, plants have evolved mechanisms for ROS detoxification and the most effective is the activation of the enzymatic antioxidant system, which is able to eliminate superoxide and hydro-peroxides [62]. SOD is the first line of defence against ROS, as it catalyzes the breakdown of superoxide radical ($O_2^{\bullet-}$) into H_2O_2, which is further broken down by catalase and peroxidases. The results indicated that NaCl stress increased SOD and APX activities (Figure 5). Application of Mo considerably increased SOD activity in the roots of both the control and NaCl-treated sorghum plants. This result indicates that Mo possibly plays a role in reducing ROS accumulation through induced SOD activity. NaCl stress also increased APX activity in both the roots and shoots of sorghum plants. While APX activity increased in control plants, upon Mo application, its activity significantly reduced in the shoots of NaCl-treated plants. This result correlated positively with histograms which showed decreased $O_2^{\bullet-}$ spots, whereas H_2O_2 spots were not completely cleared in NaCl-treated plants upon Mo application. Several studies indicated the effective role of Mo in inducing the antioxidant enzyme activities under abiotic stresses, including cold [39], drought [63] and heavy metal stress [42].

Accumulation of stress responsive proteins also plays an important role in protecting cells and developing tolerance against stress [64]. The induction of the HSP70 under stress correlates with its role as a molecular chaperon by preventing protein denaturation and aggregation [65]. Our results showed that HSP70, was highly induced in the shoots of sorghum plants treated with NaCl only as compared to the control and Mo treatments (Figure 6). Although the effect of Mo on NaCl-treated plants was done for the first time in sorghum, the results showed that Mo improved sorghum tolerance to NaCl stress by regulating the HSP70 expression.

Table S1: Effect of Mo on the growth attributes (Fresh and dry weights) of sorghum in the absence and presence of NaCl. Data represented are mean ± S.D

Mo (µM)	NaCl (mM)	FW (mg)	DW (mg)
0	0	0.521 ± 0.030	0.046 ± 0.004
0	200	0.390 ± 0.014**	0.025 ± 0.004
0.5	0	0.570 ± 0.028	0.032 ± 0.005
0	200	0.423 ± 0.021	0.019 ± 0.001
1	0	0.315 ± 0.049***	0.020 ± 0.001
0	200	0.284 ± 0.037***	0.009 ± 0.005

Conclusions

This study demonstrated that the exogenous use of low Mo concentration (0.5 and 1 µM) is effective in alleviating the detrimental effects of salt stress on sorghum. The improved growth and well-structured (epidermis and xylem) layers under NaCl stress, is most likely attributable to Mo-mediated tolerance through the induced antioxidant enzyme activities and the regulation of HSP70 expression by 0.5 µM Mo, since HSP70 expression was not completely switched off by this low concentration. We therefore propose that exogenous application of low concentrations of Mo (e.g 0.5 µM) can effectively be used in agricultural fields for cultivation without causing any toxicity to both the plants and the soil.

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Conflicts of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Proietti I, Frazzoli C, Mantovani A. Exploiting nutritional value of staple foods in the world's semi-arid areas: risks, benefits, challenges and opportunities of Sorghum. Healthcare. 2015;3(2):172-193.
2. FAO. The future of food and agriculture- Trends and challenges. Rome, 2017. Available: http://www.fao.org/3/a-i6583e.pdf.
3. Rosenow DT, Quisenberry JE, Wendt CW, Clark LE. Drought tolerant sorghum and cotton germplasm. Agric. Water Manag. 1983;7(1-3):207-222.
4. Krishnamurthy L, Serraj R, Hash CT, Dakheel AJ, Reddy BVS. Screening sorghum genotypes for salinity tolerant biomass production. Euphytica. 2007;156:15-24.
5. Igartua E, Gracia MP, Lasa JM. Field responses of grain sorghum to a salinity gradient. Field Crops Res. 1995;42(1):407-421.
6. Nemati I, Moradi F, Gholizadeh S, Esmaeili MA, Bihamta MR. The effects of salinity stress on ions and soluble sugars distribution in leaves, leaf sheaths and roots of rice (*Oryza sativa* L.) seedlings. Plant Soil Environ. 2011;57(1):26-33.
7. Flowers TJ, Yeo AR. Breeding for salinity resistance in crop plants: where next? Aust. J. Plant Physiol. 1995;22(6):875-884.
8. Ueda A, Kathiresan A, Inada M, Narita Y, Nakamura T, Shi W, et al. Osmotic stress in barley regulates expression of a different set of genes than salt stress does. J. Exp. Bot. 2004;55(6):2213-2218.
9. Munns R, Tester M. Mechanisms of salt tolerance. Annu. Rev. Plant Biol. 2008;59:651-681.
10. Cheng Y, Qi Y, Zhu Q, Chen X, Wang N, Zhao X, et al. New changes in the plasma-membrane-associated proteome of rice roots under salt stress. Proteomics. 2009;9(11):3100-3114.
11. Cherifi K, Haddioui A, Hansali ME, Boufous EH. Growth and proline content in NaCl stressed plants of annual medic species. Int. J. Adv. Res. Biol. Sci. 2016;9:82-90.
12. Csiszár J, Lantos E, Tari I, Madoșă E, Wodala B, Vashegyi Á, et al. Antioxidant enzyme activities in Allium species and their cultivars under water stress. Plant Soil Environ. 2018;53(12):517-523.
13. Taïbi K, Taïbi F, Abderrahim LA, Ennajah A, Belkhodja M, Mulet JM. Effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in Phaseolus vulgaris L. South African J. Bot. 2016;105:306-312.
14. Ul Haq S, Khan A, Ali M, Khattak AM, Gai WX, Zhang HX, et al. Heat shock proteins: dynamic biomolecules to counter plant biotic and abiotic stresses. Int. J. Mol. Sci. 2019;20(21):5321.
15. Gutteridge JMC, Halliwell B. Free radicals and antioxidants in the year. A hystorical look to the future. Ann. N. Y. Acad. Sci. 2000;899(1):136-147.
16. Yu M, Hu C, Wang Y. Influences of seed molybdenum and molybdenum application on nitrate reductase activity, shoot dry matter, and grain yields of winter wheat cultivars. J. of Plant Nutr. 1999;22(9):1433-1441.
17. Kaiser B, Gridley KL, Ngaire-Brady J, Phillips T, Tyerman SD. The role of molybdenum in agricultural plant production. Ann. Bot. 2005;96(6):745-754.
18. Babenko ON, Brychkova G, Sagi M, Alikulov ZA. Molybdenum application enhances adaptation of crested wheatgrass to salinity stress. Acta Physiol. Plant. 2015;37:1-13.
19. Liu P, Yang YS, Xu GD, Fang YH, Yang YA, Kalin R. The effects of molybdenum and boron in soil on the growth and photosynthesis of three soybean varieties. Plant Soil and Environ. 2005;51(5):197-205.
20. Bagheri A, Jafari A. Effect of salinity and molybdenum

application on photosynthesis, nitrogenase activity and yield of barley inoculated with *Azosprillium brasilense*. Cereal Res. Commun. 2012;40(2):235-245.
21. Zhang M, Hu C, Sun X, Zhao X, Tan Q, Zhang Y, et al. Molybdenum affects photosynthesis and Ionic homeostasis of Chinese Cabbage under salinity stress. Commun. Soil Sci. Plant Anal. 2014:45(20):2660-2672.
22. Salha B, Chaabane R. Expression of some molybdenzymes genes under salt stress conditions in Chickpea, Bean and Lentil plant. Int. J. Environ. Agric. Biotech. 2016;4:748-759.
23. Farhangi-Abriz S, Faegi-Analou R, Nikpour-Rashidabad N. Foliar application of sodium molybdate enhanced nitrogen uptake and translocation in soybean plants by improving nodulation process under salt stress. Cercet. Agron. Mold. 2017;50:71-82.
24. Mulaudzi-Masuku T, Mutepe RD, Mukhoro OC, Faro A, Ndimba B. Identification and characterization of a heat-inducible Hsp70 gene from Sorghum bicolor which confers tolerance to thermal stress. Cell Stress Chaperones. 2015;20:793-804.
25. Rakgotho T, Ndou N, Mulaudzi T, Iwuoha E, Mayedwa N, Ayayi RF. Green–synthesized zinc oxide nanoparticles mitigate salt stress in Sorghum bicolor. Agriculture. 2022;12:597.
26. Arnon IA. Copper enzymes in isolated chloroplasts polyphenoloxidase in Beta vulgaris. Plant Physiol. 1949;24:1-15.
27. Khare T, Desai D, Kumar V. Effect of MgCl2 stress on germination, plant growth, chlorophyll content, proline content and lipid peroxidation in sorghum cultivars. J. stress Physiol. Biochem. 2012;8(4):169-178.
28. Kumar D, Yusuf MA, Singh P, Sardar M, Sarin NB. Modulation of antioxidant machinery in alpha-tocopherol-enriched transgenic *Brassica juncea* plants tolerant to abiotic stress conditions. Protoplasma. 2013;250:1079-1089.
29. Junglee S, Urban L, Sallanon H, Lopez-Laurie F. Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. Am. J. Anal. Chem. 2014;5(11):730-736.
30. Niu L, Zhang H, Wu Z, Wang Y, Liu H, Wu X, et al. Modified TCA/acetone precipitation of plant proteins for proteomic analysis. PLoS One. 2018;13(12):e0202238.
31. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72:248-254.
32. Giannopolitis CN, Resi SK. Superoxide dismutase. Plant Physiol. 1977;59(2):309-314.
33. Ngara R, Ndimba R, Borch-Jensen J, Jensen ON, Ndimba B. Identification and profiling of salinity stress-responsive proteins in *Sorghum bicolor* seedlings. J. Proteom. 2012;75(13):13, 4139-4150.
34. Punia H, Tokas J, Bhadu S, Mohanty AK, Rawat P, Malik A Satpal. Proteome dynamics and transcriptome profiling in sorghum [*Sorghum bicolor* (L.) Moench] under salt stress. Biotechnol J. 2020;10:412.
35. Mulaudzi T, Hendricks K, Mabiya T, Muthevhuli M, Ajayi RF, Mayedwa N, et al. Calcium improves germination and growth of Sorghum bicolor seedlings under salt stress. Plants. 2020;9(6):730.
36. Zhuo Y, Zhang Y, Xie G, Xiong S. Effects of salt stress on biomass and ash composition of switchgrass (Panicum virgatum). Acta Agric. Scand.- B - Soil Plant Sci. 2015;65(4):300-309.
37. De Oliveira AB, Mendes Alencar NL, Gomes-Filho E. Comparison between the water and salt stress effects on plant growth and development. In: Akinci S, eds. Responses of Organisms to Water Stress. Intech: United States, 2013, 67-94.
38. Batyrshina Z, Yergaliyev TM, Nurbekova Z, Moldakimova NA, Masalimov ZK, Sagi M, et al. Differential influence of molybdenum and tungsten on the growth of barley seedlings and the activity of aldehyde oxidase under salinity. J. Plant Physiol. 2018;228:189-196.
39. Al-Issawi M, Rihan HZ, Al-Shmgani H, Fuller MP. Molybdenum application enhances antioxidant enzyme activity and COR15a protein expression under cold stress in wheat. J. Plant Interact. 2016;11(1):5-10.
40. Wu S, Sun X, Tan Q, Hu C. Molybdenum improves water uptake via extensive root morphology, aquaporin expressions and increased ionic concentrations in wheat under drought stress. Environ. Exp. Bot. 2016;157:241-249.
41. Han Z, Wei X, Wan D, He W, Wang X, Xiong Y. Effect of molybdenum on plant physiology and cadmium uptake and translocation in rape (Brassica napus L.) under different levels of cadmium stress. Int. J. Environ. Res. Pub. He. 2020;7(17):2355.
42. Imran M, Hussain S, El-Esawi MA, Rana MS, Saleem MH, Riaz M, et al. Molybdenum supply alleviates the cadmium toxicity in fragrant rice by modulating oxidative stress and antioxidant gene expression. Biomolecules. 2020;10(11):1582.
43. Hameed M, Ashraf M, Naz N. Anatomical adaptions to salinity in cogon grass [*Imperata cylindrica* (L.) Raeuschel] from the salt range, Pakistan. Plant Soil Environ. 2009;322:229-238.
44. Kutschera U. The growing outer epidermal wall: design and physiological role of a composite structure. Ann. Bot. 2008;101(5):615-621.
45. Savaldi-Goldstein S, Chory J. Growth coordination and the shoot epidermis. Curr. Opin. Plant Biol. 2008;11(11):42-48.
46. Hajibagheri MA, Hall JL, Flowers TJ. Stereological analysis of leaf cells of the halophyte *Suaeda maritima* (L.) dum. J. Exp. Bot. 1984;35(10):1547-1557.
47. Bray S, Reid DM. The effect of salinity and CO2 enrichment on the growth and anatomy of the second trifoliate leaf of Phaseolus vulgaris. Can. JB. 2002;80(4):349-359.
48. Mostafa H. Effects of salinity stress on growth,

chlorophyll content and osmotic components of two basil (*Ocimum basilicum* L.) genotypes. Afr. J. Biotechnol. 2011;11(2):379-384.
49. Siddiqui H, Hayat S, Bajguz A. Regulation of photosynthesis by brassinosteriods in plants. Acta Physiol. Plant, 2018, 40-59.
50. Ashraf MA, Ashraf M. Salt induced variation in some potential physiochemical attributes of two genetically diverse spring wheat (*Tricum aestivum* L.) cultivars: Photosynthesis and photosystem Π efficiency. Pak. J. Bot. 2012;44(1):53-64.
51. Foyer CH, Lelandais M, Kunert KJ. Photooxidative stress in plants. Physiol. Plant. 1994;92:696-717.
52. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 2002;7(9):405-410.
53. Hasanuzzaman M, Nahar K, Gill SS, Fujita M. Drought stress responses in plants, oxidative stress and antioxidant defense. In: Tuteja N, Gill SS, eds. Climate change and plant abiotic stress tolerance, 2014, 209-249.
54. Bambara S, Ndakidemi A. Effects of Rhizobium inocultion, lime and molybdenum on photosynthesis and chlorophyll content of Phaseolus vulgaris L. Afr. J. Microbiol. Res. 2019;3:791-798.
55. Munns R. Comparative physiology of salt and water stress. Plant Cell Environ. 2002;25(2):239-250.
56. Chun SC, Paramasivan M, Chandrasekaran M. Proline accumulation influenced by osmotic stress in arbuscular mycorrhizal symbiotic plants. Front. Microbiol. 2018;9:2525.
57. Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A. Role of proline under changing environments: a review. Plant Signal. Behav. 2012;7(11):1456-1466.
58. Sairam RK, Tyagi A. Physiology and molecular biology of salinity stress tolerance in plants. Curr. Sci. 2004;86:407-421.
59. Rahneshan Z, Nasibi F, Moghadam AA. Effects of salinity stress on some growth, physiology, biochemical parameters and nutrients in two pistachio (*Pistacia vera* L.) rootstocks. J. Plant Interact. 2018;13(1):73-82.
60. Kumar D, Al Hassan M, Naranjo MA, Agrawal V, Boscaiu M, Vicente O. Effects of salinity and drought on growth, ionic relations, compatible solutes and activation of antioxidant systems in oleander (*Nerium oleander* L.). PLoS One. 2017;12(9):e0185017.
61. Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. J. Bot, 2012, 1-26.
62. Asada K. The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. Annu. Rev. Plant Physiol. Plant Biol. 1999;50(1):601-639.
63. Wu S, Hu C, Tan Q, Nie Z, Sun X. Effects of molybdenum on water utilization, antioxidative defense system and osmotic-adjustment ability in winter wheat (*Triticum aestivum*) under drought stress. Plant Physiol. Biochem. 2014;83:365-374.
64. Al-Whaibi MH. Plant heat-shock proteins: A mini review. J. King Saud Univ. Sci. 2011;23(2):139-150.
65. Mayer MP, Bukau B. Hsp70 chaperon: Cellular functions and molecular mechanism. Cell. Mol. Life Sci. 2005;62:670-84.