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Antioxidant responses are associated with differences in drought tolerance between maize and sorghum.

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Abstract

Drought is a major cause of decreased yield in crops worldwide. Sorghum (*Sorghum bicolor*) and maize (*Zea mays*) are two of the key crops in Africa serving as human food as well as livestock feed. For improved crop production, selection for drought resilient genotypes is imperative and the biological basis for drought tolerance ought to be fully understood to achieve such selection. Sorghum can tolerate drought better than maize and it is a key model for studying the physiological and biochemical mechanisms conferring drought tolerance. In this study, comparative analyses in terms of changes in growth, chlorophyll content, ROS content, lipid peroxidation level and the activity of antioxidant enzymes were investigated. Exposure to drought triggered ROS generation in both plant species. However, sorghum showed less cell damage under water deficit compared to maize. Furthermore, differences in antioxidant enzyme activity between maize and sorghum were identified. Our findings reveal differences in and association between the physiological and biochemical responses of maize and sorghum to drought, which may be relevant for breeding drought tolerant crops.

1. INTRODUCTION

Beside the hazardous impacts on the environment, climate change is challenging socioeconomic development (He et al., 2019). Climate change and variability disturbs the basic elements of agriculture (soil moisture, heat and sunlight) as it leads to disparities in temperature, precipitation and the frequency of climate extremes such as drought (Raza et al., 2019). Drought is one of the biggest challenges affecting the globe. The lack of drought mitigation strategies and limited understanding of drought resilience, declined agricultural productivity, increases food shortages and global food insecurity (Iqbal et al., 2020). Exposure of plants to drought conditions induces oxidative stress and reduces stomatal conductivity, which results in decreased leaf internal carbon dioxide (CO2) and leads to over-formation of reactive oxygen species (ROS) (Xie et al., 2019). Oxidative damage via ROS generation imposes adverse changes in fundamental processes of plant growth and development, including seed germination, plant height, stem girth, leaf area, number of leaves, flowering and fruit production (Fahad et al., 2017). To counter ROS-triggered alterations, plants rely on ROS-scavenging systems which include enzymatic antioxidants such as superoxide dismutase (SOD), glutathione reductase (GR), peroxidase (POD), ascorbate peroxidase (APX), and catalase (CAT), or non-enzymatic antioxidants including carotenoids, ascorbic acid (AsA) and glutathione (GSH) (Ren

et al., 2020). The degree to which these defense systems are activated under drought stress notably varies among plant species, and even between different genotypes of the same plant species (Hasanuzzaman et al., 2020). Drought-induced oxidative stress causes lipid peroxidation as indicated by the increase in malondialdehyde (MDA) content. Thus, it is essential for plants to maintain greater levels of antioxidants in order to cope with drought stress (Laxa et al., 2019). Maize and sorghum are closely related species which are members of the Panicoideae subfamily in the family of Gramineae (Kellogg, 2001). Maize is the most important crop in the world (Li et al., 2016). In Africa, more than 300 million people depend on maize as the major staple (IITA, 1967), and is the main food crop for 50 % of the population in Sub-Saharan Africa (CGIAR, 2012). Under sufficient water supply, maize achieves high grain yield (Trout and DeJonge, 2017). However, maize is sensitive to drought stress (Meng et al., 2016). In contrast, sorghum is a drought resilient crop and the availability of its full genome sequence puts it in the lead as a key model system for deciphering the physiological and biochemical mechanisms of drought tolerance (Azzouz-Olden et al., 2020). Sorghum is the second major crop in Africa (Mundia et al., 2019). Besides being used as an animal feed, it is processed as traditional food such as semi-fermented bread, porridges, dumplings and couscous (Taylor and Belton, 2002). With the growing world population, it becomes crucial to improve and sustain crop performance under water limited conditions to ensure food security. Therefore, screening for drought adaptive responses through comparative studies in drought sensitive and drought tolerant crops is vital. It is on this basis that this study compared the physiological and antioxidant responses between maize and sorghum.

2. MATERIALS AND METHODS

2.1. Plant growth and drought treatment

Seeds of sorghum (*Sorghum bicolor* L.) Moench cv. Super Dan] and maize (*Zea mays* L.) cv. Border King] were surface sterilized and then imbibed in aerated 10 mM calcium sulphate for 16 hours prior to germination. Germinated seeds were sown in Perspex tubes (100 x 10 cm), filled with 18 L of Promix Organic (Windell Hydroponics, South Africa) saturated with water containing 1% fertilizer (v/v) [Nitrosol®, Envirogreen (Pty) Ltd)]. Seedlings were grown until the V1 stage of development by irrigating the tubes every two days with 500 ml of water. The plants were grown in controlled greenhouse under 16/8 h day/night cycle with average temperature of 25˚C. The photosynthetic photon flux density was 400 $\mu mol.m^{-2}.s^{-1}$. At the V1 stage, control plants continued to receive 500 ml of water every second day until the day of harvest (V8 growth stage), while water-deprived plants were provided with 20% (100 ml) of that amount of water on a weekly basis until the V3 stage of development. Thereafter, the water-deficit plants were subjected to complete water deprivation until the symptoms of drought stress (leaf rolling or oldest leaves turning brown) were observed. At this point, the growth parameters of control and water-deprived plants were measured and the four youngest leaves were harvested. The harvested leaves were immediately frozen and ground into a fine powder using liquid nitrogen and then stored at -80°C. Cell viability and O˙2 were determined using fresh leaves.

2.2. Trichloroacetic Acid (TCA) Extraction

A protein-free extract was obtained by homogenizing 5 volumes of 6% trichloroacetic acid (TCA) with 100 mg of powdered plant material. The homogenate was then centrifuged at 13000 x g for 15 minutes at 4˚C and the supernatant was used for measuring the MDA and H2O2 content.

2.3. Determination of lipid peroxidation degree

Lipid peroxidation was determined by the method of Dhindsa et al. (1981). To a 200 µl aliquot of TCA extract, 400 µl of a solution containing 0.5% thiobarbituric acid (TBA) and 20% TCA was added. The mixture was boiled at 90 ˚C for 20 minutes and then cooled on ice. After centrifugation at 12 000 x g for 5 minutes, 200 µl of supernatant was transferred into a microtiter plate and the absorbance was recorded at 532 nm and 600 nm using a POLARstar Omega microtiter plate reader (BMG Labtech, Offenburg, Germany). The data obtained were used to calculate the MDA content based on the extinction coefficient of 1.56 x 105 cm-1M-1.

2.4. Evaluation of Cell Viability

Cell viability was measured according to the method of Sanevas et al. (2007). A 1 cm2 cutting

from the second youngest leaf was stained in 0.25% Evans Blue in the dark for 30 minutes at room temperature. The leaf material was then transferred into an Eppendorf tube filled with distilled water and incubated overnight at room temperature to eliminate any free dye. After the water was discarded, Evans Blue was extracted by incubating the samples in 1.5 ml of 1% sodium dodecyl sulfate (SDS) at 65°C for 1 hour. Samples were then centrifuged at 13000 x g for 5 minutes and the absorbance of the supernatant was measured at 600 nm on a POLARstar Omega microtiter plate reader (BMG Labtech, Offenburg, Germany).

2.5. Reactive oxygen species (ROS) measurements

2.5.1. Hydrogen peroxide content

A 50 µl aliquot of TCA extract was mixed with 100 µl potassium iodide (KI) and 50 µl of potassium phosphate (K2HPO4), pH 5.0, to a total volume of 200 µl. A standard curve with known concentrations of H2O2 was prepared by mixing 10 µM H2O2 with varying volumes of dH2O, 0.5 M KI and 20 mM K2HPO4, to give a total volume of 200 µl. The absorbance was recorded at 390 nm and the extinction coefficient 39.4 mM-1.cm-1 was used to calculate the concentration of hydrogen peroxide in the sample.

2.5.2 Superoxide

Superoxide content was quantified according to Able et al. (1998), with modifications. A leaf disc (1 cm2) and a 4 cm cutting of the root (from the tip) collected from fresh plant tissue were incubated in a 50 mM potassium phosphate buffer (KPO4) containing 10 mM potassium cyanide (KCN), 10 mM H2O2, 2% sodium dodecyl sulphate (SDS), 80 µM Thiazolyl Blue Tetrazolium Blue (MTT), for 20 minutes at room temperature. The plant tissue was then crushed with a small pestle and centrifuged at 13000 x g for 10 minutes. A 200 µl aliquot of the obtained supernatant was loaded onto a microtiter plate. Absorbance was recorded at 570 nm and the extinction coefficient 13.0 mM−1 cm−1 for the MTT was used to calculate super oxide content.

2.6. Total Protein Extraction and quantification

A 200 mg sample of plant tissue was homogenized in 400 µl of protein extraction buffer [40 mM phosphate buffer at pH 7.4; 1 mM ethylenediaminetetraacetic acid (EDTA) and 5% (w/v) polyvinylpolypyrrolidone (PVPP)]. The homogenate was centrifuged at 13000 x g for 20 minutes at 4˚C and the supernatant was used as enzyme source for APX, CAT, SOD and GR assays. The concentration of protein in the extracts was estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.7. Assays of antioxidant enzyme activities

For native in-gel assays, the staining of all enzymes was done using non-denaturing discontinuous gel electrophoresis formed of 15 % separating gel [except for CAT (in which case the separating gel was a 7.5 % gel)] and 5 % stacking gel. The gels were electrophoresed with a running buffer containing 25 mM Tris, 192 mM glycine, and additionally (in the case of APX) 2 mM ascorbic acid (AsA) at 4˚C and 80 mV. All gels were run until the loading dye reached the bottom edge of the gel except for CAT (the gel was allowed to run for 2 hours after the dye ran off the gel to allow for good separation of isoforms).

2.7.1. Assay of APX activity

Following electrophoretic separation, the ascorbate peroxidase gels were stained according to Seckin et al. (2010) in order to determine the activity of each of the APX isoforms in maize and sorghum. The ascorbate peroxidase total activity was determined by the method of Nakano and Asada (1981). APX activity was estimated by measuring the decrease in absorbance at 290 nm as ascorbate was oxidised and using the extinction coefficient of 2.8 mM-1 cm-1 for ascorbate. One unit of APX activity was defined as the amount of protein required to hydrolyse 1 µmole of H2O2/minute.

2.7.2. Assay of CAT activity

The staining of catalase gels was performed as described by Yamashita et al. (2007). The catalase total activity was measured by following the consumption of hydrogen peroxide as described by Lück (1965). The calculations were done by monitoring the decrease in absorbance at 240 nm (ε = 39.4 mM-1 cm-1), one unit of enzyme is the amount necessary to convert 1 µmole of H2O2/ minute.

2.7.3. Assay of SOD activity

Superoxide dismutase (SOD) activity staining was assayed according to Beauchamp and Fridovich (1971). One unit of SOD activity represented the amount of enzyme required to inhibit 50 % of NBT.

2.7.4. Assay of GR activity

Following native PAGE separation, Glutathione reductase (GR) activity staining was done as reported by Rao et al. (1996). The total activity of GR was estimated by the method of Foyer and Halliwell (1976). GR activity was expressed as µmol of NADPH oxidized/minute per mg of protein.

2.8 Statistical analysis

Data generated were subjected to a one-way analysis of variance (ANOVA) and significance was determined by the Tukey-Kramer test at a 5% level of significance using GraphPad Prism 6.0.

3. RESULTS AND DISCUSSION

3.1. Reduction in growth under drought is more severe in maize than in sorghum

Exposure of crops to drought can adversely affect their growth and yield (Hussain et al., 2019). Under drought stress, maize suffered a greater reduction of shoot length (approximately 29 %) than sorghum which showed only 16 % decrease in shoot length (Fig. 1. a). Both maize and sorghum exhibited a significant increase in their root length under drought as shown in Fig. 1. b. However, the increase in root length was even greater in sorghum (approximately 44 %) than in maize (approximately 14 %), similarly to the study reported by Wright et al. (1983). Drought decreased the shoot fresh weight in both maize and sorghum by approximately 84 % and 77 % respectively (Fig.1. c). The longer root length in sorghum is likely an adaptive response in order to reach deeper soil layers to acquire moisture.

3.2. Oxidative stress is higher in maize than sorghum under water deprivation

Fig. 2 illustrates hydrogen peroxide content, superoxide content and the level of lipid peroxidation (MDA content) in maize and sorghum. Drought stress increased the acummulation of H_2O_2 in the leaves of maize and sorghum, while no marked changes in H_2O_2 content were observed in the roots of both species (Fig. 2. a and b). However, maize plants showed a much higher increase in H_2O_2 content, as it increased by approximately 70%, than the approximately 55% increase in H_2O_2 content observed in sorghum. Under drought stress, $O_2^{\cdot-}$ content was increased by approximately 38.6%

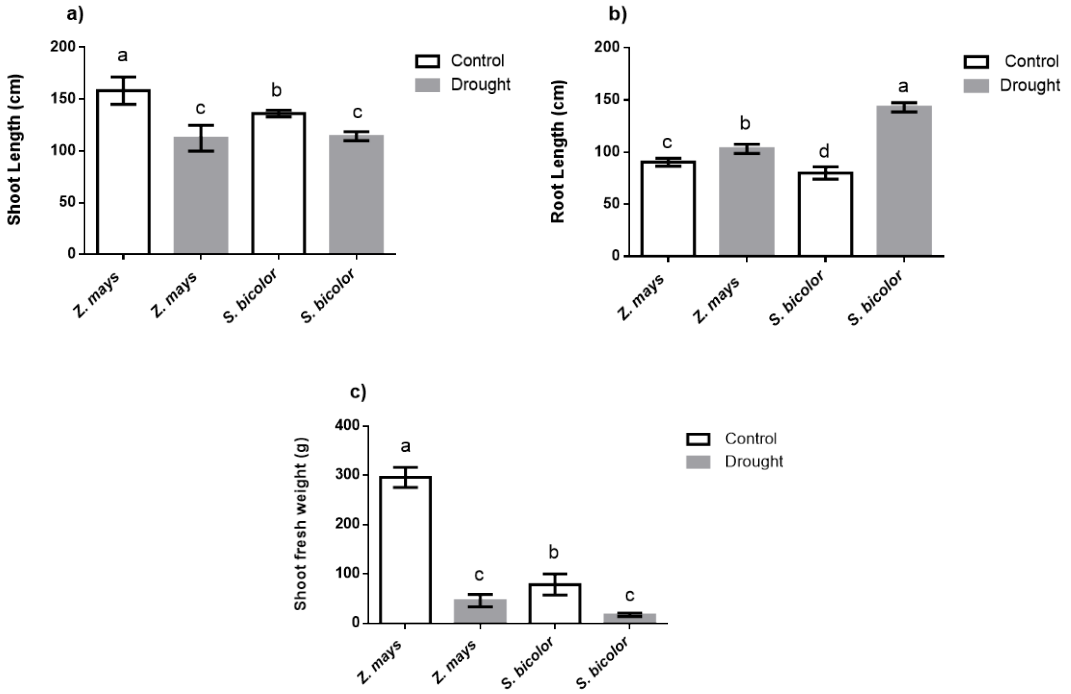

Fig. 1. The effect of drought stress on shoot length (a), root length (b) and shoot fresh weight (c) in *Zea mays* and *Sorghum bicolor*. The stress parameters of well-watered and water-deprived plants were determined at V8 stage of growth. Values are means ± SE of ten plants, $P \leq 0.05$.

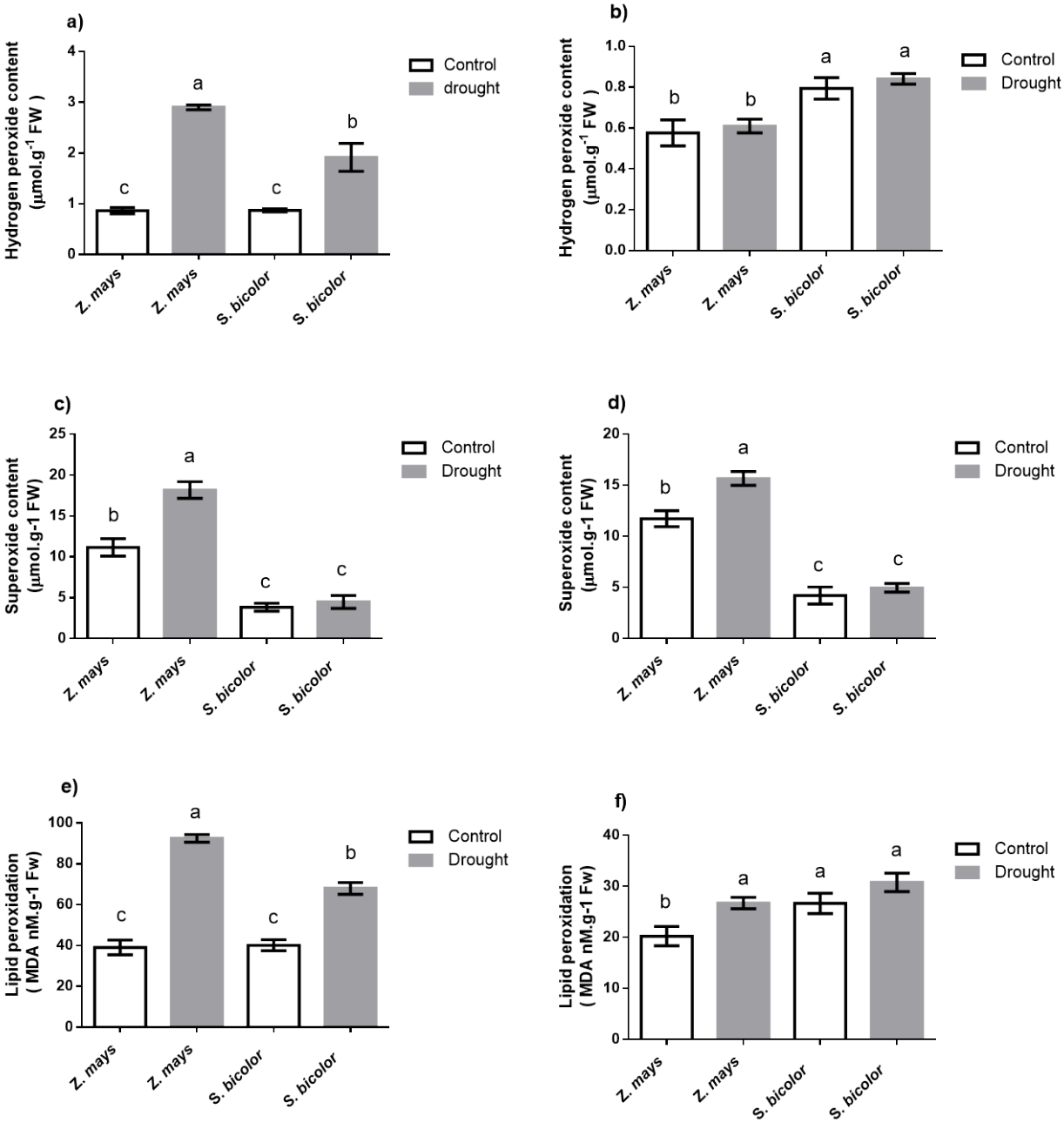

Fig. 2. The degree of oxidative stress exhibited in *Zea mays* and *Sorghum bicolor* under well-watered and water-deprived conditions. Hydrogen peroxide (H2O2) content in leaves (a) and roots (b), and O2-˙ in leaves (c) and roots (d) were measured as the indication of ROS accumulation; and lipid peroxidation in leaves (e) and roots (f) was measured as an indication of oxidative stress. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P \leq 0.05$.

in the leaves of maize, whereas no significant change in $O_2^{-\cdot}$ content was observed in sorghum (Fig. 2. c). Similarly, an increase of approximately 25% in $O_2^{-\cdot}$ content in the roots occurred in water-deprived maize, whereas no significant difference in $O_2^{-\cdot}$ was observed in sorghum (Fig. 2. d). Drought increased MDA content by approximately 58% in maize leaves and by approximately 41% in sorghum leaves (Fig. 2. e). In the roots (Fig. 2. f), maize plants showed a 70% increase in MDA content in response to drought while sorghum plants showed a 55% increase in MDA content.

The above results show that maize suffers greater oxidative damage than sorghum under the same drought conditions. The drought-

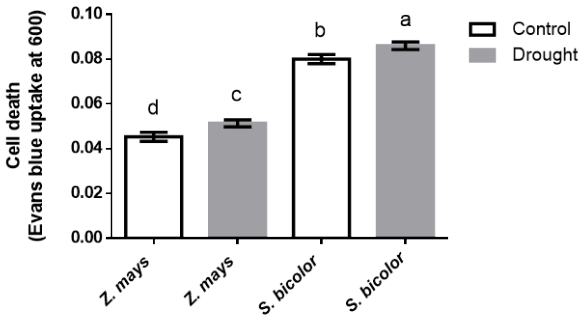

Fig. 3. The effect of drought stress on cell viability in *Zea mays* and *Sorghum bicolor*. Data presented are means (±SE) of three independent experiments (n=3). Different letters above error bars denote mean values that are significantly different at $P \leq 0.05$.

induced accumulation of H_2O_2 and $O_2^{-\cdot}$ content demonstrated here are the cause of the higher lipid peroxidation and the differences in their accumulation between maize and sorghum partly explain the differing degree of tolerance to drought stress between these two species.

Oxidative stress occurs when the critical balance between ROS formation and ROS scavenging by antioxidant enzymes is disturbed due to decreased antioxidant enzyme activity or excess formation of ROS, or both (Poljsak et al., 2013). This excessive ROS accumulation is caused by a wide range of environmental stresses, including drought (Cruz de Carvalho, 2008).

3.3. Drought stress induces cell death in maize and sorghum

Drought stress affects photosynthesis and enhances photorespiration, disrupts normal cell homeostasis and leads to increased formation of reactive oxygen species (ROS) (Miller et al., 2010). The excess ROS may cause severe oxidative stress and ultimately cell death (Sharma et al., 2012). However, an effective ROS scavenging system and low levels of cell death under drought conditions are associated with plant tolerance to drought stress (Laxa et al., 2019).

Fig. 3. shows the level of cell death as determined by Evans Blue uptake. Under drought, maize showed nearly 12 % increase in the level of cell death, while cell death in sorghum was approximately 7 % higher when compared to their relative controls. The more pronounced increase of cell death in maize could be attributed to the higher oxidative stress in maize compared to sorghum as a result of excessive ROS accumulation under drought conditions (Fig. 2. e and f), similar findings were described for salinity stress by Keyster et al. (2013).

3.4 Drought stress significantly alters the activity of antioxidant enzymes

During ROS-triggered oxidative stress, plants regulate their antioxidant defense system to scavenge ROS, thus avoiding excessive ROS accumulation and contributing to plant survival

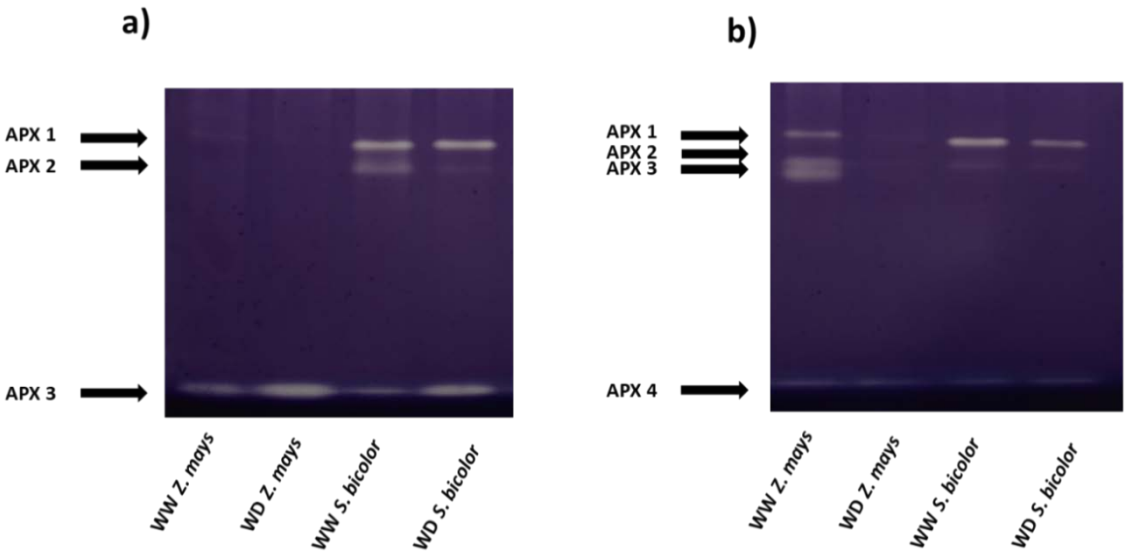

Fig. 4. Changes in ascorbate peroxidase (APX) isoforms in response to drought stress in the leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor*. WW represents well-watered, WD represents water-deprived. White bands in dark-blue background signify isoforms of APX.

(Huang et al., 2019). Based on APX native PAGE activity staining, four isoforms were detected in maize while only three isoforms were identified in sorghum (Fig. 4.). As a result of exposure to drought, ascorbate peroxidase isoform activity was differentially regulated between maize and sorghum. Interestingly, drought increased APX total activity by approximately 38% in the leaves of maize and by approximately 19% in the leaves of sorghum when compared to their respective controls (Fig. 5. a). However, in response to drought, APX total activity was decreased by approximately 73% in the roots of maize, whereas it remained unchanged in sorghum (Fig. 5. b). The increase in APX activity could be attributed to efforts to avoid oxidative damage caused by the excessive generation of H2O2. Similar findings were reported by Chugh et al. (2011), who observed a significant increase in APX activity in maize when exposed to water deficit stress. The greater increase of APX activity in the leaves of maize could be a result of the higher increase of H2O2 content in maize than in sorghum under drought.

According to the native PAGE analysis, three CAT isozymes were detected in the leaves of maize and sorghum. In the roots, only one CAT isoform was identified in maize, while two CAT isoforms were detected in sorghum. The activity of all CAT isoforms was significantly attenuated in both maize and sorghum in response to drought stress (Fig. 6.). In response to drought, the total CAT activity was decreased by approximately 45% in the leaves of maize and by approximately 33% in the leaves of sorghum (Fig. 7. a). Similarly, an approximately 41% and 66% reduction in CAT activity was observed in the roots of maize and sorghum plants, respectively, under water deprivation (Fig. 7. b). The results obtained above are in agreement with a study done by Bakalova et al. (2004) in wheat and Pan et al. (2006) in liquorice. Decreased catalase activity could be the reason for the observed increase in H2O2 content in response to drought.

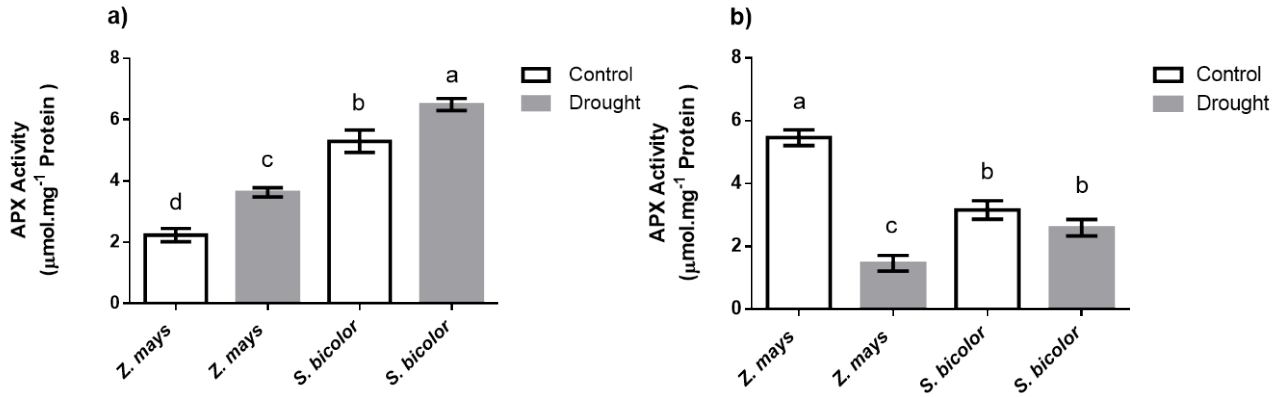

Fig. 5. Determination of the total APX activity in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* by spectrophotometry. Error bars represent the means ± SE; n= 3. Different letters indicate statistically

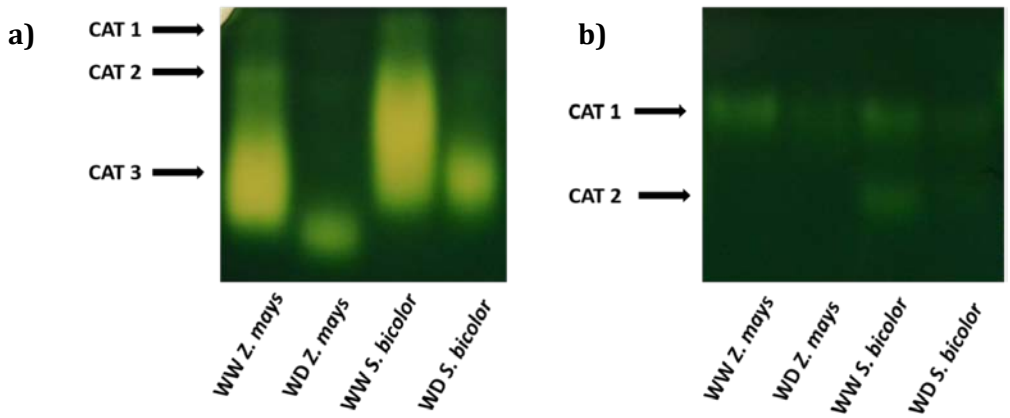

Fig. 6. Catalase isoforms activity was decreased under water stress in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor*. WW represents tissue obtained from well-watered plants, WD represents tissue obtained from water-deprived plants.

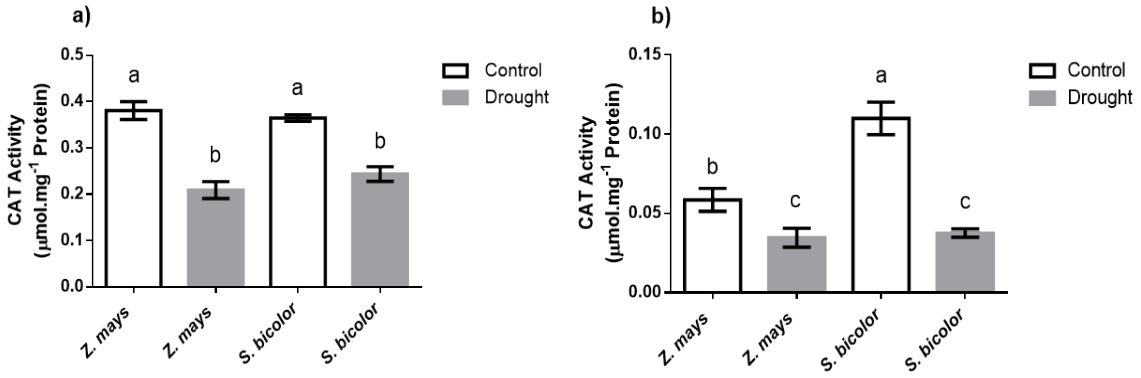

Fig. 7. Determination of total catalase activity in leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor* by a spectrophotometry assay. Error bars represent the means ± SE; n = 3. Different letters indicate statistically different means ($P \leq 0.05$).

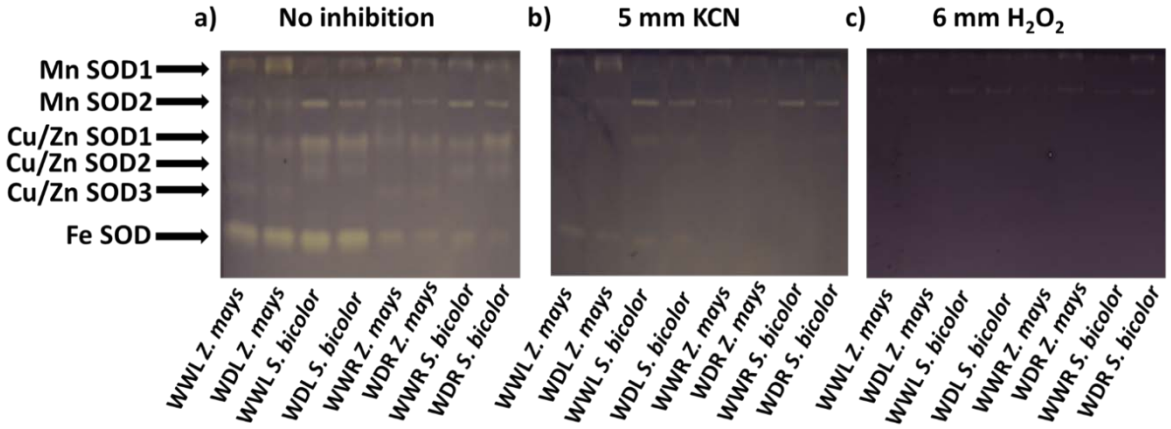

Fig. 8. Changes in superoxide dismutase (SOD) isoenzyme activities in native gels in leaves and roots of *Zea mays* and *Sorghum bicolor* in response to drought stress. The three in-gel assays represent the detection of different SOD isoforms (a) without inhibitors, (b) treated with 5 mM KCN and (c) treated with 6 mM H_2O_2. WW represents tissue from well-watered plants, WD represents tissue from water-deprived plants, L represents leaves and R represents the roots.

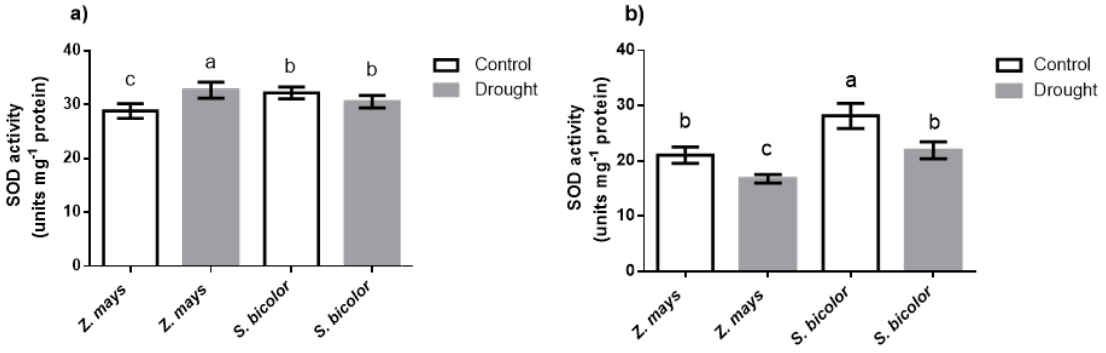

Fig. 9. Spectrophotometric determination of total SOD activity in the leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor.* Error bars represent the means ± SE; n= 3. Different letters indicate significant differences between means at $P \leq 0.05$.

Based on the sensitivity to selective SOD inhibitors (5 mM KCN and 6 mM H2O2), a total of six SOD isozymes were identified in maize and sorghum (Fig. 8.). Two isoforms were identified as manganese superoxide dismutase (MnSOD), three as copper/zinc superoxide dismutase (Cu/Zn SOD) and one as iron superoxide dismutase (Fe-SOD). As shown in Fig. 9. a, overall SOD activity slightly increased (by approximately 12%) in the leaves of water-deprived maize plants, while no significant change was observed in the leaves of water-deprived sorghum plants. The upregulation of SOD activity in maize is likely a response to the higher $O2^{-\cdot}$ accumulation in maize than in sorghum in response to drought, similarly to work described by Luna et al. (1985), where higher SOD activity was observed in maize than wheat when subjected to water deprivation. Drought decreased total SOD activity in the roots of both maize and sorghum (by 20% and 22%, respectively) (Fig. 9. b). The downregulation of SOD activity in maize roots is likely the underlying reason for the elevation of $O2^{-\cdot}$ content.

Although the SOD activity was also inhibited in sorghum roots by drought, no significant difference in $O2^{-\cdot}$ content was observed, suggesting that sorghum has superior SOD capacity than maize.

Glutathione reductase enzyme (GR) is essential for restoration of the reduced glutathione (GSH) pool by converting the oxidised glutathione disulphide (GSSG) back into the sulfhydryl form [GSH donates an electron to dehydroascorbate reductase (DHAR), which

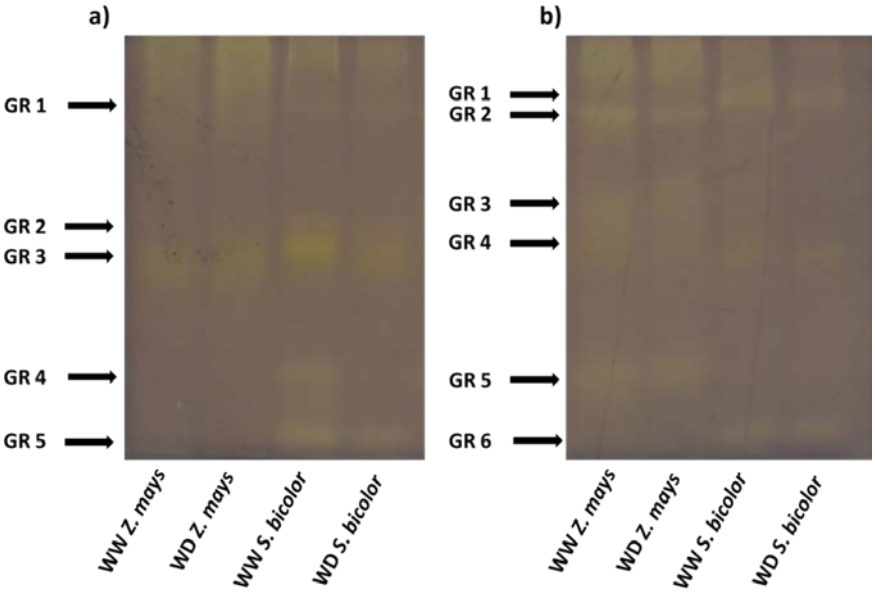

Fig. 10. Changes in glutathione reductase (GR) isozyme activity in native gels in leaves and roots of *Zea mays* and *Sorghum bicolor* in response to drought stress. WW represents well-watered plants, WD represents water-deprived plants, L represents the leaves and R represents the roots.

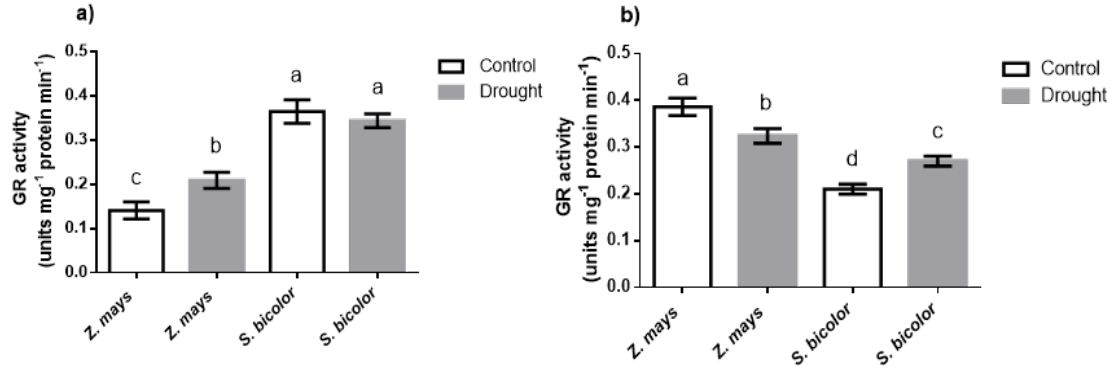

Fig. 11. Spectrophotometric determination of total GR activity in the leaves (a) and roots (b) of *Zea mays* and *Sorghum bicolor.* Error bars represent the means ± SE; n= 3. Different letters indicate significant differences between means at $P \leq 0.05$.

reduces dehydroascorbic acid (DHA) to ascorbate] (Meloni et al., 2003). Based on native PAGE gel analysis, a total of six GR isozymes are present in maize and sorghum. Drought stress significantly altered the glutathione reductase isoform activity between maize and sorghum as shown in Fig. 10. The overall activity of GR increased by approximately 33% in the leaves of maize under drought stress, whereas no statistically significant difference was observed in sorghum in response to drought (Fig. 11. a). The increase in GR activity observed in the leaves of maize is likely due to the upregulation of APX activity in the leaves of maize as the cells may be driving GR activity for reformation of both GSH and ascorbate. GR total activity in the roots of maize was decreased by approximately 16% in response to drought, while the GR activity was significantly higher (by approximately 22%) in sorghum roots (Fig. 11. b). The reduction in GR activity in the roots of maize could similarly be attributed to the downregulation of APX activity in the roots of maize as the cells may have less need for driving GR activity for reformation of both GSH and ascorbate.

4. CONCLUSION

Drought stress significantly inhibited the growth of maize and induced ROS, lipid peroxidation and cell death. In contrary, sorghum exhibited less effects on growth and oxidative stress in response to drought, which may be due to differing levels of antioxidant enzyme activity in these plant species. From our results, it appears that adaptive responses in enzymatic antioxidants in maize were less sufficient than in sorghum to prevent oxidative damage caused by drought stress. This may provide the foundation for further elucidating the differences in molecular events that determine differences in drought tolerance between maize and sorghum, likely through omics-based analyses that could assist in improvement of maize drought tolerance. This can be achieved if regulation of the expression of genes discovered to be key to sorghum tolerance can be manipulated in maize to emulate the expression profile of sorghum.

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