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Drought and salinity stress alters ROS accumulation, water retention, and osmolyte content in sorghum plants

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ABSTRACT

Abiotic stresses such as drought and salinity are major environmental factors that influence crop productivity worldwide. These adverse conditions induce osmotic stresses in plant cells by decreasing water availability, thus leading to loss of cell turgor and the accumulation of reactive oxygen species (ROS) that are detrimental to plant growth and development. To survive such harsh environmental conditions, plants must initiate intracellular and physiological signaling networks to rapidly respond and efficiently neutralize these stresses. Inefficient scavenging of ROS would lead to increased levels of cell death, thus inhibiting plant growth and reducing crop productivity. This study investigates the effect of drought and salinity stress on plant growth, water retention, oxidative damage, chlorophyll content, and proline accumulation in sorghum plants. Plant growth, biomass, and leaf chlorophyll were significantly reduced whereas the total proline content was enhanced in response to stress conditions. The significant increase in hydrogen peroxide content as a consequence of stress conditions resulted in augmented levels of lipid peroxidation, which was manifested as extensive cell death and biomass reduction.

1. Introduction

Drought and salinity are major environmental factors that influence crop productivity worldwide. These abiotic stress conditions result in biochemical changes such as the accumulation of ROS that are by-products of cellular metabolism (Pan et al., 2006). These reactive species include superoxide (O_2^-), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2) that are thought to play an important role in inhibiting plant growth and development if not carefully regulated. To avoid cellular damage due to ROS accumulation, plants produce a number of compatible solutes, non-enzymatic and enzymatic antioxidant constituents that are induced to provide secondary protection against oxidative stress (Mittova et al., 2003). Drought and salinity stress have been shown to trigger various interacting events including the inhibition of enzyme activities in metabolic pathways (Binzel and Reuveni, 1994; Tsugane et al., 1999). However, the complete response of plants to abiotic stress conditions such as drought and salinity has not been systematically elucidated.

Compatible solutes, such as proline, are known to accumulate under abiotic stress conditions in many plant species (Krasensky and Jonak, 2012; Hayat et al., 2012). They have been shown to play a major role in osmotic adjustment in crops such as potato (Büssis and Heineke, 1998), whereas in others like tomato (Pérez-Alfocea et al., 1993), it accounts for only a small fraction of the total concentration of osmotically active solutes. There is still some controversy surrounding the contribution of proline to osmotic adjustment and tolerance of plants that are exposed to unfavourable environmental conditions (Molinari et al., 2007).

Sorghum (*Sorghum bicolor L. Moench.*) is a drought-tolerant crop with high resistance to saline–alkaline soils and may serve as an alternative summer crop for biofuel production in areas where irrigation water is limited. Sweet sorghum is characterized by high sugar content, mainly sucrose, fructose, and glucose, in the juice of the stalks, from which ethanol can be easily produced and used as biofuel. For this reason, sweet sorghum has also become a popular energy plant throughout the world (Mastrorilli et al., 1999). Despite the potential of sweet sorghum as an alternative energy crop (Smith and Buxton, 1993; Steduto et al., 1997), the ability of various sweet sorghum cultivars including Sugargraze (a sweet sorghum hybrid) to grow under soil salinity and water deficiency conditions has not been sufficiently elucidated.

Although several biochemical and physiological changes have been shown to be involved in abiotic stress acclimation process in various grass species, little is known about the responses of sweet sorghum plants to ROS accumulation that leads to oxidative damage and ultimate cell death. In order to clarify the plant's response to drought and salinity stress, we describe the changes in plant growth, ROS accumulation,

Abbreviations: ANOVA, Analysis of variance; DMSO, Dimethylsulfoxide; DW, Dry weights; FW, Fresh weights; H_2O_2, Hydrogen peroxide; MDA, Malondealdehyde; ROS, Reactive oxygen species; RWC, Relative water content; TCA, Trichloroacetic acid.

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oxidative damage coupled with physiological and biochemical changes, and the extent of cell death.

2. Materials and methods

2.1. Plant growth, treatments, and experimental design

Seeds of Sorghum (*Sorghum bicolor*) cv Sugargraze (purchased from Agricol Pty Ltd., Brackenfell, South Africa) were surface-sterilized in 0.35% sodium hypochlorite for 10 min and rinsed with distilled water. Seeds were germinated (four seeds per pot) in 20 cm plastic pots containing a mixture of double grow potting soil and double grow weed-free compost in a volume ratio of 1:1. The field capacity (100%) of each pot was determined by saturating the soil with distilled water. All pots were covered in plastic sheets and allowed to drain for 48 h. Pot weights were recoded after 48 h of drainage and the soil were dried for 24 h at 105 °C. The soil moisture content at field capacity was calculated as the difference between the soil weight after drainage and soil weight after drying. Germinated seedlings were well watered (maintained at field capacity) and grown on a 25/19 °C day/night temperature cycle under a 16/8 h light/dark cycle, at a photosynthetic photon flux density of 300 μmol photons.m^{-2}.s^{-1} during the day phase until they reached the V3 stage (four fully expanded leaves and one emerging leaf) of vegetative growth. Plants were grown in a completely randomized design to eliminate the effect of variations in environmental conditions at different positions in the growth room. Plants at the same developmental stage and of similar height were selected for all experiments.

For treatment with NaCl to impose salinity stress, plants were supplied with ±100 ml distilled water (to maintain field capacity) containing NaCl at a final concentration of 100 mM (at intervals of 3 days between each treatment) for a period of 16 days. Control plants were treated in a similar manner except that distilled water without NaCl was supplied. Drought stress was imposed (at the V3 stage) by allowing plants to progressively dry by withholding water for a period of 16 days.

2.2. Growth analysis

Growth analysis was performed on sorghum plants by measuring root length, shoot length, leaf fresh weight (FW), and dry weight (DW). The DW was determined by drying leaf tissue in an oven at 60 °C for 48 h as described by Valentovic et al. (2006).

Relative water content (RWC) of leaf tissue was measured as described by Sumithra et al. (2006) using the following formula:

$$RWC(\%) = [(FW\text{-}DW)/FW] \times 100$$

2.3. Leaf chlorophyll content

Chlorophyll content was estimated based on a modified method described by Hiscox and Israelstam (1979). Freshly harvested leaf tissue was cut into pieces of ±0.5 cm^{-2} in size. Leaf tissue (100 mg per plant) was mixed with 5 ml of dimethylsulfoxide (DMSO) and incubated at 65 °C for 3 h. The absorbance of an aliquot of the leaf-DMSO extract (200 μl) was recorded at 645 nm and 663 nm, with DMSO used as a blank.

2.4. Total free proline content

Total free proline content was estimated using a modified method described by Khare et al. (2012). Fresh leaf samples from each treatment (0.1 g each) were homogenized in 0.5 ml of 3% (*w*/*v*) sulphosalicylic acid using a mortar and pestle. About 0.2 ml of each homogenate was mixed with 0.2 ml of glacial acetic acid to which 0.2 ml of ninhydrin was added. The reaction mixture was boiled in a water bath at 100 °C for 30 min and immediately cooled in an ice bath. After cooling, 0.4 ml of toluene was added to the reaction mixture. After thorough mixing, the chromophore containing toluene was separated and absorbance of red color developed was read at 520 nm against toluene blank on UV–visible spectrophotometer (ChemitoSpectrascan, UV 2600).

2.5. Protein isolation

Plant extracts for biochemical assays were obtained by grinding leaf tissue (0.4 g) into a fine powder in liquid nitrogen. The tissue was homogenized in 1 ml of 10% trichloroacetic acid (TCA. The homogenate was centrifuged at 12,000 × *g* for 15 min and used to determine hydrogen peroxide and malondialdehyde (MDA) content. Protein concentrations for all assays were measured in extracts derived from homogenizing buffer as described by the manufacturer for the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories, Inc., Hercules, CA).

2.6. Hydrogen peroxide content

Hydrogen peroxide content was determined based on a method previously described by Velikova et al. (2000). The reaction mixture contained 75 μl of the TCA extract, 5 mM K_2HPO_4, pH 5.0 and 0.5 M KI. Samples were incubated at 25 °C for 20 min and absorbance readings of the samples were recorded at 390 nm. Hydrogen peroxide content was calculated using a standard curve based on the absorbance ($A_{390\ nm}$) of H_2O_2 standards.

2.7. Lipid peroxidation

Products of lipid peroxidation (reflective of MDA content) were estimated using the thiobarbituric acid (TBA) method as described by Buege and Aust (1978). For these measurements, 1 ml of TCA extract was mixed with 4 ml of 0.5% TBA (prepared in 20% TCA). The mixture was incubated at 95 °C for 30 min and cooled in ice for 10 min. The specific absorbance of products was recorded at 532 nm and nonspecific background-absorbance at 600 nm was subtracted from the readings. The concentration of MDA was calculated using a molar extinction coefficient 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} of fresh weight.

2.8. Evaluation of cell viability

Cell viability was measured in the leaves and roots of sorghum plants using a modified method described by Sanevas et al. (2007). Fresh leaf and root tissue (0.1 g) from sorghum plants was harvested and stained with 0.25% (*w*/*v*) Evans Blue dye for 15 min at room temperature. The leaves and roots were washed for 45 min in distilled water to remove surface-bound dye, followed by extraction of the Evans Blue stain (taken up by dead cells) from leaf and root tissue using 1% (*w*/*v*) SDS, after incubation for 1 h at 55 °C. Absorbance of the extracts was measured at 600 nm to determine the level of Evans Blue uptake by the leaf and root tissue.

2.9. Statistical analysis

All experiments described were performed three times independently, with measurements taken from ten (plant growth measurements) or five (for all other experiments) different plants for each treatment in each of the three independent experiments. For statistical analysis, one-way analysis of variance (ANOVA) test was used for all data, and means (for three independent experiments) were compared according to the Tukey–Kramer test at 5% level of significance, using GraphPad Prism 5.03 software.

3. Results

3.1. Plant growth and development

Plant growth and development was significantly influenced by the stress conditions as manifested by a reduction in shoot and root length including leaf fresh and dry weights (Fig. 1A–D). Leaf fresh was reduced by ±44% as a consequence of drought-induced stress whereas salinity stress contributed to a 47% reduction in leaf fresh weight (Fig. 1A). A similar phenomenon was observed for leaf dry weight where drought and salinity reduced leaf dry weights 57% and 60%, respectively (Fig. 1B).

Shoot length was reduced by ±32% when plants were exposed to drought stress, while salinity stress reduced shoot length by ±36% (Fig. 1C). The reduction in root length was not as pronounced as for the shoot length albeit significant. Drought stress reduced root length by ±13%, whereas salinity stress had an even more adverse effect on root growth by reducing root length by ±17% when compared to untreated plants (Fig. 1D).

3.2. Effects of drought and salinity stress on water retention, osmolyte accumulation, and chlorophyll content

Long-term exposure of sorghum plants to drought and salinity stress condition significantly altered relative water content (RWC) and proline accumulation. Relative water content was reduced by ±23% in response to drought and salinity treatments (Fig. 2). Total proline content in the same treatments was significantly enhanced compared to the control plants. An increase of 37% was observed in the drought-stressed plants, whereas an even higher increase of 63% was observed in response to salinity stress (Fig. 3). Photosynthetic pigmentation was significantly reduced by drought and salinity treatments as manifested by the reduction of 28% and 65%, respectively, in total chlorophyll content compared to the control plants (Fig. 4).

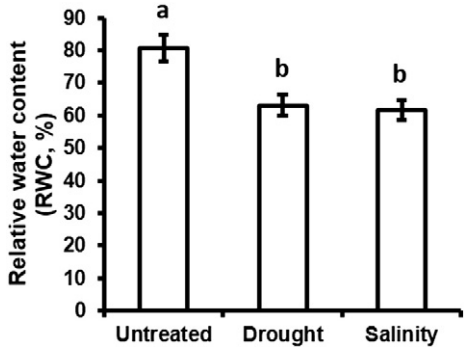

Fig. 2. The effect of drought and salinity treatments on relative water content in sorghum plants. Different letters on bars indicate statistically different means ($P < 0.05$).

3.3. Hydrogen peroxide content

Hydrogen peroxide content in the leaves and roots of sorghum plants were significantly higher in response to drought and salinity stress compared to untreated controls (Fig. 5A). Leaf H_2O_2 content was 2-fold higher than the roots H_2O_2 content in response to drought and salinity stress. An increase of 113% in H_2O_2 content was measured in the leaves of drought-stressed plants, whereas an even higher increase of 149% was observed in the salinity treatment (Fig. 5A).

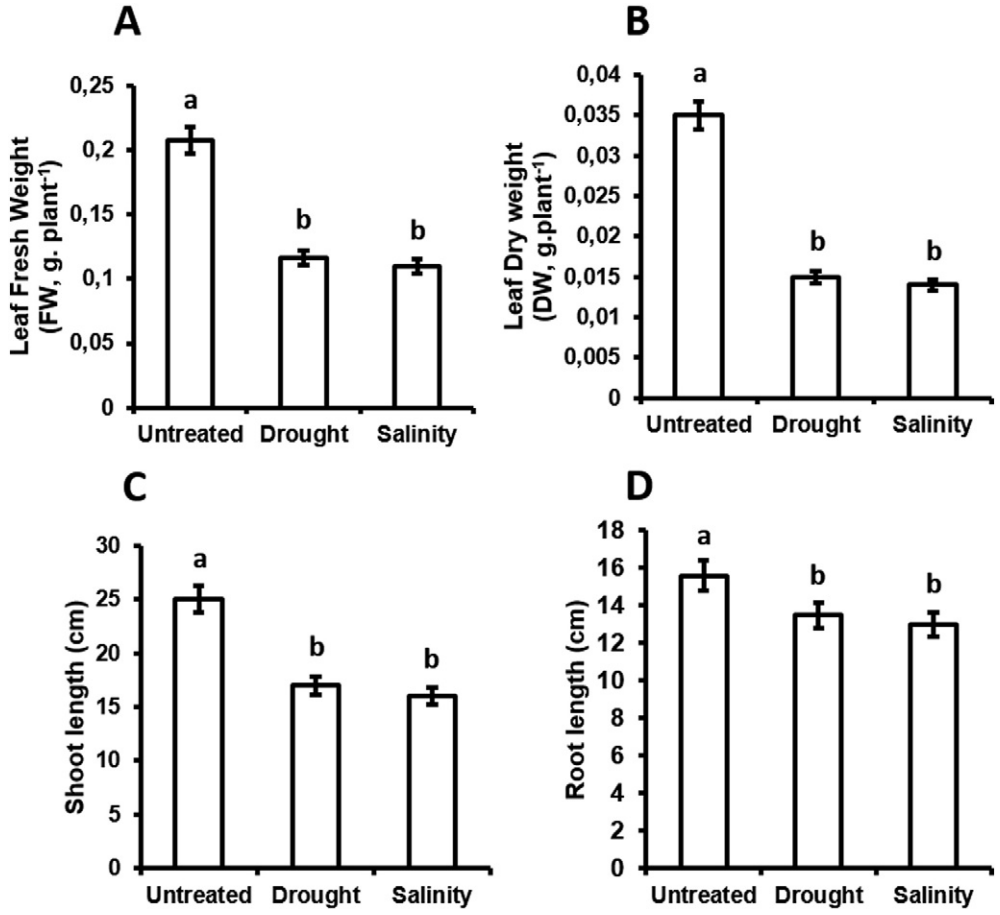

Fig. 1. The effect of drought and salinity stress on leaf fresh weight (A), leaf dry weight (B), shoot length (C), and root length (D) of sorghum plants. Bars with different letters signify statistically different means ($P < 0.05$).

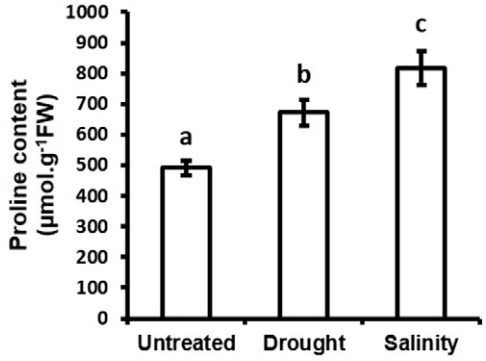

Fig. 3. The effect of drought and salinity treatments on proline accumulation in sorghum plants. Different letters on bars indicate statistically different means ($P < 0.05$).

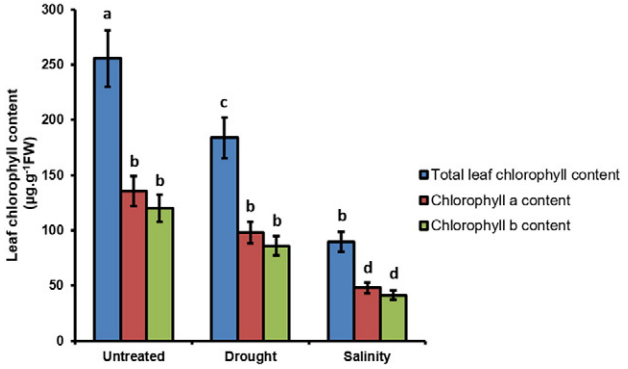

Fig. 4. The effect of drought and salinity treatments on photosynthetic pigments in sorghum plants. The photosynthetic pigments measured in the experiment include chlorophyll a, chlorophyll b, and total chlorophyll contents. Different letters on bars indicate statistically different means ($P < 0.05$).

Although a significant increase in H_2O_2 content for roots was observed in both treatments, this increase was not as pronounced as observed in the leaf tissue (Fig. 5B). Root H_2O_2 content in drought-stressed plants was 38% higher than in untreated control plants, whereas a 34% increase in H_2O_2 content was measured in salt stressed plants (Fig. 5B).

3.4. Lipid peroxidation and cell viability

MDA is a final product of lipid peroxidation and its content has been regarded as an indicator of oxidative stresses in plants (Mittler, 2002). Significant changes in MDA content in both leaves and roots to drought and salinity stress were observed when compared to their respective controls (Fig. 6A and B). MDA content in leaves and roots of sorghum plants was significantly increased as a consequence of drought and salinity treatments (Fig. 6A and B). Leaf MDA content was enhanced by 94% in the drought treatment compared to the untreated controls with an even higher increase observed in the salinity treatment (106%) (Fig. 6A). No significant difference in the level of MDA content was observed in the leaves of drought- and salinity-stressed plants. A similar trend (albeit different degrees of change) as observed in the leaves for both treatments was also observed for the roots. Root MDA content was significantly increased by 98% in the drought treatment, whereas a 116% increase in MDA content was observed for the salinity treatment when compared to the untreated controls (Fig. 6B).

Drought and salinity treatments caused a drastic loss in leaf and root cell viability (Fig. 7A and B). This is shown by the significant increase in Evans Blue uptake in both tissues. In response to drought stress, Evans Blue uptake in the leaves of sorghum plants was increased by 100% compared to untreated controls, whereas an even higher increase of 119% was observed in leaves of salinity-stressed plants (Fig. 7A and B). A similar trend (albeit different degrees of change) in Evans Blue uptake as observed in the leaves for both treatments was also observed for the roots. The uptake of Evans Blue in response to drought and salinity treatments increased by 53% and 55%, respectively, when compared to the untreated controls. Therefore, no significant difference in Evans Blue uptake was observed in the roots of drought- and salinity-stressed treatments (Fig. 7B).

4. Discussion

This study described the influence of drought and salinity stress on sorghum growth, water retention, and various biochemical responses. The results showed that long-term drought and salinity stress significantly reduced plant growth and development as manifested by reduced leaf fresh and dry weights coupled with reduced shoot and root length (Fig. 1A–D). The reduction in plant growth observed in this study is a common response to drought and salinity stress as plant growth is one of the most important agricultural indices of stress tolerance as indicated by different studies (Parida and Das, 2005).

Drought and salinity stress reduced water retention in sorghum plants although the degree of changes between these treatments were insignificant (Fig. 2). Relative water content is considered as one of the easiest agricultural parameters that can be used to screen plants for drought tolerance. Drought-tolerant plant species have been shown to keep high RWC compared with drought-sensitive species (Boutraa et al., 2010).

Chlorophyll is the main color agent responsible for photosynthesis. In this study, we have shown that the chlorophyll content (shown as chl_a, chl_b) in the leaves of sorghum plants was significantly reduced in both treatments with the highest reduction being observed in the salinity stress treatment (Fig. 4). Reduction of chlorophyll content due

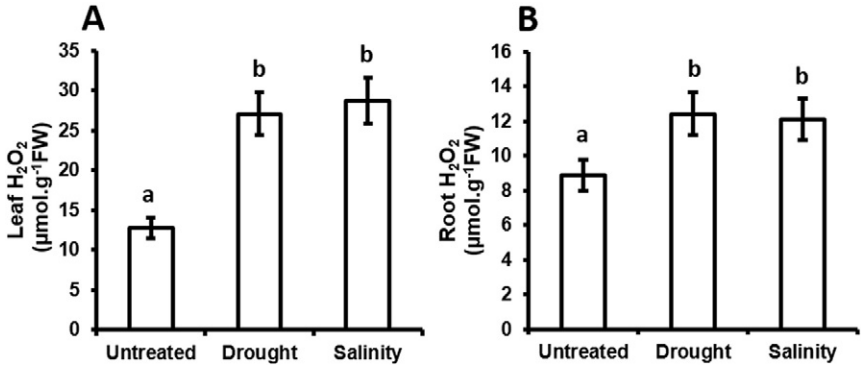

Fig. 5. The influence of drought and salinity stress on H_2O_2 content in sorghum plants. Changes in H_2O_2 content in leaves (A) and roots (B) of sorghum plants in response to stress treatments. Bars with different letters signify statistically different means ($P < 0.05$).

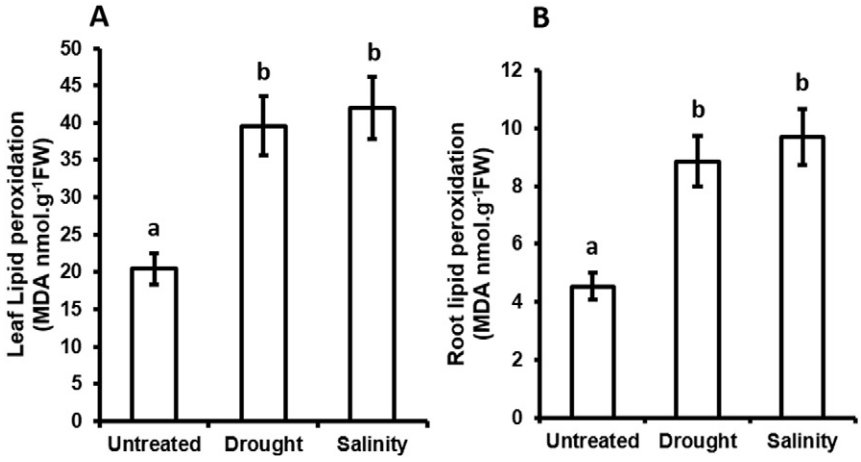

Fig. 6. The effect of drought and salinity treatments on lipid peroxidation in leaves (A) and roots (B) of sorghum plants. Bars with different letters signify statistically different means ($P < 0.05$).

to salinity stress is very common in salt-sensitive plant species because of salt toxicity which mostly causes burning of leaves or other succulent parts and degradation of other pigments too. A decrease in chlorophyll content as affected by water deficit (drought stress) produces reactive oxygen species (ROS) such as O_2^- and H_2O_2 that leads to lipid peroxidation and consequently, chlorophyll destruction (Smirnoff, 1993; Foyer et al., 1994). Interestingly, the reduction in chlorophyll contents observed in this study was more pronounced in the salinity stress treatment than the drought stress treatment. This suggests that salinity stress was more severe to sorghum plants than drought stress, a phenomenon previously described by Soussi et al. (1998) albeit for potato plants. According to Poljakoff and Gale (1975), the ability to synthesize more chlorophyll under stress conditions is a good criterion for the species stress tolerance.

The accumulation of osmoprotectants such as proline in stressed plants has been documented as a primary defence response to maintain the osmotic pressure in the cell (Desingh and Kanagaraj, 2007; Koca et al., 2007; Veeranagamallaiah et al., 2007). The capacity to accumulate proline under stress conditions has been correlated with stress tolerance in several plant species (Maggio et al., 2002; Claussen, 2005). Although proline content was augmented under both drought and salinity stress conditions (Fig. 3), it was not sufficient to reduce the oxidative damage caused by accumulation of H_2O_2 and the consequent increase in lipid peroxidation. Lipid peroxidation has been previously associated with cellular damage caused by various environmental stress conditions (Huang et al., 2009). In this study, we have shown that stress treatments significantly enhance lipid peroxidation and that this increase is directly linked to ROS accumulation that causes membrane instability. The increase in oxidative damage caused by augmented levels of H_2O_2 to drought and salinity stress was manifested as extensive cell death (Fig. 7A and B) coupled with reduced plant biomass (Fig. 1A and B).

In consideration of all the results obtained from this study, we conclude that progressive drought and salinity stress treatments significantly reduced sorghum growth and development by differentially modulating various biochemical responses. Furthermore, we have proven that a direct relationship exists between stress-induced oxidative damage (caused by enhanced H_2O_2 levels) and osmolyte accumulation with stress tolerance being controlled by the efficient scavenging of ROS.

Author contribution statement

XN did all the experiments presented in this article. AK and BN conceived and designed the research. All authors wrote, critically read, and approved the manuscript.

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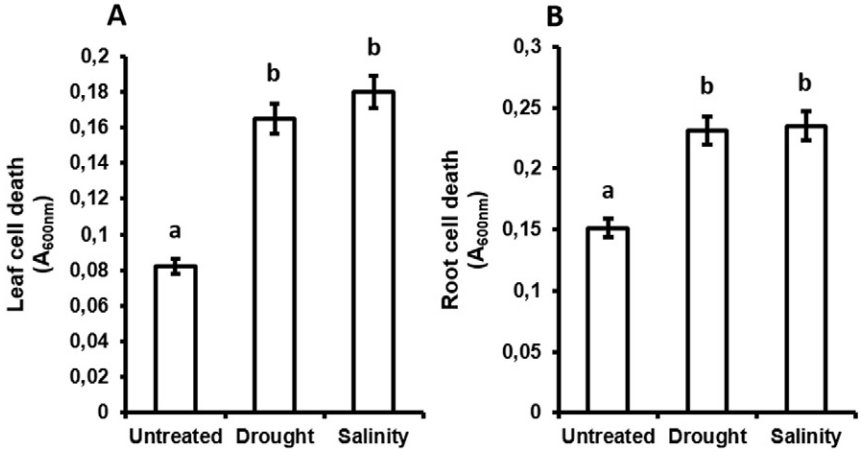

Fig. 7. The influence of drought and salinity stress on cell viability in leaves (A) and roots (B) of sorghum plants. Different letters on bars indicate statistically different means ($P < 0.05$).

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