Protection Mehcanism Against Drought In *Axsonopus Compressus*: Insight of Physio-Biochemical Traits, Antioxident Interplay and Gene Experssion

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Abstract

Drought is a major environmental constraint that affects plant growth and internal physio-biochemical features. The present study was conducted to evaluate the performance of three different *Axonopus compressus* accessions, i.e., A-38, A-58, and A-59 under well-watered (WW), low drought (LD), moderate (MD) and severe drought (SD) conditions at field capacity of 100, 80, 60, and 40%, respectively. Results indicated that drought-induced higher production of proline and soluble sugar (SS) up to 40 and 41% respectively, than control. Drought stress caused excessive production of H_2O_2 while the highest value (10.15µmol g^{-1} FW) was observed in the A-38 under SD. However, the lowest enzymatic (SOD, POD, CAT, and APX) activity were observed in A-38 than A-58 and A-59 respectively, in the SD. In A-58 the efficient enzymatic and nonenzymatic defense systems hinder the severe damage while stunted growth occurred in *Axonopus compressus* accessions at SD which were more pronounced in the A-38. Overall, the performance of all *Axonopus compressus* accessions under drought stress was recorded as A-58>A-59>A-38. The qRT PCR expression analysis also revealed highest expression of drought responsive genes in A-58 and reinforced the findings of physiological data. These results suggested the plant's ability to maintain its functions during drought induction could be used for further investigation under scarce water for developing drought tolerance.

Background

Drought stress alters the physio-biochemical mechanisms that drastically affect growth, development, and biomass accumulation in plants. Plant-water relations, photosynthetic gas exchange, cell turgidity, and various other metabolic functions and gene expression are largely affected by drought stress [1−3]. Moreover, membrane proteins, lipids, and nucleic acids are also affected by the overproduction of reactive oxygen species (ROS), such as superoxide anions (O_2^-), singlet oxygen (O^{-1}_2), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2), ultimately leading to cell death [4, 5]. To negate the deleterious effects of drought, plants have a well-developed and innate behavior to synthesize different solutes/osmolytes to maintain cell water balance. These osmolytes prevent drought-induced damage to plants by regulating various physiological mechanisms [6−8]. For instance, proline formation under drought, not only aides to maintain cell turgidity but also engaged with extinguishing free radicals, maintaining sub-cellular structures, and intermediating redox potential [4, 6, 9].

Plants have an excellent enzymatic and non-enzymatic antioxidant defense system to cope with ROS-induced oxidative stress [7, 10, 11]. Both enzymatic, viz., ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), as well as non-enzymatic, viz., reduced glutathione (GSH), oxidized glutathione (GSSG) antioxidants minimize the oxidative damage under drought stress conditions. Hussain et al. [12] found that GSH protects the chloroplasts from ROS damage by increasing the reduced glutathione to oxidized glutathione ratio (GSH/GSSG). Moreover, the enhanced activities/levels of both enzymatic and non-enzymatic antioxidants might ameliorate the effect of drought by ROS- quenching mechanism [5, 13, 14]. The sustained growth and development of different species under drought stress were linked to augmented antioxidant activities [5, 15]. The plant confronts

long-term drought stress by improved enzymatic and non-enzymatic antioxidants that assure plant tolerance against drought stress [13, 16], and activation of cellular mechanisms [17], which are controlled by different gene. The drought-responsive genes such as MAPK1, DREB2, WRKY1, NAC1, and MYB5, etc are well-known for their crucial role in drought tolerance [18–22]. Similarly, PIP gene family such as OsPIP2;1 and OsPIP2;2 in rice, may constitutively control the water transport during drought [23]. WRKY transcription factors, previously referred to as essential governing bodies of biotic stress in biogical system, have already been documented to provide abiotic stress tolerance in plants [20, 24, 25]. The function of WRKY transcription factors as mitigator of abiotic stresses was also revealed by its constitutive expression in plants, which conferred its vulnerability to water deficit stresses [20, 25, 26]. The NAC genes were also discovered for involvement in abiotic stress responses in crop plants. For example, OsNAC1, OsNAC6 and/or SNAC2 demonstrated overexpression in rice results in enhanced ability to tolerate blast disease, drought, low temperature and salinity [18, 27, 28]. The adverse effects of stress on plants were minimized by the overexpressing of OsNAC5 in transgenic rice plants [29–31]. Moreover, the MYB family also found to gave overexpression in plants to increased tolerance against abiotic stress such as; drought and salt stress [32], as evident by greater proline content, increased antioxidant activities, and reduced REL and MDA values in transgenic plants under unfavorable environment conditions [33].

For the evaluation, screening and selection of drought-tolerant accessions as well as for agronomic and genetic engineering, the knowledge of tolerance mechanisms and identification of most effective antioxidants in plants must be known in detail at different drought regimes [34, 35].

Axonopus compressus [Carpetgrass], is one of the important perennial warm-season turfgrasses, native to South-America. It has many distinct characteristics, such as easy propagation, spreading type nature, and low management made it popular throughout the world [34]. By virtue of these traits, carpet-grass is widely used for soil protective cover, planted as turf in lawns, highway sides, sports fields, and road-side areas throughout world [36–38]. Axonopus compressus is extensively distributed in the tropical and subtropical climatic areas in China (27N–27S), such as Guangxi, Guangdong, Guizhou, Fujian, Hainan, and Yunnan [39]. Therefore, the present study was conducted to assess the drought-induced changes in morpho-physiological attributes and expression levels of different drought-responsive genes in order to check the drought tolerance potential of the Axonopus compressus. This study will be helpful to get a better understanding of Axonopus compressus tolerance mechanism(s) against drought stress.

Results

Drought stress hampers the chlorophyll contents and carotenoids

Drought stress and cultivars significantly ($P < 0.05$) affected the production of photosynthetic pigments, i.e., Chl a, Chl b, total chlorophyll (Chl a + b), and carotenoids, whereas no interaction was found statistically significant ($P < 0.05$). The photosynthetic pigments decline rates were higher with an increase in drought stress with the highest rate at severe water deficit in all Axonopus compressus cultivar (Fig. 2).

The values of percentage reduction in Chl a, Chl b, and total chlorophyll content (Chl a + b) were recorded as 8–27, 34–77, and 17–43%in A-58, 12–25, 48–74, and 22–39% in A-59 and 19–33, 44–73, and 25–43% in A-38, as compared with control. A-58 gave significantly higher values 1.423, 0.667 and 2.092 of the Chl a, b and a + b respectively, while minimum values were found in the A-38. Moreover, the change in chlorophyll contents due to drought was more severe at higher levels of drought in all Axonopus compressus accessions. Overall, the A-58 performed better in terms of the chlorophyll a, b and a + b contents than A-59 and A-38. While the magnitude of drought stress on photosynthetic pigments across cultivars was recorded as followed A-58 > A-59 > A-38 (Fig. 2a-d).

Drought stress inhibited growth and leaf water status of Axonopus compressus.

Significant effects were observed in morphological traits and leaf water potential due to Drought stress in Axonopus compressus, whereas no interaction effect was found significant except in case of leaf length and area. The highest values 46.13, 4.30, 0.97, and 4.71 cm of morphological traits i.e; stem length, leaf length, leaf width, and leaf area respectively, were observed in A-58. Moreover, greater value of leaf water potential was also observed in A-58 (Table 2). The highest decrease 36, 11, 32, and 15% of stem length, leaf length, leaf width, and leaf area respectively due to drought induction was observed in A-38 (Table 2). The reductions in morphological traits were increased with an increase in drought levels from low to high drought in all accessions. Overall, the result showed A-58 proved tolerant than other two accessions (A-38 and A-59) and drought-induced damage was more intense in A-38 than those in A-59 or A-58.

Drought stress-triggered oxidative damage and osmolyte accumulation.

Drought-induced oxidative stress in terms of enhanced H_2O_2 production, lipid peroxidation, and membrane damage; though levels were fairly higher at severe drought for all A-58, A-59 and A-38 (Fig. 3). The production of H_2O_2 was 33.09-100.88, 32.90-93.06 and 40.61-125.05% in A-58, A-59 and A29 respectively, Similarly, electrolyte leakage and MDA production were enhanced linearly with increasing drought level, maximum at severe drought level i.e., 21.03 and 110.26% (for A-58), 33.55 and 123.26% (for A-59), 37.72 and 149.59% (for A-38). Drought stress affected TBARS accumulation significantly (P < 0.05) but the cultivars were remained statistically similar (P > 0.05). Overall, the rate of oxidative stress was higher at high drought stress and was more prominent in A-38 than A-58 and A-59.

Both drought stress and accessions affected total phenols and proline accumulation significantly (P < 0.05) whereas, no interaction was found statistically significant (P < 0.05) (Fig. 4a,c). While in case of the soluble sugars and protein the interactions were found statistically significantly (P > 0.05). Higher values 40.03 and 67.31, 38.84 and 62.53, 31.09 and 68.08 of proline and total phenols were estimated in the A-58, A-59, and A-38 respectively,under severe drought. In addition, both soluble sugar and soluble proteins accumulation were increased with an increase in drought induction in all accessions. Furthermore, increase of soluble sugar and soluble proteins accumulation were ranged from 15.95–41.29 and 17.13–78.59%, 3.57–38.11 and 11.64–60.59%, 13.54–22.84 and 17.46–54.67% in A-58, A-59 and A-38 respectively (Fig. 4b, d).

Drought-induced regulations of enzymatic and non-enzymatic antioxidant activity.

Drought stress and accessions significantly (P < 0.05) affected enzymatic (SOD, POD, CAT, and APX) and non-enzymatic (GSH, GSSG, total glutathione contents (GSH + GSSG) whereas, GSH/GSSG levels were found non-significant regarding stimulation of the antioxidants. Furthermore, the drought × accession interaction was extended up to significant level (P < 0.05) for SOD, CAT, POD, APX, GSSH and total glutathione contents (GSH + GSSG).

For A-58 the activities of CAT, POD, SOD, and APX increased by 7.23, 42.67, 30.32, and 27.57% under severe drought, as compared with control. Whereas, the activities of the enzymes were changed by -8.31 and − 35.13, 33.57 and − 19.58, 30.19 and − 4.29, 19.56 and − 47.51% for the A-59 and A-38 in severe drought (Fig. 5a-d). Antioxidant activities were found higher at high drought level while decreased abruptly as drought level increased form medium to high, especially in A-38. Furthermore, activity of SOD increased 7.85 and 10.20% till medium to high drought level (for A-58 and A-59 respectively), while the activity decreased 7.90% in A-38 for medium to high drought level. POD activity increased with increase in drought severity (maximum at medium drought level i.e., 44.49 and 39.34% in A-58 and A-59, respectively), CAT activity enhanced by 56.15% up to medium drought-level then decreased with an increase in drought level (in A-58) and for A-59 highest (41.77%) activity at medium drought level. The activities of APX increased with an increase in drought-levels in both A-58 and A-59 with highest values of 27.57 and 19.56% respectively, while, in A-38, the APX activity decreased with increase in drought stress with highest value (13.14%) at low drought level. Overall, antioxidant enzymatic activities were found higher in A-58 than both A-59 and A-38 (Fig. 5).

Furthermore, drought significantly changed (P < 0.05) GSH, GSSG and total glutathione (GSH + GSSG) in the Axonopus compressus leaves. For A-58, plants exposed to severe drought level accumulated highest (18.31%) GSH contents, similar trends were observed for GSSG (9.09 and 7.23%) and total glutathione (GSH + GSSG) (10.71 and 8.66%) contents in both A-59 and A-38. Nonetheless, GSH/GSSG ratio was found non-significant in all accessions (Fig. 6a-d).

Effect of Drought Stress on the expression level of the drought-responsive genes and transcription factors in A. Compressus

TFs determine the expression of genes in plants. When drought and high-temperature stress disorders happen, the plant TFs expression promotes to alter the expression of downstream responsive genes that improve the ability to resist stress. The stress responses in plants are strongly linked to TFs i.e., MYB, WRKY, NAC, and DREB; that support plants to normalize their functioning in unfavorable drought conditions. Drought stress influenced the TFs expression in A. compressus during the experiment (Fig. 7).

In the current study, the MYB and WRKY1 gene average expression levels under drought stress were 2.89 fold and 1.9 fold higher in A-58 than those in the control A. compressus plants respectively. While for the DREB and NAC genes the 1642.83 fold and 39.96-fold higher values were observed in A-58 during drought treatment than well water control. The expression levels of drought-responsive genes (PIP1, ABI5,

and MAPK1) were higher under drought stress treatments when compared to those in the untreated control A. compressus (Fig. 7A, B, C). The PIP1 and ABI5 show elevated expression with values of 5.96 fold and 12.98 fold higher than well water control (Fig. 7E, F). Similarly, the MAPK also showed significant expression level value of 5.43 folds than well water control (Fig. 7G).

Discussion

The accession-specific metabolic alteration was found in Axonopus compressus under drought stress. These variations in metabolic responses to drought also appeared as changed crop morphological traits. Differential metabolic and biochemical responses among various plant species have been widely reported in previous studies, which provide valuable insight into the mechanisms underlying responses to different traits of interest [43, 50–55]. So, encompassing the inherit variation of Axonopus compressus accessions, a worthy tool to enquire about complex stresses related mechanisms. Roy et al. [50] studied the effect of the abiotic stress on the physiological and biochemical responses in different turf grasses. It was observed that the Axonopus compressus is sensitive to abiotic stresses. These results provide a basis that stress conditions affect the performance of Axonopus compressus and it may act differently under various abiotic stress conditions.

In the present study, it was found that chlorophyll contents (Figure. 2) were decreased while cell damage and H_2O_2 production (Figure. 3c) were increased in response to drought. Previous work on water deficit studies revealed that chlorosis, leaf discoloration, and chlorophyll damage occurred as consequences of drought [56, 57]. The leaf water potential was also found positively related to drought resistance in Axonopus compressus (Table 2). Similarly, the leaf length and area of the Axonopus compressus were also affected by limited water supply. Drought stress promotes un-stabilization of the plasma membrane, decreases cell turgor, thus cell damage occurs with the ultimate decrease in the photosynthetic activity and falsifies the light-capturing apparatus, phenomena that largely related to drought stress [58]. Drought also triggered ROS (mainly H_2O_2) burst and underlying oxidative damage as demonstrated by Miller et al. [59] and Mittler et al. [60]. The highest activity of ROS and membrane destruction were recorded in A-59 after A-38 while lowest activity was observed in the A-38 which shows its resistive ability against drought (Fig. 3c).

In the current study, the effect of drought on H_2O_2 accumulation and membrane damage was more obvious in A-38, which represented its sensitive nature to drought (Fig. 3c). Furthermore, A-38 showed increased EL and lipid peroxidation under drought that is similar to the findings of Anjum et al. [5]. This is resulting from more production of H_2O_2, and MDA and in-efficient scavenging capacity of antioxidant defense in the A-38. Drought stress ruptures the plasma membrane and causes cell turgor imbalance. Consequently membrane damage occurs and leading to plant death, that intrinsically linked with the outburst of drought triggered ROS [5, 61]. Sustaining the normal functioning of cell membranes structure and cellular activities is critical under stress conditions and thus greatly influences the plant stress tolerance. Similarly, Pawar et al. [62] revealed that EL increased drastically under drought stress compared to well-watered conditions in chickpea cultivars.

Drought triggered the osmolytes bio-synthesis and accumulation in all Axonopus compressus. Proline, soluble sugar, total phenolic contents, and proteins concentrations were predominantly higher in A-38 than A-59 and A-38under drought conditions (Fig. 4a-d). It has been revealed that proline biosynthesis and accumulation were significantly affected under moderate to SD stress [5] and enhanced soluble sugars production under deficit water supply in plants [34, 63].

Drought stress boosts ROS biosynthesis and accumulation processes. It also severely damages the biological organic molecules and membranes system. Likewise, lipid peroxidation (MDA) buildup is also served as an indicator of oxidative damage triggered by ROS. Recently, Sanchita et al. [58]; Abid et al. [61] and Anjum et al. [5] revealed that ROS wipeout with enhanced anti-oxidant activities was associated largely with drought tolerance in Withania somnifera, corn (Zea mays) and wheat. The drought tolerance in young palm oil trees was also associated with efficient mechanisms of protection and encounter of ROS by enzymatic and non-enzymatic antioxidant activation strategies [61, 64]. The ROS counter mechanism (enzymatic and non-enzymatic) performs an efficient job against ROS in young oil palm plants during drought conditions [64]. The ROS metabolism is very complicated under stress conditions, discovered to act as molecular oxidative damage [59], and possibly ROS metabolism involved in intrinsic varying responses of Axonopus compressus to drought stress. Higher GSH in the A-58 confers the efficient resistance against the drought stress (Figure. 6a). The GSH a non-enzymatic antioxidant in plants is the prevalent defense system of antioxidants, and the active thiol-group empowers the GSH to become a dynamic scavenger of free radical in plants [60, 65]. In our study, A-58 was more efficient in terms of activating the antioxidant detoxification defense system (enzymatic POD, SOD, CAT and non-enzymatic GSH, GSSG), which help the Axonopus compressus to confer with drought-induced oxidative impairment and enhanced resistance against drought. The recent development of transcriptomics, proteomics, and metabolomics enable the researchers to develop some new methods that gave a real reflection of these complex stress respondent mechanisms in the plant [25, 66, 67], and our recent study will be helpful for the further omics-based investigation.

Expression analysis of the drought-responsive genes reinforce the results of the physiology and biochemical studies of the A. compressus. All the genes showed significant increase in expression level as compared to the control treatment except in A-38. In A-38, the expression level of WRKY, DREB and MAP kinase genes was not increased as compared to control treatment. The BdWRKY24 was also negatively regulated under drought stress [68–70], which showed the same trends as in our study. While the MAP kinase also did not display significant expression in the A-38, these results are in compliance with previous reports [71], for the non-significant modification of expression level of MAPK in leaf of the Solanum tuberosum. These results indicate the practical divergence of MAPK genes during plant growth and development when it came to spatial and/or temporal transcript accumulation patterns. Kidokoro et al. [72] revealed the expression of the GmDREB1 gene was induced by all tested stresses including drought. However, the GmDREB1E;2 and GmDREB1H;1 expression was not altered in response to stress. This might be the case with A-38 where DREB1expression was not significantly altered in drought stress. In the present study, we found that drought-responsive genes were expressed at a markedly higher level in A-58 than others, indicating that A-58 might have the potential to regulate the drought stress and develop

tolerance against water deficit conditions. Efforts to identify genes and physio-biochemical response for the drought tolerance would help to understand the evolution of protein functions in A. compressus.

Conclusion

This study concluded that drought conditions might have severe consequences on Axonopus compressus by changing its internal physio-biochemical mechanisms and inhibiting the biosynthesis of photosynthetic pigments. Among the studied Axonopus compressus accessions the A-58, A-59, and A-38 proved tolerant, medium, and sensitive, respectively. The A-58 proved superior with respect to percentage photosynthetic pigment, morphological traits, electrolyte leakage, and ROS production. The qRT PCR analysis also showed best results of A-58. Although drought stress affected all the Axonopus compressus accessions, the effect was more pronounced at the severe drought stress. This dire natural variation for drought resistance will be a foundation for understanding physiological, metabolic and biochemical mechanisms and omic-pathways of drought stress tolerance in Axonopus compressus.

Methods

Plant Material and Growth Conditions

The experiment was conducted in a greenhouse at the Institute of Tropical Agriculture and Forestry, Hainan University, China (latitude 20° 03' 22.80" N, longitude 110° 19' 10.20" E). The propagation material of three Axonopus compressus accessions, i.e., A-58, A-38, and A-59 was acquired from the germplasm resource maintained at the Hainan University. The healthy and homogenous vegetative stems with three nodes were selected for the further propagation of plant. The Hoagland nutrient solution containing 2.4 mM Ca $(NO_3)_2$, 1.0 mM KH_2PO_4, 3.0 mM KNO_3, 1.0 mM $MgSO_4$, 0.5 mM NaCl, 23.1 µM H_3BO_3, 4.6 µM $MnCl_2$, 0.38 µM $ZnSO_4$, 0.16 µM $CuSO_4$, 0.052 µM H_2MoO_4, 44.8 µM $FeSO_4$(EDTA) was used to irrigate the plants during experiment. The plants had been cultivated under the normal irrigated condition for 25 days, and then drought treatments were imposed by skipping the irrigation and take field capacity to the prescribed levels [5]. The average night /day temperature (T) of the greenhouse was a range of 26–31 ∘C during the growth period, while the relative humidity (RH) was 60–88% in the morning and 50–75% in the afternoon.

Drought Treatments and Experimental Design

The plants were grown under well water conditions for twenty-five days in all three groups of the experiment. On 26th days after planting (DAP), three different drought stress levels, i.e., low drought (LD, 80% field capacity (FC)), moderate drought (MD, 60% FC) and severe drought (SD, 40% FC) had been established for two group of experiment out of three, while a check with 100% FC was kept for treatment comparison [5]. The drought stress was regularly monitored on the pot weight basis. The prescribed drought treatments were allowed to persist for two weeks. The pots were arranged in factorial-RCBD

design in triplicate with four pots per replicate. The pictorial description of the layout is prescribed in Fig. 1.

For the experiment of drought-responsive gene-expression analysis, we applied 15% (m/v) poly-ethylene glycol-8000 (PEG-8000) drought stress treatment to three weeks old uniformly grown plants. A. compressus leaves for drought-responsive gene expression analysis were harvested at 24-h after the application of drought stress treatment and rapidly frozen in liquid nitrogen, and stored at − 80 ∘C for further analysis.

Determination of chlorophyll and carotenoid contents

Fresh leave samples (0.1 g) were extracted with 8 ml of 95% ethanol and put in the dark place for overnight at room temperature for chlorophyll content determination. The samples were subsequently filtered with Whatsman's filter paper to remove chaff and the chlorophyll (Chl a, Chl b and total Chl a + b) and carotenoids were estimated in the filtrates by using a spectrophotometer. The absorbance was recorded at 470, 645 and 663 nm and content were estimated according to the formula of [40].

Estimation of hydrogen peroxide, malondialdehyde and electrolyte leakage

The hydrogen peroxide (H_2O_2) contents were assayed according to [41] with minor modifications. Fresh leaves samples (.1 g) were smashed in pre-cold mortar and pestle and positioned on an ice bath and homogenized with one-milliliter of 0.1% trichloroacetic acid (TCA) and centrifuged at 12000 g for 15 minutes. The reaction mixture contained the 0.5 ml potassium phosphate buffer (pH 7.0) and 1-milliliter potassium iodide (1 M) and the 0.5 milliliter aliquot of the leave extracts and then read sample absorbance at 390 nm in triplicate while H_2O_2 values were expressed as mmol g^{-1} FW.

The malondialdehyde (MDA) contents were estimated according to the methods devised by [42]. Fresh leaves samples (0.1 g) were homogenized in two ml 0.5% thiobarbituric acid (TBA) solution in 20% trichloroacetic acid (TCA) and warm it to 100 °C in the hot water bath at for 30 min. The boiled samples were then turned cold to room temperature in an ice-cooled water bath and centrifuged at 10,000 × g for five-minutes. The absorbance of the reaction mixtures was read at 532 nm, 600 nm in triplicate. The MDA values of the reaction solutions were estimated as: MDA content = and the final value was denoted as mmol g^{-1} FW. To determine electrolyte leakage (EL), fresh leaves samples (0.1 g) were rinsed thrice with distilled water, then dip in deionized water (8 ml) and nursed at 25 °C for 2 h and the first value of electrical conductivity (EC_1) was recorded with an EC meter. Then samples were incubated in a hot water bath at 90 °C for one hour and cooled down to room temperature for recording second EC (EC_2). The EL of the samples were computed by the following formula (EL (%) = EC_1/EC_2 × 100).

Determination Protein, proline and soluble sugars

The leaf proteins were estimated using G-250 [43]. The fresh leaves samples (0.1 g) were homogenized in 50 mM Na-phosphate buffer with 7.0 pH containing 2% polyvinylpyrrolidine (PVP) and 1 mM EDTA-Na2.

The mixture was then centrifuged at 8,000 g for 20 min at 4 ⬚ centigrade. The absorbance of the reaction mixture was taken at 595 nm and final protein contents were expressed as mg g-1 FW.

The Leaf proline was estimated according to Bates et al. [44] by using ninhydrin reagent. The extraction was made in a reaction mixture containing two ml toluene and the absorbance of the red chromophore in the toluene fraction was estimated at 520 nm. The amount of proline was assessed by the standard curve method and expressed as mg g^{-1} FW. For soluble sugars determination, fresh leaves sample (0.1 g) was put into the test tubes containing 10 ml of distilled water and 60 min at 100 C in a boiling water bath. After making the mixture cooled in an ice bath, 0.5 ml of boiled samples vortex with three milliliters of pure anthrone and absorbance were taken at 620 nm and denoted as mg g^{-1} FW [45].

Bioassays of enzymatic and non-enzymatic anti-oxidants

Fresh leaves sample (0.1 g) were homogenized in five ml of 50 ml Na- phosphate buffer (pH 7.8) with chilled mortar and pestle and homogenate were centrifuged at 8000 rpm at 4 C for 20 min and the supernatant aliquot was used for the enzymatic assay. Superoxide dismutase (SOD, EC 1.15.1.1) was determined according to Zhang et al. [46] by following the inhibition of photochemical reduction due to nitro blue tetrazolium (NBT). The reaction mixture consists of 1.75 ml of Na- phosphate buffer (pH 7.8), 0.3 ml of 130 mmol/L Met buffer, 0.3 ml of 750 mmol l1 NBT buffer, 0.3 ml of 100 mmol l^{-1} EDTA-Na$_2$ buffer, 0.3 ml of 20 mmol l^{-1} lactoflavin and 0.05 ml of enzyme extract. The reaction mixture and enzyme extract mixed and placed in the LUX-4000 for 20 min then read the absorbance at 560 nm.

The activity of peroxidase (POD, EC 1.11.1.7) was determined by using guaiacol according to the methods advised by [47] with minor modifications. The reactions mixture contained 1 ml of sodium phosphate buffer (pH 7.8), 0.95 ml of 0.2% guaiacol, 1 ml of 0.3% H_2O_2, and 0.05 ml enzyme extract. The absorbance was read at 470 nm. One unit of POD activity was the amount of enzyme that caused the decomposition of one mg substrate. Catalase activity (CAT, EC 1.11.1.6) was measured according to the method of Aebi, [48]. The reaction mixture contained 1.95 ml sodium phosphate buffer (pH 7.8), 1 ml of 0.1 M H_2O_2 and 0.05 ml aliquot of the supernatant. The absorbance was read at 240 nm. One unit measure of enzyme activity (U) was the decomposition of 1 M H_2O_2 at A_{240} in one min duration in one gram of fresh leaves samples. Ascorbate peroxidase (APX, EC 1.11.1.11) was estimated by using the "APX determination kit" purchased from Nanjing Jiancheng Bioengineering Institute, China. The methods directed by the manufacturer were followed while the absorbance was read at 290 nm. The glutathione family (reduced glutathione GSH, oxidized glutathione GSSG) was assayed by using kits encoded as A006-1 for GSH and A061-2 for GSSG, from Nanjing Jiancheng Bioengineering Institute, China (www.njjcbio.com). The instructions were carefully compiled and the absorbance was read at 420 nm and 412 nm, respectively. Total glutathione was computed by the addition of both GSSG and GSH (GSSG + GSH). The GSH/GSSG ratio is determined by dividing the GSSG to GSH value.

cDNA synthesis and Quantitate Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

For qRT-PCR, the leave samples of each treatment that were saved at -80, used to extract the RNA with RNAprep Pure Plant Kit (TIANGEN). The mRNA from each treatment was converted into cDNA using MONAD MonScript™ RTIII All-in-One Mix V2 (with dsDNase) in a 20 μL reaction volume that subsequently served as the template for qRT-PCR. The drought-related gene sequences of plant species belongs to grass family are PIP1, MAPK1, NAC1 (KU886332.1), WRKY1 (AF140554.1), DREB2 (EF672101.1), and MYB5 (AJ133638.1) were searched on NCBI; they were blast with the gene sequences of other species i.e. Triticum aestivum (taxid:4565), Zea mays (taxid:4577), Oryza sativa (taxid:4530), Avena sativa (taxid:4498), Hordeum vulgare (taxid:4513), Cynodon dactylon (taxid:28909), Sorghum bicolor (taxid:4558), and Leymus chinensis (taxid:52714) and the homologous sequences of the genes from species were downloaded. The primers were designed in the conserved regions of sequences after verification by using MEGA software (Center for Evolutionary Medicine and Informatics, The Biodesign Institute, McAllister Ave, Tempe, AZ, USA). The qRT-PCR primers were designed using Primer 5.0 software (Premier Biosoft, Palo Alto, CA, USA) (Table 1). The Actin gene was used as internal references for all the qRT-PCR analyses [49]. The applied bio-system 7500 was used for the qRT-PCR using the following protocol: 00:30 min at 95 °C, and followed by 45 cycles of 05 s at 95 °C, 34 s at 60 °C, and 00:15 s at 95 °C.

Abbreviations

WW:Well-watered; LD:Low drought; MD:Moderate drought; SD:Severe drought; SOD:Superoxide dismutase; POD:Peroxidase; CAT:Catalase; APX:Ascorbate peroxidase; H_2O_2:Hydrogen peroxide; O_2^-:Superoxide anions; O-12:Singlet oxygen; OH^-:Hydroxyl radicals; ROS:Reactive oxygen species; GSH:Reduced glutathione; GSSG:oxidized glutathione; GSH/GSSG:Glutathione to oxidized glutathione ratio; TCA:Trichloroacetic acid; TBA:Thiobarbituric acid; MDA:Methylenedioxyamphetamine; RH:Relative humidity; DAP:Days after planting; FC:Field capacity; RCBD:Randomized complete block design; EC:Electrical conductivity; PVP:Polyvinylpyrrolidine; FW:Fresh weight; NBT:Nitro blue tetrazolium; EDTA:Ethylene Diamine Tetraacetic Acid; cDNA:Complementary Deoxyribonucleic acid

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Declaration of conflicting interests

The author(s) announced no potential conflicts of interest with regards to the research, authorship, and/or publication of this manuscript.

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Competing interests

The authors declare that they have no competing interests

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Tables

Table 1 | List of the primers used for the qRT-PCR in expression analysis of the drought-responsive genes.

ID	Gene	Primer	(5' to 3')
01	DREB2	DREBF	AGTCTCTGAAACGATCAGGCG
02		DREBR	CCTCTTTGCACCCTGTGGAT
03	ABI5	ABI5F	GTGGGTTGAGGGGAAGGAAG
04		ABI5R	TCCCGGTTCTTGATCATCCG
05	MYB1	MYBF	TGCTCGTCAACTACATCGCC
06		MYBR	CTCTTGCCTGTGCGTTTCAG
07	MAP kinase1	MAP kinaseF	GAGCTGCTGCTCAACTCCAC
08		MAP kinaseR	CTCCATGAAGATGCAGCCGA
09	NAC1	NACF	ATGAGGAGGGAGAGGGACG
10		NACR	CAGGTAGTGCTCCACCAGC
11	PIP1	PIPF	GTCGCCACCTTCCTCTTCC
12		PIPR	CGCACTTGGAGTTGGACTTG
13	WRKY1	WRKYF	TCCCGAGGTACCACCAGATG
14		WRKYR	TTGGGGAACTGGGTAGGGAT
15	Actin	ActinF	ATGTTGCCATCCAGGCTGTG
16		ActinR	TAAGTCACGTCCAGCGAGGT

Figures

TABLE 2 | Agronomic traits influenced by different drought stress levels in *Axonopus compressus* accessions

| Accession No.	Treatments	Stem length (cm)	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Water potential (MPa)
A-58	Ck	46.13 ±2.14[a]	4.30 ±0.03[a]	0.97 ±0.03[a]	4.71±0.06[a]	-06.80 ±0.51[a]
	LD	39.67 ±0.33[bc]	4.27 ±0.04[a]	0.90 ±0.05[ab]	4.61±0.03[ab]	-11.06 ±1.45[ab]
	MD	36.67 ±0.67[cd]	4.27 ±0.03[a]	0.87 ±0.03[abc]	4.58±0.03[ab]	-14.93 ±0.75[bc]
	SD	30.60 ±0.67[ef]	4.23 ±0.03[a]	0.83 ±0.06[abc]	4.52±0.06[ab]	-18.21 ±1.23[cd]
	Means	38.27	4.27	0.89	4.60	-12.75
A-59	Ck	44.63 ±0.70[ab]	4.30 ±0.12[a]	0.93 ±0.02[a]	4.67 ±0.10[a]	-07.91 ±0.31[a]
	LD	38.00 ±0.58[cd]	4.20 ±0.06[ab]	0.83 ±0.03[abc]	4.49 ±0.03[ab]	-11.06 ±1.12[ab]
	MD	34.50 ±0.76[de]	4.10 ±0.06[abc]	0.83 ±0.06[abc]	4.40 ±0.02[bc]	-16.80 ±0.71[cd]
	SD	28.83 ±1.09[f]	3.95 ±0.03[cd]	0.73 ±0.03[cd]	4.17 ±0.06[cd]	-20.02 ±1.00[cd]
	Means	36.49	4.14	0.83	4.43	-13.95
A-38	Ck	43.60 ±0.74[ab]	4.27 ±0.02[a]	0.93 ±0.03[a]	4.65 ±0.03[ab]	-08.04 ±0.39[a]
	LD	37.17 ±0.83[cd]	4.17 ±0.03[abc]	0.77 ±0.03[bcd]	4.39 ±0.03[bc]	-11.40 ±2.56[ab]
	MD	34.33 ±0.88[de]	3.98 ±0.02[bcd]	0.67 ±0.03[d]	4.13 ±0.05[cd]	-18.73 ±0.51[cd]
	SD	27.83 ±0.93[f]	3.80 ±0.06[d]	0.63 ±0.02[d]	3.94 ±0.07[d]	-21.10 ±1.72[d]
	Means	35.73	4.05	0.75	4.28	-14.82
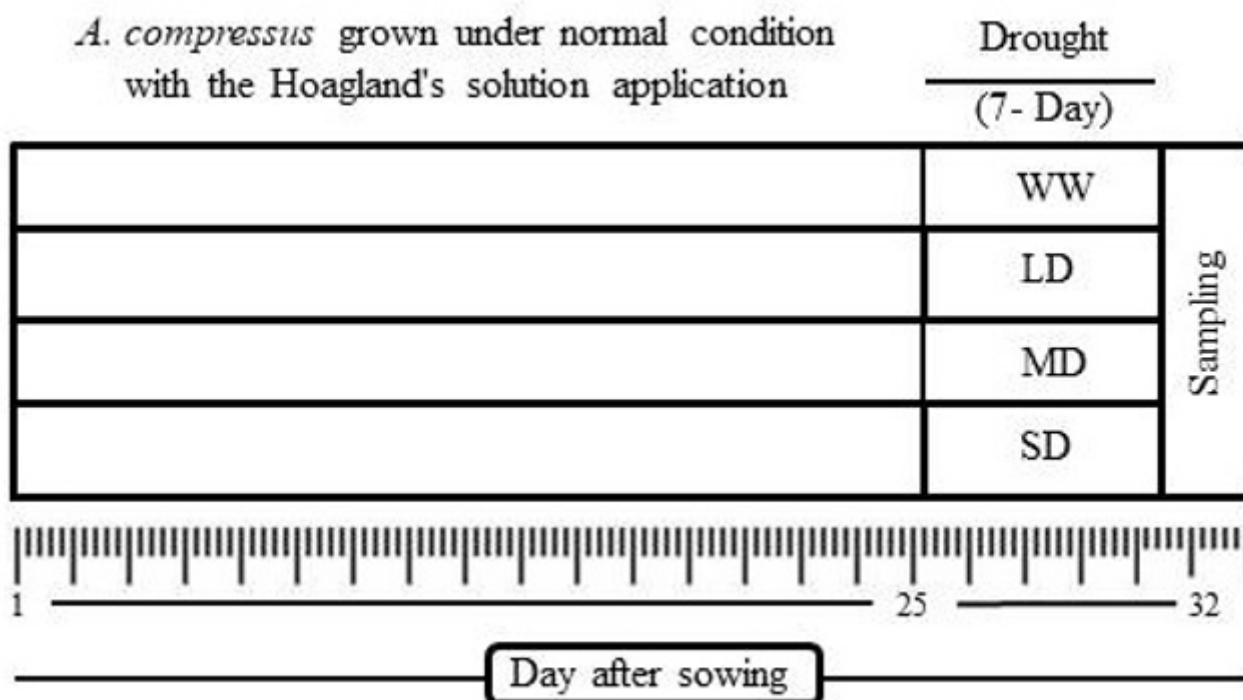
Figure 1

Experimental design of the study: three A. compressus was grown under normal conditions for 25 days then drought stress treatments were applied by withholding irrigation till the soil field capacity (FC) reached the desired levels i.e. the severe drought(SD); 40%, Moderate drought(MD); 60% and low drought(LD); 80%. The drought treatments were maintained for 7 days by weighing the pots and compensating the water lost to the desired FC and then followed by re-watering. while well-watered (WW) control was watered as in the normal condition.
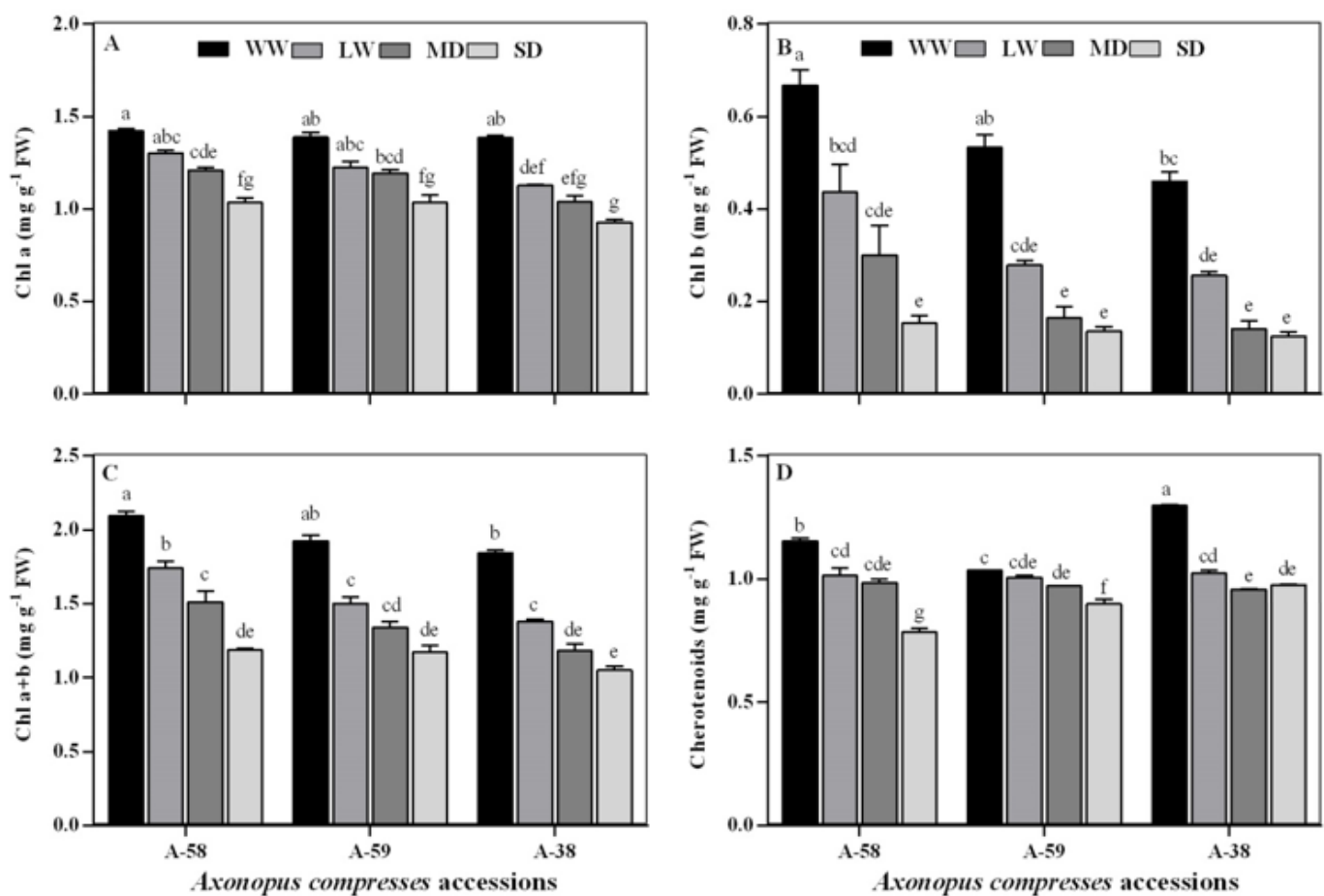
Figure 2

Effect of various drought stress levels on the (A) Chlorophyll a, (B) Chlorophyll b, (C) Chlorophyll a+b, (D) Carotenoids c, in three Axonopus compressus accessions. Capped bars above means represent ±SE of three replicates. Means with letters in common don't denote the significant differences among treatment at P < 0.05. Ck, control (100% FC); LD, low drought (80% FC); MRD, moderate drought (60% FC); and SD, severe drought (40% FC); FC, Field capacity.
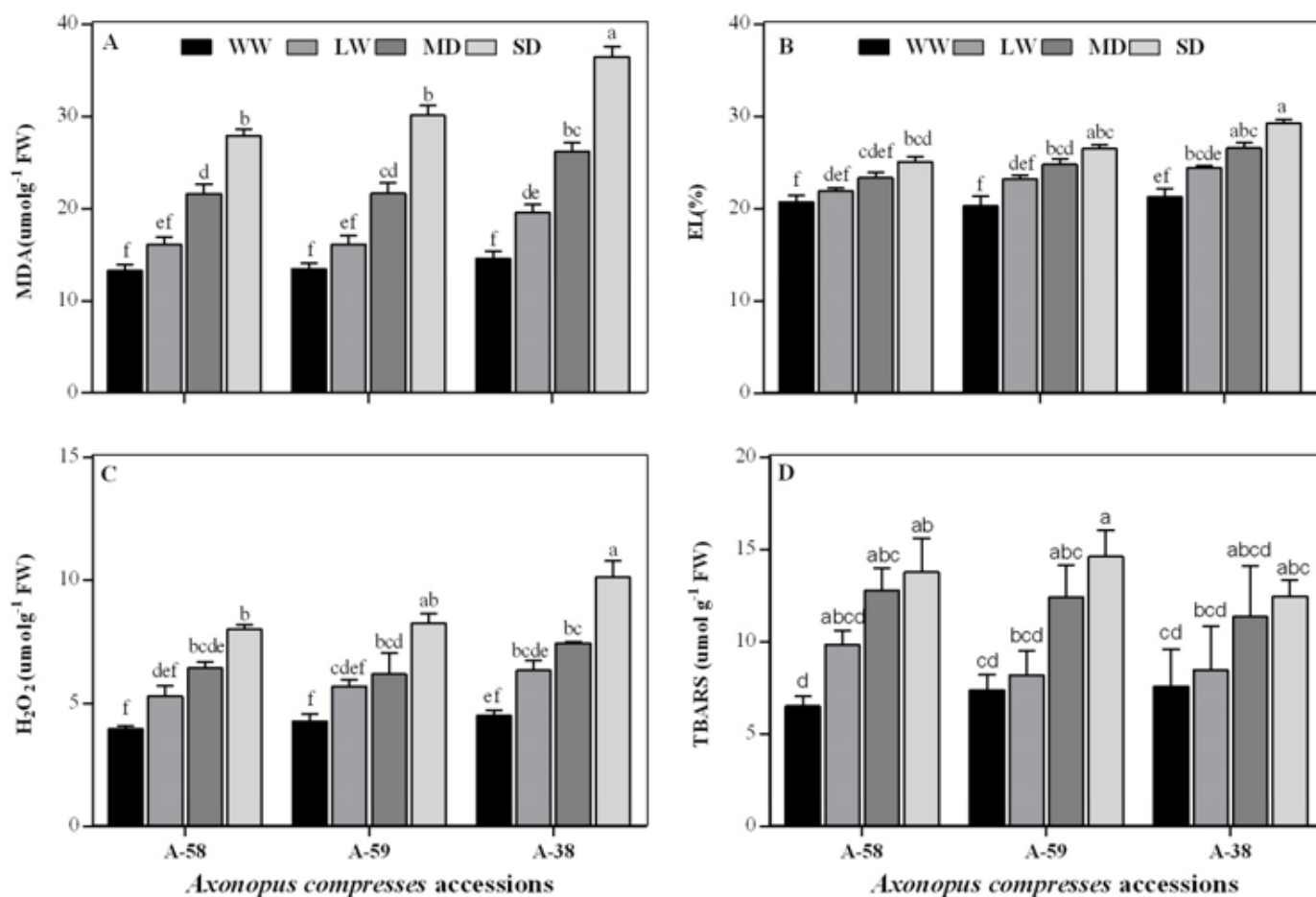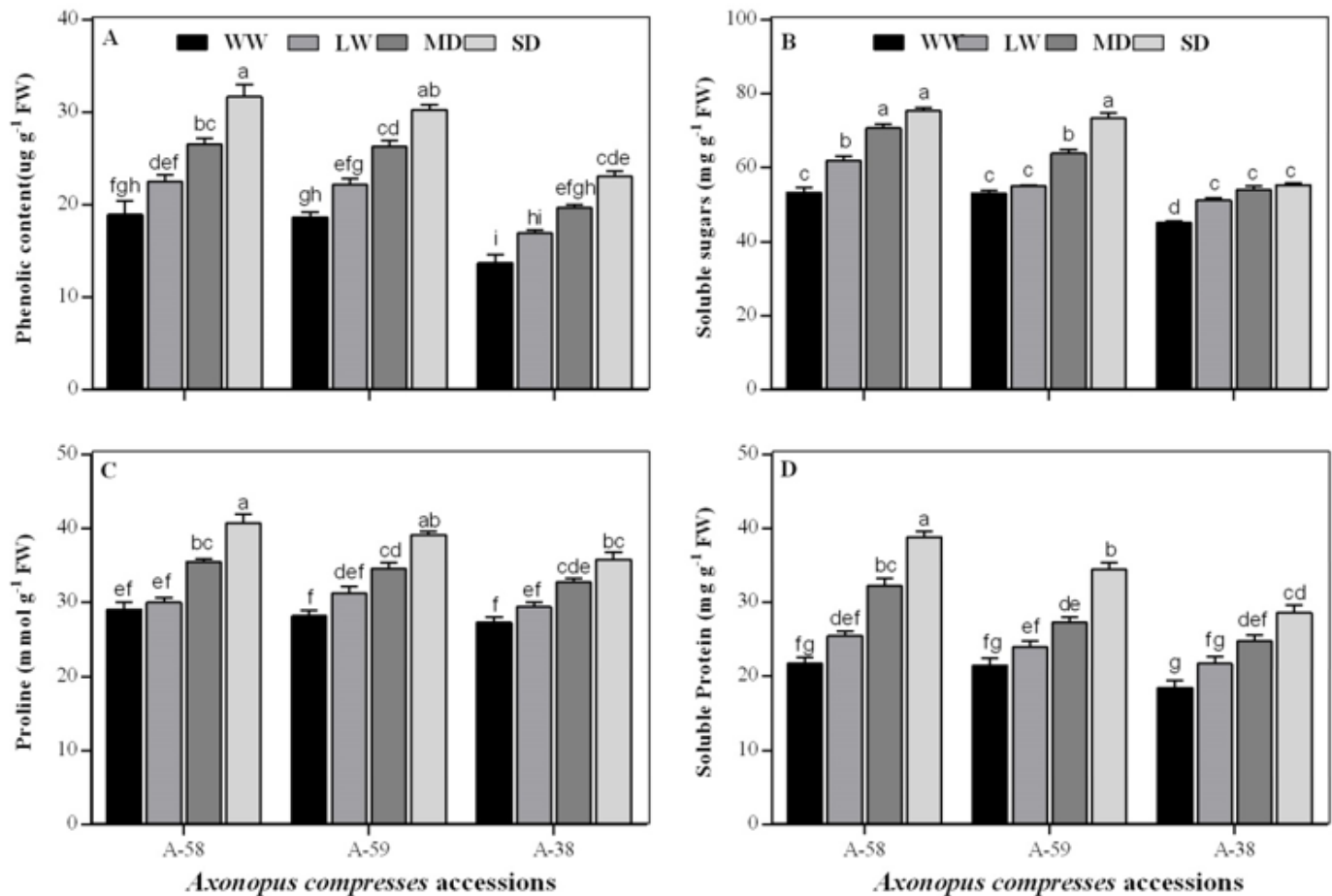
Figure 3

Effect of various drought stress levels on the production of (A) Malondialdehyde (MDA), (B) electrolyte leakage (EL), (C) hydrogen peroxide (H2O2), (D) thiobarbituric acid reactive substances (TBARS), in three Axonopus compressus accessions. Capped bars above means represent ±SE of three replicates. Means with letters in common don't denote the significant differences among treatment at P < 0.05. Ck, control (100% FC); LD, low drought (80% FC); MRD, moderate drought (60% FC); and SD, severe drought (40% FC); FC, Field capacity.
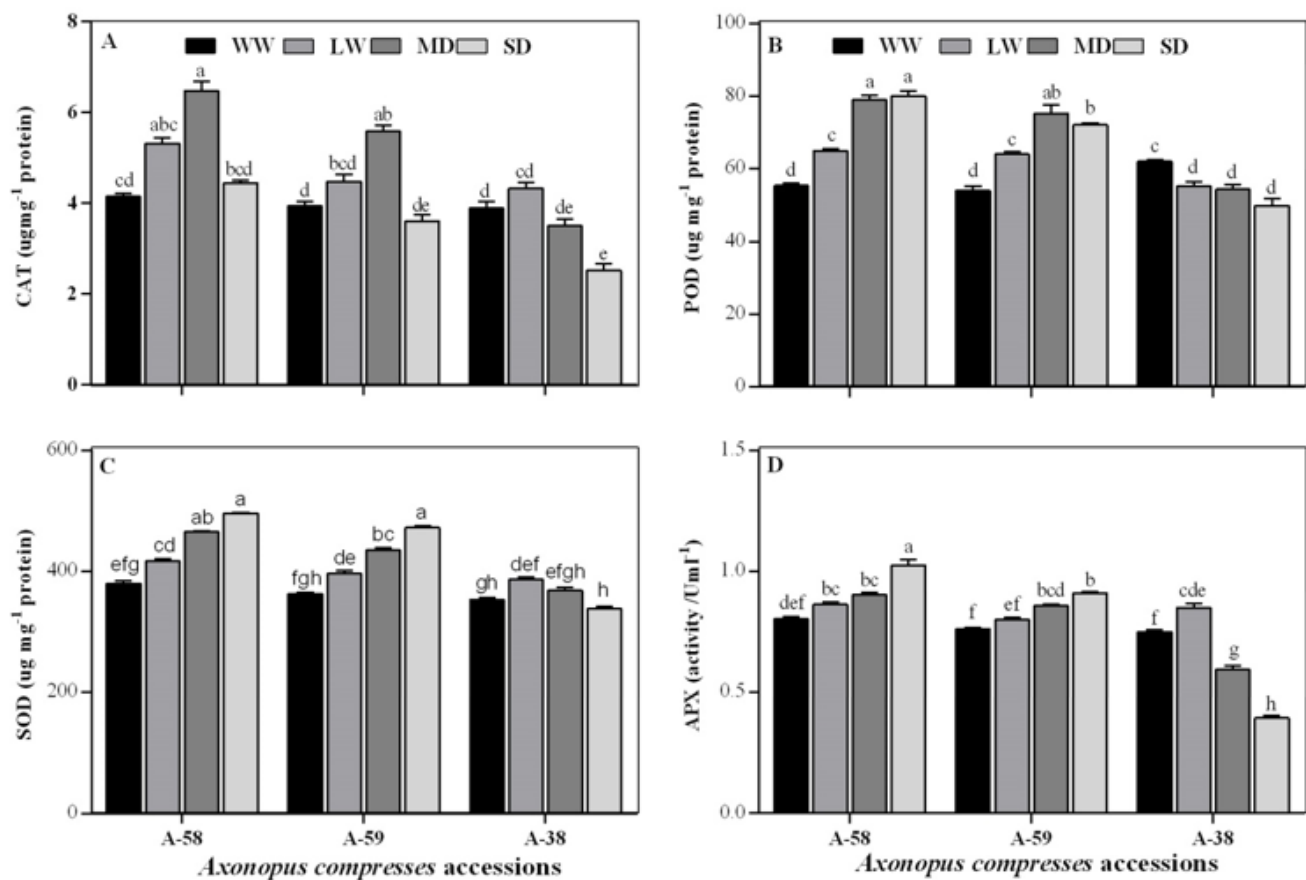
Figure 4

Effect of various drought stress levels on the accumulations of (A) phenolic contents, (B) soluble sugars, (C) free proline, (D) soluble protein in three Axonopus compressus accessions. Capped bars above means represent ±SE of three replicates. Means with letters in common don't denote the significant differences among treatment at P < 0.05. Ck, control (100% FC); LD, low drought (80% FC); MRD, moderate drought (60% FC); and SD, severe drought (40% FC); FC, Field capacity.

Figure 5

Effect of various drought stress levels on the activities of (A)) catalase (CAT), (B) peroxidase (POD), (C) superoxide dismutase (SOD), (D) Ascorbate peroxidase (APX) in three Axonopus compressus accessions. Capped bars above means represent ±SE of three replications. Means with letters in common don't denote the significant differences among treatment at P < 0.05. Ck, control (100% FC); LD, low drought (80% FC); MRD, moderate drought (60% FC); and SD, severe drought (40% FC); FC, Field capacity.
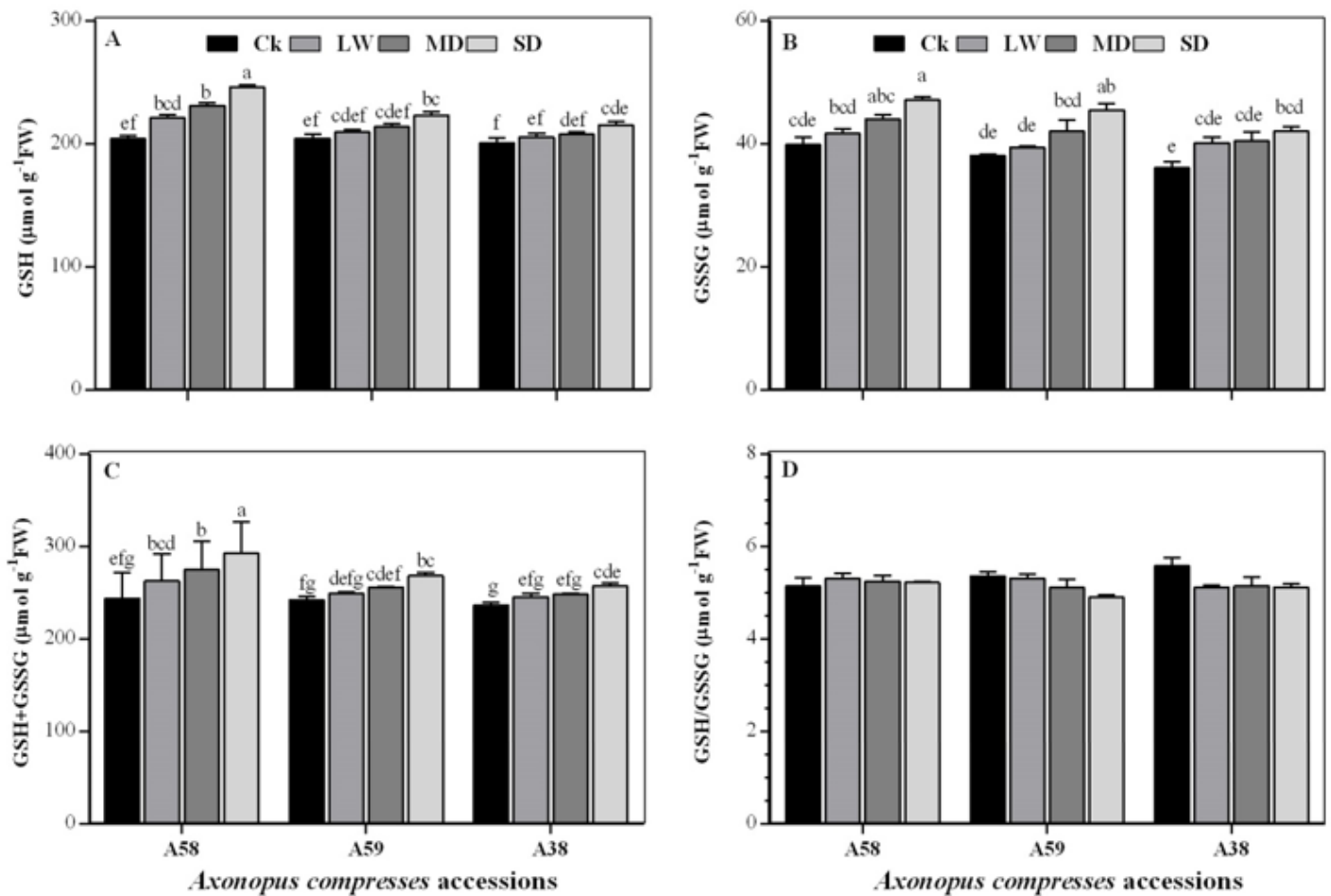
Figure 6

Effect of various drought stress levels on (A) reduced glutathione (GSH), (B) oxidized glutathione (GSSG), (C) Total glutathione (GSH+GSSG), (D) GSH/GSSG ratio in three Axonopus compressus accessions. Means with letters in common don't denote the significant differences among treatment at P < 0.05. Capped bars above means represent ±SE of three replicates. Ck, control (100% FC); LD, low drought (80% FC); MRD, moderate drought (60% FC); and SD, severe drought (40% FC); FC, Field capacity.
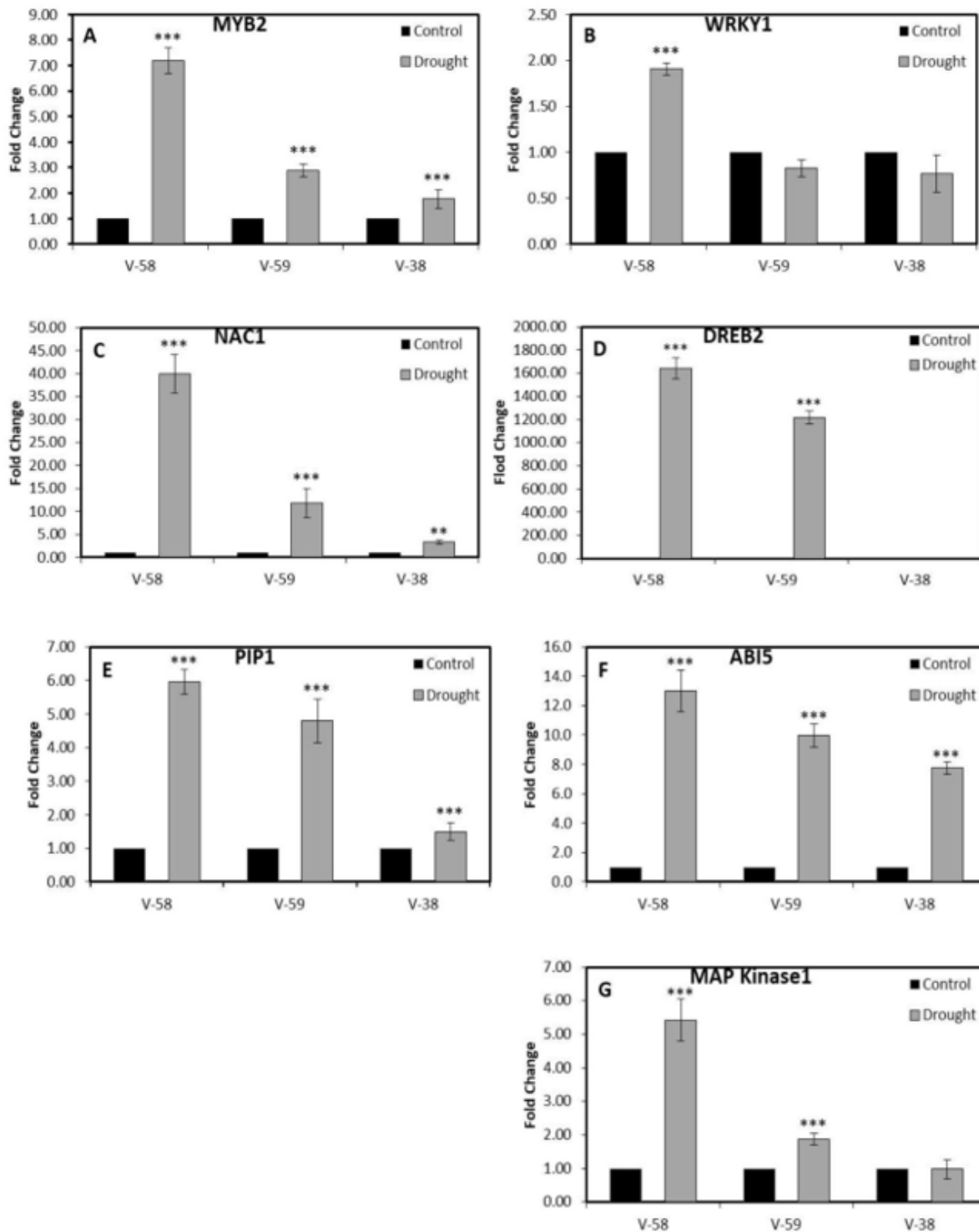
Figure 7

Effect of drought treatment on expression of drought-responsive genes in leaves of A. compressus. The bars showed relative expression of protein kinase genes (A) MYB2, (B) WRKY1, (C) NAC, (D) DREB2, (E) PIP1 (F) ABI5, and (G) MAP Kinase1 with values represent mean ±standard deviation (n = 3).