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Full Length Article

Impact of Drought and Calcium Sulfate on Antioxidants, S-assimilation, Ecophysiology and Growth of Tomato (*Lycopersicon esculentum*)

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Abstract

Drought poses threats to the production of tomato (*Lycopersicon esculentum* Mill.). Tomato plants need a large amount of water for proper growth, development and productivity. We screened four important tomato varieties for their potential to withstand drought. The variety that resisted the drought most 'SNU' was studied for drought and calcium sulfate (gypsum; hereinafter called CS) induced changes in growth photosynthesis, antioxidant and metabolite levels. Tomato plants were divided into four sets viz. 1. Set 1: Control, Set 2: Drought, 3: Control+Gypsum (2% w/v CS, calcium sulfate), and 4. Drought+CS. Gypsum CS (2% w/w). Thus plants of Set 3 and Set 4 were provided with a supply of additional sulfur and calcium. Plants were grown for 80 days under controlled conditions and estimations were made at 35 and 70 days after treatment (DAT). Drought induced oxidative stress in tomato estimated as thiobarbituric acid reactive substance (TBARS); however, gypsum treated plants suffered lesser damage. Loss to chlorophyll contents was lesser when gypsum was applied. Ascorbate-glutathione antioxidant system was affected by drought but operated finely with gypsum treatment. Drought-exposed plants accumulated higher amount of osmolyte proline in the presence of CS. The ratio of reduced forms of ascorbate and glutathione shifted towards oxidized forms; levels of glutathione increased under gypsum-application both at 35 (flowering stage) and 70 (fruiting stage) DAT. Gypsum treatment elevated the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT), glutathione S-transferase (GST) and ATP-Sulfurylase (ATPS). In tomato fruits, contents of β-carotene, lycopene and ascorbic acid increased under drought, but average fruit weight decreased significantly. To conclude, water stress induced oxidative stress but tomatoes elevated its antioxidant system, sulfur metabolism and osmolyte defense minimize the losses incurred by the drought in a better way when gypsum was applied both at 35 DAT and 70 DAT. This study highlighted the critical importance of calcium and sulfur, applied as gypsum in proper growth and improved defense system of tomato plant besides improving fruit quality.

Keywords: Tomato; Drought; Sulfur; Calcium; Antioxidants

Introduction

Tomato is a very important crop of world including Saudi Arabia (KSA). It serves as a source of nutrition besides contributing to national economy (Agarwal and Rao, 2000; Rizwan *et al*., 2010; Mozos *et al*. 2018). During shortage of crop, KSA had to import tomato from time-to-time (Grindle *et al*. 2015). For farmers, tomato serves as cash crop thus helping in a quick recovery of their investments with profits (Kelley and Boyhan, 2017). As a mere fact, tomato requires large amounts of water for its proper growth and production. KSA is a dry land country with scanty water availability and ample sandy soils. Hence, it is often difficult to cope up with the harsh environmental conditions and drought is of major concern among them (Schlaepfer *et al*., 2017).

It is known that abiotic stresses affect tomato growth and production adversely. Klunklin and Savage (2017) exposed tomato to drought and found that growth and concentrations of various constituents were affected. Carotenoids are synthesized and stored in the plastids and plays important roles in plant metabolism (Sun *et al*., 2017). Since lycopene is among the most important carotenoids in tomato, its concentration should persist under stress episodes. At plant level, numerous parameters determine the potential of any crop to withstand water deficit stress. Such parameters include the level of osmolyte and cellular antioxidant elevation to counter the negative water potential and oxyradicals etc. (Ahmad *et al*. 2017).

Adding any fertilizer to the soil can increase the water holding capacity and improve nutritional value. A source of calcium and sulfur, commonly known as gypsum has been shown to improve defense of the plant against stress (Al-Huqail *et al*. 2017). Gypsum is a natural source of calcium (Ca) and sulfur (S) which is recommended for application in

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agricultural soils. It improves both soil quality and growth of plants (Chen and Dick, 2011; Tirado-Corbalá *et al*., 2017). In plants, role of Ca varies such as in growth and development, cellular signaling (Edel *et al*., 2017) and other metabolic functions (Wang and Komatsu, 2017). S also interacts with several other nutrients; for example, in plastids (Przybyla-Toscano *et al*., 2018) and metabolism of nitrogen-fixing rhizobia in nodules (Kalloniati *et al*., 2015). S plays important interactive roles with other nutrients including iron (Fe), such as to synthesize Fe-S clusters, and part of enzyme cofactors (Qureshi *et al*., 2010; Bashir *et al*., 2013).

Proline has long been considered as a marker of dehydration in plants (Bashir *et al*. 2013). Serving as an osmoprotectant, proline has been suggested to counter free oxyradicals directly (Szepesi and Szőllősi, 2018). It has also shown as degradation product of proteins as well as reservoir of nitrogen under stress conditions or interconversion of other amino acids; e.g., glutamate is converted to proline by the activities of P5CR (Δ1-pyrroline-5-carboxylate reductase) and P5CS (Δ1-pyrroline-5-carboxylate synthetase) in plants (Ashraf *et al*., 2018).

Drought is reported to produce oxidative stress in plants including tomato (Martinez *et al*., 2018). To counter the oxidative stress, plants use cellular antioxidant system (both enzymatic and non-enzymatic). Both the antioxidant systems run in well-coordination to keep oxidative stress under control. Major players of non-enzymatic antioxidants include ascorbic acid and glutathione, constituting ascorbate-glutathione (Bagheri *et al*., 2017). The major enzymatic players are ascorbate peroxidase and glutathione reductase. However, first line of defense is present in chloroplast, mitochondria and cytoplasm in three isomeric forms called as superoxide dismutase (SOD) (del Rio *et al*., 2018). SOD converts superoxide to hydrogen peroxide (H_2O_2), which is then broken by catalase (CAT) into water and oxygen. The ascorbate-glutathione system also removes H_2O_2 from the cell. Cat is absent in the chloroplast hence later system is the major H_2O_2 scavenging system in the chloroplast. Since proper running of ascorbate-glutathione system requires NADPH(H) it is mostly accumulated in the chloroplasts because of reduced availability of atmospheric CO_2. The availability of atmospheric CO_2 is reduced because of the fact that drought induces closure of stomata (Tombesi *et al*., 2015). This accumulated NADPH(H) thus serves a source to regenerate oxidized forms of ascorbate and glutathione to reduced form, and removing H_2O_2 efficiently (Tuzet *et al*., 2018).

Glutathione S-transferases (GSTs) are directly involved in protecting plants against the environmental stresses besides helping in regular growth and development (Bashir *et al*., 2013). Previously known as ligandins, GSTs are enzymes ubiquitous in nature and catalyze the addition of reduced glutathione (GSH) to substrates helping in tagging for vacuolar sequestration (Edwards *et al*., 2000). These enzymes also cause peroxidation and isomerization and can also protect cells against H_2O_2-induced cell death by inhibiting the c-Jun N-terminal kinase (Sheehan *et al*. 2001).

One of the potential pathways to improve crop yield and defense is sulfur assimilation. ATP-sulfurylase catalyzes the committed step in this pathway which synthesizes APS (adenosine 5'-phosphosulfate) from sulfate and ATP (Herrmann *et al*., 2014). This pathway is responsible for synthesis of cysteine, methionine, glutathione, biotin and thiamin (vitamin cofactors), glucosinolates, and iron-sulfur clusters (Herrmann *et al*., 2014). This is cysteine which forms methionine through a trans-sulfuration reaction (Hesse *et al.*, 2004). These amino acids are crucial for survival of plants being integral part of GSH and proteins. Both GSH and many forms of proteins are required for metal tolerance.

Comparative growth analysis of crop varieties has been an effective in predicting the environmental stress tolerance ability (Zhou *et al*., 2017). Similarly, comparative assessments of photosynthetic, growth, development, biochemical, antioxidant and molecular parameters help in unraveling of basis of stress-tolerance. The present study was conducted on a selected drought tolerant tomato variety out of many tomato varieties available in the market. Impact of drought and gypsum was studied on magnitude of oxidative stress, proline accumulation, sulfur metabolism, cellular antioxidant system, ascorbate-glutathione pathway and chlorophylls. Fruit fresh weight, lycopene and ascorbate were also analyzed.

Materials and Methods

Tomato Growth and Experimental Conditions

Screening of tomato for drought tolerance was conducted on 15-days-old plants which were subjected to 25% of water applied to control and days to achieve >50% reduction in plant fresh weight was considered as a parameter to assess drought stress tolerance marker. Sampling was done on weekly basis. Four important cultivars of tomato were tested namely MGS-S41, MA, SNU and SSSHVS22. Tomato cultivar SNU took longest duration (35 days) for >50% growth inhibition hence selected in present study as drought tolerant cultivar. Seedlings of tomato (*Lycopersicon esculentum* L. cv. SNU) were grown in a combination of Soilrite™ and organic manure (50:50, w/w). After ten days of growth, the seedlings were transferred to the pots (0.5 kg of Soilrite™ added with 0.5 kg organic manure per pot). Sixty pots were divided into four sets of 15 each viz. 1. Set 1: Control, Set 2: Drought, 3: Control+Gypsum (2% w/v CS, calcium sulfate), and 4. Drought+CS. Gypsum CS (2% w/w) was added to the pots of Set 3 and Set 4 to provide a supply of additional sulfur and calcium. An initial screening for selection of comparatively drought-tolerant tomato genotype was performed.

TBARS, Hydrogen Peroxide, Superoxide Anions and Osmolytes

The content of thiobarbituric acid rreactive substances

(TBARS) was estimated in tomato leaf the method of Heath and Packer (1968). A 0.5 g of fresh tomato leaf was homogenized in 5 mL of 0.1 % (w/v) TCA (trichloroacetic acid) and spun at 7826 *g* for 10 min. A mixture was formed containing 0.5 mL of supernatant and 2 mL of 0.5 % (w/v) TBA followed by a heating for 30 min at 99°C. The content was cooled and spun at 1957 *g* for 5 min. The supernatant was read at 532 nm and corrected for unspecific turbidity after subtraction from the value obtained at 600 nm. Total content of TBA reactive substances was estimated using coefficient of absorbance of 155 mM^{-1} cm^{-1}.

To measure H_2O_2 in using the method of Wolff (1994), fresh leaf was immediately ground to a paste and spun at 7826 *g* for 5 min. The reaction was performed with 180 µL of sample, 20 µL of methanol and 1.8 mL of xylenol orange reagent and read at 560 nm. Assay was based on ferrous ion oxidation in the presence of the ferric ion indicator xylenol orange.

Production of superoxides was measured as described by Elstner and Heupel (1976). An aqueous paste of 0.2 g fresh leaf sample was prepared and spun at 7826 *g* for 5 min. Supernatant was used for estimation of production rate of superoxides through monitoring of nitrite formation from hydroxylamine. The absorbance in the aqueous solution was recorded at 530 nm and measured against the standard curve of NO_2^-. The superoxide production rate was expressed as nmol g^{-1} DW min^{-1}.

Proline is an imino acid that serves as an indicator of plant osmotic status. The content of free proline was estimated by the method of Bates *et al.* (1973) in the fresh leaf of tomato. Leaf sample (1 g) was ground in 20 mL of 3% (w/v) sulphosalicylic acid. The extract was spun at 7826 *g* at 4°C for 10 min. A 4 mL of supernatant was added with 4 mL of acid ninhydrin reagent followed by addition of 2 mL of glacial acetic acid. The mixture was boiled at 100°C for 30 min in a water-bath. The reaction was immediately stopped using an ice-bath. In the cool mixture, 8 mL of toluene was added. Two distinct layers were formed; the upper layer was aspirated, warmed to room temperature and read at 520 nm for estimation of free proline. The corresponding concentration of proline was determined against the standard curve of L-proline and expressed in mg g^{-1} DW.

Ascorbate (AsA), dehydroascorbate (DHA), total ascorbate (AsA + DHA) and AsA:DHA ratio were estimated by the method of Law *et al.* (1983). One g fresh tomato leaf was homogenized in 4 mL of extraction buffer (0.1 M Na-phosphate, pH 7, 1 mM EDTA). Homogenate was spun at 7826 *g* for 10 min. The supernatant was divided in two parts for the estimation of total ascorbate and ascorbate (reduced form). To 0.8 mL of supernatant, 0.4 mL of 10% (w/v) TCA was added in both the sets. After 5 min 0.02 mL of 5 M NaOH was added. A mixture of 400 µL of supernatant, 400 µL of Na-phosphate buffer (150 mM, pH 7.4) and 400 µL of DDW was prepared. Reduced form of ascorbic acid was determined in the second set. To 400 µL of supernatant, 400 µL of Na-phosphate buffer and 100 µL of 10 mM dithiothritol (DTT) was added. The mixture was left at room temperature for 15 min and added with 100 µL of 0.5% (w/v) N-ethylmaleimide. The samples were incubated at 24°C for 1 min. To each sample, 800 µL of 10% (w/v) TCA, 800 µL of 44% (v/v) H_3PO_4, 800 µL of 4% (w/v) bipyridyl and 400 µL of 3% (w/v) $FeCl_3$ were added, followed by 1h incubation at 37°C. The absorbance was read at 525 nm.

Glutathione is one of the major products of sulfur assimilation pathway. It occurs in forms. Content of reduced (GSH) and oxidised (GSSG), total glutathione (GSH + GSSG) and GSH:GSSG ratio were determined by the method of Anderson (1985). One g fresh tomato leaf was ground in 4 mL of 5% (w/v) sulphosalicylic acid, spun at 7826 *g* at 4°C for 10 min. An equal amount of reaction buffer (0.1 M Na-phosphate, pH 7, 1 mM EDTA) and 0.04 mL of 0.15% (w/v) 5,5-dithiobis-2-nitrobenzoic acid (DTNB) were added to the supernatant. The samples were incubated at room temperature for 2 min and read at 412 nm. In the same samples, glutathione reductase mediated reaction was performed using 0.04 mL of 0.4% (w/v) NADPH. The reaction was performed at 25°C for 30 min and read at 412 nm to determine the total glutathione. All calculations were made on dry weight (DW) basis and expressed as µg −SH g^{-1} DW.

Antioxidant Enzymes Assay

Superoxide dismutase (SOD) activity was measured through estimation of supernatant ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT). SOD assay was performed by the method of Dhindsa *et al.* (1981). A 0.2 g tomato leaf was homogenized in 2 mL of extraction buffer (0.5 M Na-phosphate buffer, pH 7.3, 3 mM EDTA) followed by a spun at 11269 *g* for 5 min at 4°C. The assay of 0.1 mL enzyme extract was performed in a reaction buffer (0.1 M Na-phosphate buffer, pH 7.5) containing appropriate amounts of 20 mM methionine, 1 M $NaHCO_3$, 2.25 mM NBT, 3 mM EDTA and, 60 µM riboflavin. The reaction was performed under 15 W inflorescent lamp at 28°C. A 50% protection of NTB from photo-reduction was considered as one unit of enzyme activity.

Ascorbate peroxidase (APX) was measured by measuring a fall the in concentration of ascorbic acid using the method of Nakano and Asada (1981). One g tomato fresh leaf was grinded in 5 mL of extraction buffer (0.1 M potassium-phosphate, pH 7, 3 mM EDTA) and spun at 7826 *g* for 10 min at 4°C. APX activity was measured at 290 nm for 5 min in 0.1 mL of enzyme extract in a buffer containing appropriate amounts of 0.5 mM ascorbate, 0.1 mM H_2O_2 and 0.1 mM EDTA. One EU exhibits the amount of APX that degraded 1 µmol of ascorbate per min.

GR assay was performed by the method of Rao (1992). One g fresh tomato leaf was ground in 5 mL of extraction buffer (0.1 M Na-phosphate, pH 7.0, 3 mM EDTA) and spun at 7826 *g* for 15 min/4°C. GR activity was

assayed in the supernatant mediated by NADPH and read at 340 nm. A 100 µL supernatant was assayed in a reaction mixture containing appropriated volumes of 0.2 mM NADPH and 0.5 mM GSSG at 25°C for 5 min. One enzyme unit exhibits the quantity of GR enzyme necessary to decomposed 1 μmol of NADPH per min.

Method of Aebi (1983) was used to estimate the levels of CAT. One g of fresh tomato leaf was ground in 5 mL of extraction buffer (0.5 M Na-phosphate, pH 7.3) followed by a spin at 7826 g/4°C for 15 min. CAT activity was measured in 100 µL of supernatant by reading the fall in H_2O_2 concentration at 240 nm in a reaction buffer (0.4 M Na-phosphate, pH 7.2) for 5 min. One EU represents the amount of catalase required to decompose 1 µmol of H_2O_2 per min.

Method of Habig and Jacoby (1981) was used to estimate the activity of Glutathione S-transferase (GST). One g of fresh tomato leaf was ground in 5 mL of extraction buffer (0.5 M Na-phosphate, pH 7.4) followed by a spin at 7826 *g*/4 °C for 15 min. GST activity was measured at 340 nm in 100 µL of supernatant in a reaction mixture (100 mM potassium phosphate buffer, pH 6.5), 1 mM EDTA, 1 mM reduced glutathione, 1 mM 1-chloro 2, 4-dinitrobenzene (CDNB). One EU presents the amount of GST producing GS-DNB complex min^{-1} mg^{-1} protein.

ATP-sulfurylase Activity

The *in vitro* ATP-Sulfurylase (ATPS) activity was performed by the method of Wilson and Bandurski (1958). One g of fresh tomato leaf was ground in 10 mL of extraction buffer (120 mM Tris–HCl, pH 8.0) containing appropriate amounts of $MgCl_2$, KCl, DTE and $MgCl_2 \cdot 6H_2O$. The homogenate was spun at 7984 *g*/4°C for 15 min. The activity of ATPS was determined in 40 µL of the supernatant in 200 µL of reaction mixture (150 µL of 0.5 M Tris buffer, pH 8.0, 200 µL DDW). An appropriate amount of $MgCl_2 \cdot 6H_2O$ (40 mM), Na_2ATP, Na_2MoO_4, and inorganic pyrophosphatase was added accordingly. The assay mixture was incubated at 33°C for 30 min followed by the addition of appropriate amount of 2.5% ammonium molybdate solution and reducing agent (3 g of $Na_2SO_3.7H_2O$, 1 g of 1-amino-naphthol sulfonic acid, 6 g of $Na_2S_2O_5$) was added. Solution was read 660 nm after 20 min. A standard curve of KH_2PO_4 was used as a reference. One unit represents the amount of ATP-sulfurylase required for conversion of 1 µmol of APS and PPi to ATP mg^{-1} protein min^{-1}.

Protein Estimation for Enzyme Activities

Total soluble protein in 1 g of fresh leaf was estimated using Bradfords' reagent (Bradford, 1976). A standard curve of bovine serum albumin (BSA) was used as a reference. The soluble protein content thus obtained was used to calculate the content of soluble protein on dry weight basis to represent enzyme activities.

Photosynthetic Pigments

Pigments were estimated as mentioned in Klunklin and Savagae (2017). For chlorophyll and carotenoid estimation, 300 mg of fresh tomato leaf was ground in 80% chilled acetone. Absorbance of supernatant was read at three different ODs viz. 663 nm, 645 nm and 470 nm. The contents of photosynthetic pigments were estimated in fresh leaf samples and calculated according to Lichtenthaler and Buschmann (2001). Pigment content was expressed in mg g^{-1} fresh weight.

Determination of Lycopene Content

Lycopene extraction and determination was performed as mentioned in Alda *et al*. (2009). Freshly harvested tomato fruits were used in determination and estimation of lycopene content. One g of fresh fruit sample was ground and added with 50 mL of hexane:acetone:ethanol in a separating funnel for 1 h then 20 mL of DDW was added and sample was further agitated for 2 min. Two layers, polar and non-polar were formed. The absorbance of lycopene containing hexane layer (upper) was read at 472 nm and 502 nm.

Tomato Fruit Fresh Weight and Size Determination

The tomato fruits were harvested and weighed immediately to record fresh weight (g per fruit) as mentioned in Kausar *et al*. (2012). The sizes of tomato fruits from all treatments were measured using vernier caliper.

Statistical Analysis

Experiment was designed according to Randomized Block Design (RBD). Standard error (SE) represents all the estimates of sample variability. Statistical differences between pairs of means were analyzed using Student's *t* test to identify statistical differences at a confidence level of ≥ 95% through ANOVA for each set of data. Thus, the data are means ± SE from five replicates (n = 5) of at least three independent experiments. NS = non-significant, $*P \leq 0.05$, $**P \leq 0.01$.

Results

Drought-induced Oxidative Stress

The foliar health of tomato was a clear indication of the adverse impact of drought stress or its mitigation by CS treatment (Fig. 1). A comparative visual assessment made the effect of combinations of CS and drought clearly. The leaf of control was green; greener in presence of CS. Drought stress resulted into a reduced size and pale leaf color, lesser when CS was supplied during drought stress. Drought stress increased TBARS levels by 193 and 195% at 35 and 70 days after treatment (DAT), respectively.

In the presence of CS, the levels at 35 DAT and 70 DAT were declined to 70 and 32%, respectively, in comparison to control (Fig. 2).

Drought exposure of tomato increased H_2O_2 levels by 59 and 44% at 35 DAT and 70 DAT, respectively. When CS was present, the levels at 35 DAT and 70 DAT were declined to 30 and 19%, respectively, in comparison with control (Fig. 3).

Drought exposure at 35 DAT and 70 DAT increased production rate of superoxides anions by 292 and 155%, respectively. In the presence of CS, the levels were declined to 277 and 39% as compared to control at 35 DAT and 70 DAT, respectively (Fig. 4). However, CS application to control plants also increased concentration of superoxide levels by 50% at 35 DAT, but declined -13% below control at 70 DAT.

Treatment of drought increased the accumulation of free proline by 293% and 176%, respectively, as estimated on 35 DAT and 70 DAT, respectively. When CS was also supplied, the levels further increased to 244 and 188% as compared to control at 35 DAT and 70 DAT, respectively (Fig. 5). However, CS application to control plants also increased proline accumulation by 74 and 10% at 35 DAT and 70 DAT, respectively.

Exposure to water deficit declined the AsA content by approximately 36 and 59% on 35 DAT and 70 DAT, respectively. At 35 DAT and 70 DAT, the content of dehydroascorbate (DHA) content, however, increased by 93 and 70%, respectively. When CS was applied, the levels of AsA decreased by 34 and 45% on 35 DAT and 70 DAT, respectively. The DHA increased by 121% at 35 DAT and 43% at 70 DAT. In control plants, CS made no significant difference (Table 1). A general decline in total ascorbate content was recorded under drought stress. Interestingly, drought declined AsA:DHA ratio by 67 and 70% at 35 DAT and 70 DAT, respectively. In the presence of CS, this decline was 70 and 62% at 35 DAT and 70 DAT, respectively (Table 1).

Water deficit stress increased the content of glutathione (GSH) non-significantly (P>5%) at 35 DAT and 16% at 70 DAT. In the presence of CS, this increase was 65% at 35 DAT and 51% at 70 DAT. In the presence of CS, a general increase of 32% at 35 DAT and 28% at 70 DAT was noted. The levels of reduced-form of glutathione (GSSG) increased by 73% at 35 DAT and 116% at 70 DAT under drought. When CS was present, drought increased this level by 293% at 35 DAT and 272% at 70 DAT (Table 2). A non-significant (P>5%) decline in GSSG content, when CS was supplied to control plants, was noted. Water deficit resulted in an increase in total (GSH+GSSG) content 18% at 35 DAT and 33% at 70 DAT. This increase was 97% at 35 DAT and 88% at 70 DAT with CS application. An alteration in GSH:GSSG ratio was also noticed; drought stress declined GSH:GSSG ratio by 39% at 35 DAT and 46% at 70 DAT, respectively. When CS was applied, this decline was 51% at 35 DAT and 59% at 70 DAT, respectively. CS treatment to control plants increased GSH:GSSG ratio by 43% at 35 DAT and 61% at 70 DAT, respectively (Table 2).

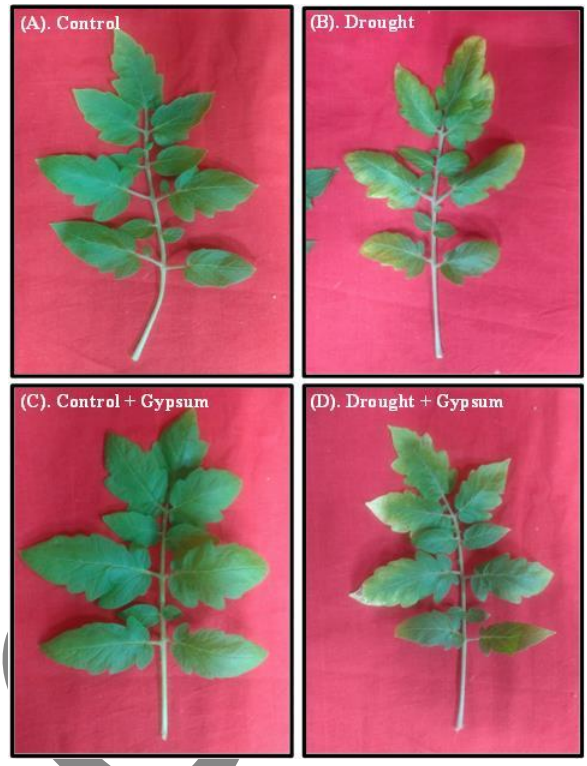

Fig. 1: Impact of drought stress on physical appearance of tomato leaves. The ameliorative effects of calcium sulfate (gypsum, CS) were clearly visible. A, Control; B, Drought, C, Control+Gypsum; Drought+Gypsum

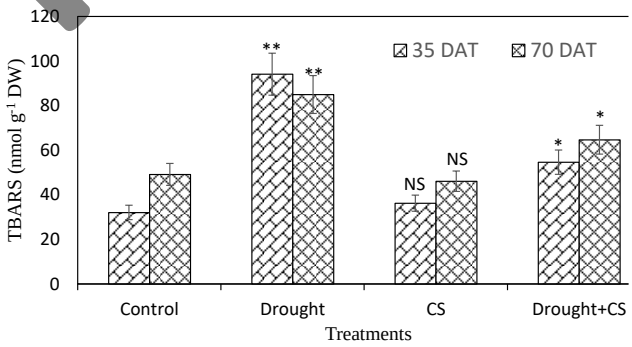

Fig. 2: Impact of drought stress on magnitude of oxidative stress (nmol g^{-1} DW TBARS) and ameliorative effects of calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P \leq 0.05$ and **$P \leq 0.01$ compared to control

Activities of Antioxidative Enzymes

Drought stress caused an increase in SOD activity by 157% at 35 DAT and 82% at 70 DAT. When CS was present, the levels were increased to 185% at 35 DAT and 97% at 70 DAT as compared to control (Fig. 6). Drought exposure

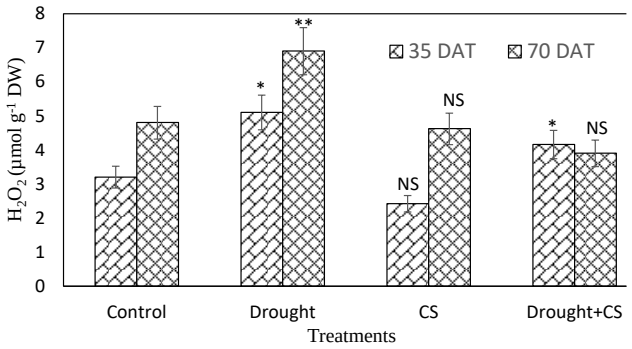

Fig. 3: Impact of drought stress on H_2O_2 concentration (µmol g^{-1} DW) and ameliorative effects of calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P\leq$ 0.05 and **$P\leq$ 0.01 compared to control

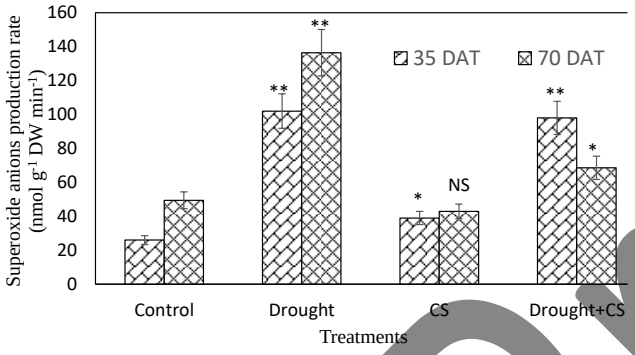

Fig. 4: Impact of drought stress on superoxide anions formation rate (nmol g^{-1} DW) and ameliorative effects of calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P\leq$ 0.05 and **$P\leq$ 0.01 compared to control

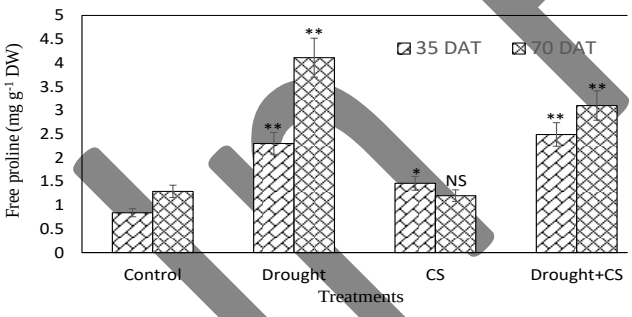

Fig. 5: Impact of drought stress on free proline accumulation (mg g^{-1} DW) and further modulations by calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P\leq$ 0.05 and **$P\leq$ 0.01 compared to control

increased the activity of enzyme APX by 123% at 35 DAT and 144% at 70 DAT. In the presence of CS, the levels were increased to 213% at 35 DAT and 126% at 70 DAT as compared to control (Fig. 7). An increase of 15-16% was observed when control plants were supplied with CS. Exposure of tomato to drought led to an increase in GR activity by 77% at 35 DAT and 79% at 70 DAT. When CS was present, the levels were increased to 109% DAT and 141% DAT in comparison to control (Fig. 8).

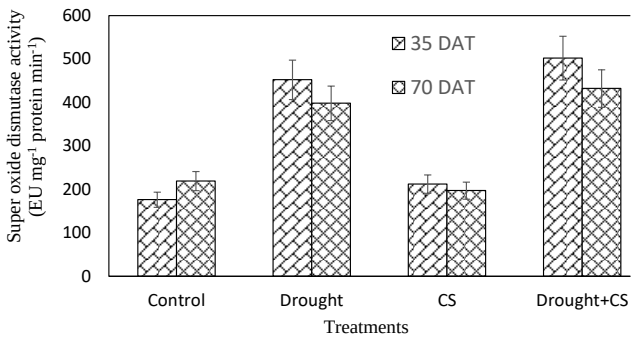

Fig. 6: Impact of drought stress on superoxide dismutase (SOD) activity (EU mg^{-1} protein min^{-1}) and further modulations by calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P\leq$ 0.05 and **$P\leq$ 0.01 compared to control

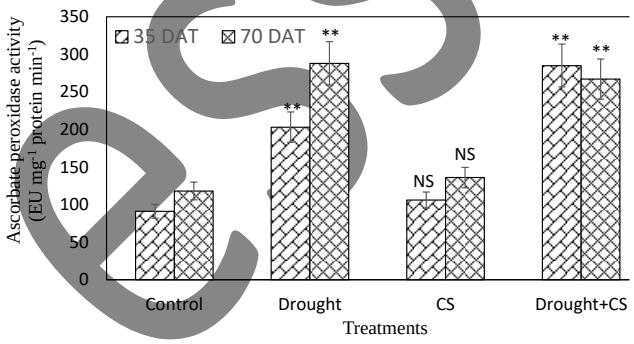

Fig. 7: Impact of drought stress on ascorbate peroxidase (APX) activity (EU mg^{-1} protein min^{-1}) and further modulations by calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P\leq$ 0.05 and **$P\leq$ 0.01 compared to control

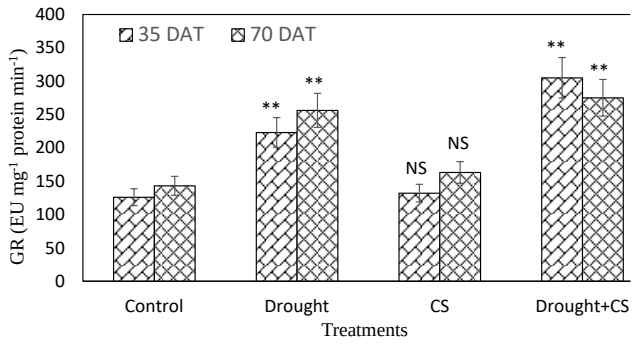

Fig. 8: Impact of drought stress on glutathione reductase (GR) activity (EU mg^{-1} protein min^{-1}) and further modulations by calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P\leq$ 0.05 and **$P\leq$ 0.01 compared to control

A non-significant increase at both stages was observed when control plants were supplied with CS. Activity of catalase (CAT) enzyme increased under drought stress. CAT activity increased by 55% at 35 DAT and 111% at 70 DAT. When CS was present, increase in CAT activity was 109% at 35 DAT and 141% at 70 DAT (Fig. 9). An increase of 23%, at 35 DAT, was observed when control plants were supplied with CS.

Table 1: Changes in contents of ascorbic acid (AsA), dehydroascorbic acid (DHA) and total ascorbic acid (AsA+DHA) under different combinations of gypsum and drought in leaf of tomato

Parameter	35 DAT				70 DAT			
	Control	Drought	Control+Gypsum	Drought+Gypsum	Control	Drought	Control+Gypsum	Drought+Gypsum
AsA	5.05±0.402	3.26±0.293	5.15±0.0318	3.32±0.231	6.98±0.712	2.86±0.280	7.25±0.867	3.83±0.247
	(00)	(–35%)**	(+2%)^{NS}	(–34%)**	(00)	(–59%)**	(+4%)^{NS}	(–45%)**
DHA	0.89±0.098	1.72±0.189	0.88±0.101	1.97±0.246	1.22±0.071	2.07±0.188	1.39±0.124	1.75±0.171
	(00)	(+93%)**	(–1%)^{NS}	(+121%)**	(00)	(+70%)**	(+13%)^{NS}	(+43%)**
AsA+DHA	5.94±0.683	4.98±0.587	6.03±0.732	5.29±0.571	8.20±0.918	4.93±0.428	8.64±0.812	5.58±0.519
	(00)	(–16%)*	(+1.51)^{NS}	(–11%)^{NS}	(00)	(–40%)**	(+5%)^{NS}	(–32%)*
AsA:DHA	5.67	1.89	5.85	1.68	5.72	1.38	5.21	2.19
	(00)	(–67%)**	(+3%)^{NS}	(–70%)^{NS}	(00)	(–76%)**	(–9%)^{NS}	(–62%)**

Values are expressed in mg g^{-1} DW. The values are the mean and standard error of the mean (mean ± SE), n = 5; *$P \leq 0.05$; **$P \leq 0.01$; NS, non-significant. Numbers in parentheses are per cent variations with respect to control. Gypsum = 2% of soil, Drought = –50% water of Control

Table 2: Changes in different attributes of thiols including GSH, GSSG, total (GSH+GSSG) and GSH:GSSG ratio under different combinations of gypsum and drought in leaf of tomato

Parameter	35 DAT				70 DAT			
	Control	Drought	Control+Gypsum	Drought+Gypsum	Control	Drought	Control+Gypsum	Drought+Gypsum
GSH	398±26	417±31	524±54	655±76	483±46	561±41	617±52	729±83
	(00)	(+5%)^{NS}	(+32%)*	(+65%)**	(00)	(+16%)^{NS}	(+28%)*	(+51%)**
GSSG	92±5.6	159±9.2	85±7.8	312±28.3	96±6.7	207±21.6	76±7.3	357±29
	(00)	(+73%)**	(–8%)^{NS}	(+293%)**	(00)	(+116%)**	(–21%)*	(+272%)**
GSH+GSSG	490±39	576±62	609±43	967±112	579±47	768±61	693±76	1086±132
	(00)	(+18%)*	(+24%)*	(+97%)**	(00)	(+33%)*	(+20%)*	(+88%)**
GSH:GSSG	4.32	2.62	6.16	2.10	5.03	2.71	8.11	2.04
	(00)	(–39%)**	(+43%)**	(–51%)**	(00)	(–46%)**	(+61%)**	(–59%)**

Values are expressed in µg −SH g^{-1} DW. The values are the mean and standard error of the mean (mean ± SE), n = 5; *$P \leq 0.05$; **$P \leq 0.01$; NS, non-significant). Numbers in parentheses are per cent variations with respect to control. Gypsum = 2% of soil, Drought = –50% water of Control

Changes were also noted in activity of GST enzyme. GST activity was deceased by the exposure of drought; GST activity deceased by 51% at 35 DAT and 41% at 70 DAT. However, when CS was present, the levels were increased by 50% at 35 DAT and 51% at 70 DAT (Fig. 10). An increase of 17% at 35 DAT and 22% at 70 DAT was observed when control plants were supplied with CS.

ATPS Activity

Changes were also noted in activity of ATP-sulfurylase enzyme. Drought exposure decreased the ATPS activity by 37% at 35 DAT and 4% at 70 DAT. However, in presence of CS, the levels were increased by 133% at 35 DAT and 171% DAT in comparison to control (Fig. 11). An increase of 99 and 200% was observed when control plants were supplied with CS, at 35 DAT and 70 DAT, respectively.

Chlorophyll Pigments

Drought exposure decreased the content of chlorophyll a (chl a) by 43% and 39% at 35 DAT and 70 DAT, respectively. This content showed lesser decreased, i.e., 24% at 35 DAT and similarly 24% 70 DAT, in presence of CS. A general increase of 25% at 35 DAT and 15% at 70 DAT was noted in control plants in the presence of CS. The levels of chlorophyll b (chl b) decreased by 40% at 35 DAT and 34% at 70 DAT under drought stress. When CS was present, drought stress decreased this level by 21% at 35 DAT and similarly 21% at 70 DAT (Table 3). An increase of 23% at 35 DAT and 14% at 70 DAT in chl b content was observed in the presence of CS. Drought stress resulted in a decrease of 42% at 35 DAT and 38% at 70 DAT in total (chl a+chl b) chlorophyll content. This decrease was 23% at 35 DAT and 18% at 70 DAT in the presence of CS. A general increase of 24% at 35 DAt and 14% at 70 DAT was noted. An alteration in chl a:chl b ratio was also noticed; drought declined this ratio non-significantly. CS treatment to control plants made no significant difference in this ratio (Table 3).

Attributes of Tomato Fruit

Drought stress increased carotenoid contents by 14%, however there was a decline of 11% when CS was applied in drought stressed plants. A non-significant (P>0.05) increase of 7% was noted in CS treated control plants (Table 4). Interestingly, content of lycopene was increased in fruits of drought stressed plants by 30%, and 46% when CS was present (Table 4). Total ascorbate content was also increased by 32% (under drought) and 25% (under drought and CS treatment). A rise of 17% ascorbate was noted when CS was provided to control plants (Table 4). A drastic decrease of 66% caused by drought was observed in tomato weight. This decrease was a bit lesser, 54%, when CS was provided to drought-stressed plants. CS supply to control plants, however, caused an increase of 26% (Table 4).

The sizes of tomato fruits were reduced by drought stress, improved by the treatment of CS and affected less when CS was present during drought stress (Fig. 12). The central axis (vertical diameter) decreased from 43 mm (Control) to 32 mm (drought). Application of CS improved this value to 49 mm from 43 mm of control. When CS was present during drought stress, value improved from 32 to 38 mm. Similarly, horizontal diameter was 32 mm (drought stress), 49 mm (CS application) and 38 mm (CS application during drought stress) as compared to 43 mm of control tomato fruits (Fig. 12).

Discussion

Drought induced oxidative stress in tomato as reflected by increased levels of TBARS (Fig. 2). TBARS is an indicator of oxidative stress in the cells. The concentration of two oxyradicals viz. H_2O_2 and superoxide anion further complimented the data of oxidative stress in tomato leaf under drought. Increase in oxidative stress could be due to closure of stomata, NADPH accumulation and lesser availability of CO_2 (Demidchik, 2015). Present study clearly showed ameliorative effects of CS against the drought-induced oxidative stress in tomato. This could be attributed to the fact that both Ca and S play central role in the plant defense (Bashir *et al*., 2013; Demidchik and Shabala, 2017).

Accumulation of free proline under drought is well known and is considered as universal phenomenon (Fu *et al*., 2018). A similar accumulation was seen under drought stress. However, a limited increase in proline accumulation in presence of CS could be due to a better homeostasis and perhaps improved moisture retention by soil.

Glutathione S-Transferase (GST) catalyzes the conjugation of the compounds produced in oxidative damage to the reduced glutathione for detoxification (Al-Huqail *et al.,* 2017). Decline in the activity of GST under drought might be due to limited availability of GSH. In the presence of CS activity of GST was improved and further increased under drought. From the data it can be inferred that availability of reduced form of GSH is detrimental to the activity of GST (Islam *et al*., 2017).

ATP Sulfurylase (ATPS) catalyzes the activation of sulfate prior to its reduction which is first committed step of sulfate assimilation (Prioretti *et al*., 2014). ATPS was declined by drought exposure which could be due the fact that plant shifts its metabolism towards tackling of oxidative and osmotic stress (Bashir *et al*., 2013). However, when higher amount of sulfate, CS, was present ATPS activity seems to be contributing in general growth and development. The CS, in fact, made plant ready with ATPS level which was not influenced by drought.

Superoxide dismutase (SOD) is an enzyme well known for its action against superoxide radical and often termed as frontline defence enzyme (del Rio *et al*., 2018). It occurs in three isoforms ranging from chloroplast to mitochondria to cytoplasm. Our data in tomato clearly indicate that activity of SOD was upregulated under drought and further increased in the CS supplied plants, however, this increase was not so prominent. It could be inferred that tomato relies on upregulation of SOD even under the lesser supply of S and Ca.

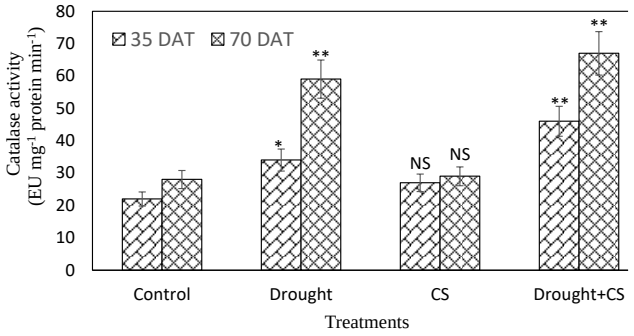

Fig. 9: Impact of drought stress on catalase (CAT) activity (EU mg^{-1} protein min^{-1}) and further modulations by calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P \leq 0.05$ and **$P \leq 0.01$ compared to control

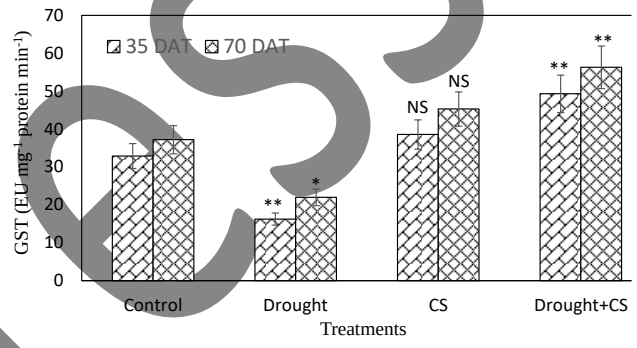

Fig. 10: Impact of drought stress on glutathione S-transferase (GST) activity (EU mg^{-1} protein min^{-1}) and further modulations by calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P \leq 0.05$ and **$P \leq 0.01$ compared to control

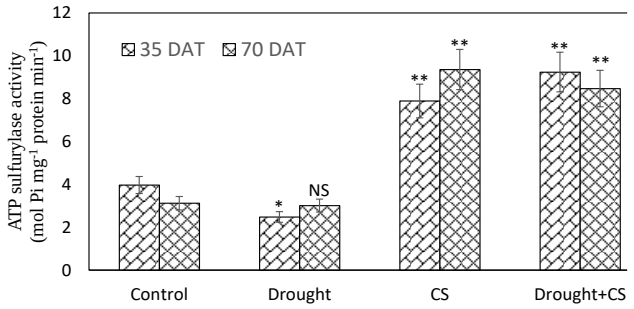

Fig. 11: Impact of drought stress on ATP-Sulfurylase activity (EU mg^{-1} protein min^{-1}) and further modulations by calcium sulfate (gypsum, CS). n = 5, NS = non-significant, *$P \leq 0.05$ and **$P \leq 0.01$ compared to control

Table 3: Changes in contents of chlorophyll a (Chl a), chlorophyll b (Chl b) and chlorophyll total (Chl a + Chl b) under different combinations of gypsum and drought in leaf of tomato

Parameter	35 DAT				70 DAT			
	Control	Drought	Control+Gypsum	Drought+Gypsum	Control	Drought	Control+Gypsum	Drought+Gypsum
Chl a	1.38±0.08	0.79±0.04	1.72±0.16	1.05±0.08	1.78±0.09	1.09±0.06	2.04±0.15	1.47±0.12
%Change	(00)	(–42.7%)**	(+24.6%)*	(–23.9%)*	(00)	(–38.7%)**	(+14.6%)^{NS}	(–23.91%)*
Chl b	0.43±0.04	0.26±0.01	0.53±0.05	0.34±0.03	0.58±0.09	0.38±0.02	0.66±0.09	0.46±0.06
%Change	(00)	(–39.5%)**	(+23.2%)*	(–20.9%)*	(00)	(–34.5%)*	(+13.8%)^{NS}	(–20.7%)*
Chl a+Chl b	1.81±0.09	1.05±0.06	2.25±0.12	1.39±0.18	2.36 ±0.18	1.47±0.06	2.70±0.12	1.93±0.18
%Change	(00)	(–42%)**	(+24.3%)*	(–23.2%)*	(00)	(–37.7%)*	(+14.4%)^{NS}	(–18.2%)*
Chl a:Chl b	3.21±0.24	3.04±0.21	3.24±0.26	3.09±0.27	3.07±0.22	2.87±0.21	3.09±0.32	3.19±0.38
%Change	(00)	(–5.3%)^{NS}	(–0.9%)^{NS}	(–3.7%)^{NS}	(00)	(–6.5%)^{NS}	(+0.6%)^{NS}	(–3.9%)^{NS}

Values are expressed in mg g^{-1} FW. The values are the mean and standard error of the mean (mean ± SE), n = 5; *$P \leq 0.05$; **$P \leq 0.01$; NS, non-significant. Numbers in parentheses are per cent variations with respect to control. Gypsum = 2% of soil, Drought = –50% water of Control

Table 4: Changes in contents of carotenoid and lycopene under different combinations of gypsum and drought in tomato fruits

Parameter	70 DAT			
	Control	Drought	Control+Gypsum	Drought+Gypsum
Carotenoid	6.43±0.348	7.32±0.412	6.86±0.452	7.12±0.692
%Change	(00)	(+14%)NS	(+7%)NS	(–11%)NS
Lycopene	32.02±2.92	41.56±4.47	34.42±2.65	46.85±4.12
%Change	(00)	(+30%)*	(+7%)NS	(+46%)**
Total Ascorbate	3213±386	4239±551	3776±408	4065±345
%Change	(00)	(+32%)*	(+17%)*	(+25%)*
Average weight (g)	57.4±7.8	19.3±2.9	72.55±8.4	36.1±3.2

Values are expressed in µg g^{-1} DW. The values are the mean and standard error of the mean (mean ± SE), n = 5; *$P \leq 0.05$; **$P \leq 0.01$; NS, non-significant. Numbers in parentheses are per cent variations with respect to control. Gypsum = 2% of soil, Drought = –50% water of Control

Ascorbate peroxidase (APX) and glutathione reductase (GR) work in coordination to maintain the dynamics of ascorbate-glutathione pathway for removal of H_2O_2 that is often accumulated because of SOD activity. Though there are many other enzymes contributing in ascorbate-glutathione pathway, APX and GR activity serves as good markers of successful response of this antioxidant pathway. Under drought, activities of both APX and GR were increased and influenced slightly by the presence of CS. Similarly, catalase (CAT) activity was increased under drought which was clearly indicated that these all enzymes were contributing in coordinated manner to curtail oxidative stress. Since CAT is absent in the chloroplasts, H_2O_2 removal could be attributed to ascorbate-glutathione pathway whereas increased CAT activity could be assigned to cytoplasmic and peroxisomal H_2O_2 (Mhamdi *et al*., 2010). Presence of CS further helped plants to tackle oxidative stress, through elevation in activities of antioxidant enzymes, in tomato plants.

Non-enzymatic components of ascorbate-glutathione pathway viz. oxidized, reduced and total ascorbate and glutathione reflects the contribution of these compounds in plant defence (Lou *et al*., 2018). A clear-cut shift of reduced form of AsA to DHA and GSH to GSSG was indicative of oxidative stress mediated conversion from reduced to oxidative forms of ascorbate and glutathione, respectively. Contrary to ascorbate, glutathione content was increased indicating the mobilization of glutathione in other metabolic defence reactions. In fact, when CS was present with control the glutathione content got increased but ascorbate.

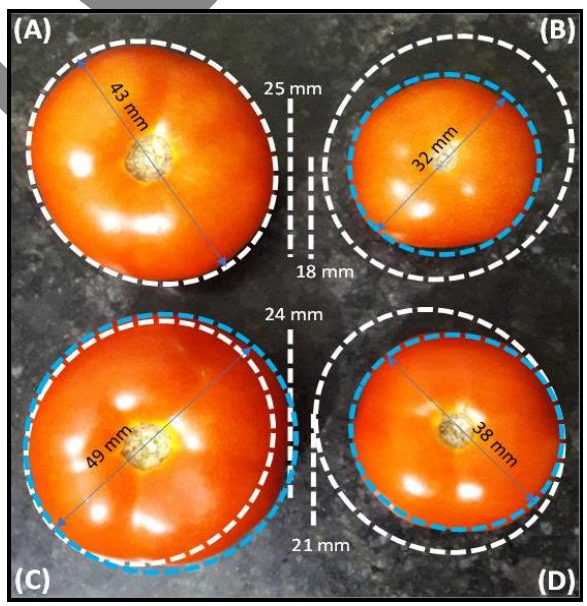

Fig. 12: Changes in tomato fruits under A. Control, B. Drought stress, C. Control+Gypsum) and D. Drought stress+Gypsum. Dotted circles are indicative of changes in the shape (volume) as compared to the control. Dotted lines are indicative of changes in the central axis and diameter as compared to the control. Data was obtained using Vernier callipers

The changed ratios of AsA:DHA and GSH:GSSG further support the role of CS in improvement of reduced state as also noted by other (Lou *et al*., 2018).

Increase in content of chlorophylls by CS further shows the beneficial role of Ca and S in anabolic reactions mainly photosynthesis. Besides that, presence of CS significantly avoided the loss of chlorophylls to the drought stress. Improvement of total chlorophyll (Chl a+Chl b) with maintenance of Chl a:Chl b ratio was a clear indicator of better photosynthesis (Guo *et al*., 2016) as observed in tomato under the CS treatment. Good yield of any crop is always an ultimate indicator of successful growth (Dreccer *et al*., 2019). The weight of fruits could be a better indicator of anabolic reactions in the plant. CS contributed for better quality tomatoes, alone or in the presence of drought. The role of Ca and S has also been shown in tomato and other fruit crops (Park *et al*., 2005; de Souza Silva *et al*., 2014; Rajasekar *et al*., 2017). However, decrease in carotenoids in the presence of CS need to be further worked out since carotenoid contents are slightly increased in presence of drought as also reported earlier, associated with drought tolerance (Nisar *et al*., 2015; Hayat *et al*., 2018). It was interesting to note that lycopene enormously increased under drought stress. There is a possibility that lycopene directly or indirectly contribute in mitigation of drought stress in tomato fruits. Accumulation of ascorbate in fruits under drought further support the fact tomato fruits were also equipped well by CS treatment hence tackle drought stress in a better manner.

Conclusion

Tomato varieties respond differently to the drought. A tomato variety 'SNU', comparatively tolerant to the drought, exhibited the modulation against drought at various levels as studied at two different growth stages. Drought treatment elevated oxidative stress; however, gypsum helped to mitigate it, besides lowering the concentrations of superoxide anions and hydrogen peroxide. Gypsum helped plants maintain osmoregulation. A fine-tuning of ascorbate-glutathione pathway and other antioxidant mechanisms including superoxide mutase, catalase and glutathione S-transferase, further helped in limiting the loss of chlorophylls and fresh weight of tomato caused by the drought stress. The amelioration extended to the tomato against drought stress could be largely attributed to the strengthening of S-assimilatory pathway and Ca-mediated signaling, offered by the gypsum.

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