



# Exogenous Glutathione-Mediated Drought Stress Tolerance in Rice (*Oryza sativa* L.) is Associated with Lower Oxidative Damage and Favorable Ionic Homeostasis

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## Abstract

Drought stress is a major concern for current agriculture as it limits plant growth and yield. An experiment was conducted with a drought susceptible rice (*Oryza sativa* L. cv. BRRI dhan29) to explore the potential functions and possible mechanisms of exogenous glutathione (GSH)-induced drought stress tolerance. Fifteen-day-old seedlings, GSH-pretreated or non-pretreated (0.2 mM GSH for 72 h), were subjected to PEG-induced (15% polyethylene glycol 6000) drought stress for 4 days. Rice plants exposed to drought stress displayed reduced growth, which was correlated with reduced chlorophyll content, water balance, antioxidant enzymes activities and mineral contents ( $K^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$ ), as evidenced by principle component analysis (PCA) and heatmap clustering. The PCA also revealed that hydrogen peroxide ( $H_2O_2$ ) and malondialdehyde (MDA) accumulations were strongly linked with the declined growth and development of rice plants under drought stress. Importantly, pretreatment with GSH improved the growth responses of rice plants to drought stress. GSH strengthened numerous physio-biochemical processes which were coupled with increased uptake of  $K^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$ , enhanced accumulation of soluble sugars, higher activities of enzymatic and non-enzymatic antioxidants. Moreover, GSH-pretreated seedlings under drought stress conditions had lower levels of  $O_2^-$ ,  $H_2O_2$  and MDA which indicates successful adaptation of rice seedlings to drought stress. The results of PCA strongly supported that GSH conferred drought tolerance mostly by enhancing antioxidant activities and mineral homeostasis. Collectively, our findings provide an important prospect for use of GSH in modulating drought tolerance in rice plants. Breeding strategies should therefore be undertaken to increase the GSH content of rice plants as a means to increase drought tolerance.

**Keywords** Water stress · Antioxidant · Oxidative stress · Glutathione · Rice · Ionic homeostasis

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## 1 Introduction

Drought stress is a pivotal constraints to global agricultural production, especially in warm, arid and semi-arid regions (Wang et al. 2016; Barik et al. 2019). By 2080, most of the world's population will directly or indirectly be affected by the reduction in crop yields caused by drought stress (Schmidhuber and Tubiello 2007). In recent years the incidence and harshness of drought have increased, most likely due to the global heating (IPCC 2014; Liu et al. 2018). For instance, in Bangladesh, the temperature has increased by up to 2.48 °C in the last four decades, and it will continue to rise in the future (Rahman and Lateh 2017). Moreover, annual rainfall has been estimated to reduce by 153 mm between 2010 and 2020 (Rahman and Lateh 2017). Thus, drought stress is considered as a serious threat for agriculture in Bangladesh, especially in the northwest part of the country (Habiba et al. 2012).

Water deficiency limits crop productivity by affecting plant growth and can also deteriorate the nutritional value of crops. Primarily, drought instigates osmotic imbalance in plants which interrupts many physiological aspects, such as enzymatic and non-enzymatic processes, stomatal conductance, membrane electron transport chain, photosynthesis and the production and scavenging of reactive oxygen species (ROS) (Lisar et al. 2012; Osakabe et al. 2014). ROS, a group of molecules that include the superoxide radical ( $O_2^-$ ), singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $OH^\cdot$ ), are produced at a low level in different cell organelles during normal physiological state, whereas under conditions of environmental stress, it's production increase by several folds (Demidchik 2015; Hossain et al. 2015; Mignolet-Spruyt et al. 2016; Czarnocka and Karpiński 2018). Lower amount of  $H_2O_2$  accumulation in plant cells activates defensive signaling pathways in plants, whereas higher concentrations are detrimental for cells and cause programmed cell death (Dat et al. 2000; Hossain et al. 2015), and can inactivate Calvin cycle (Halliwell 2006) and other vital enzymes (Das and Roychoudhury 2014). Overproduction of ROS can also result in damage to membrane lipids (Das and Roychoudhury 2014) and leads to the generation of malondialdehyde (MDA), which can cause protein degradation, disruption of membrane fluidity and inhibition of ion transport. Moreover, MDA is often considered as a pivotal hallmark of the level of oxidative damage in plants (Li et al. 2013; Awasthi et al. 2017; Tahjib-Ul-Arif et al. 2018a, b, c).

Plants have evolved mechanisms to restrict the detrimental consequence of drought-induced oxidative burst by scavenging excessive ROS through enzymatic, such as catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POX), glutathione peroxidase (GPX),

glutathione *S*-transferase (GST), etc., and non-enzymatic antioxidants, such as ascorbic acid (AsA), glutathione (GSH), carotenoids (Car), phenolic compounds (total phenolic compounds; TPC) and proline (Pro) (Gill and Tuteja 2010; Das and Roychoudhury 2014; Czarnocka and Karpiński 2018; Tahjib-Ul-Arif et al. 2019a, b). Under water deficit condition, plants also accumulate osmolytes, such as Pro and soluble sugars, that help to maintain cellular water balance and regulate essential ions (e.g.  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ , etc.) uptake (Ashraf and Foolad 2007; Krasensky and Jonak 2012). Otherwise, drought-induced water loss can cause changes to the cation–anion ratio that lead to a loss of cell turgor, intervention of cell division, reduction in photosynthesis, and imbalances in carbon metabolism (Liu et al. 2005; Miller et al. 2010).

Glutathione (GSH), a tripeptide ( $\gamma$ -Glu-Cys-Gly) which can function as an antioxidant and rescue plant cells from ROS-induced oxidative injury (Gill and Tuteja 2010), has numerous roles in plant cells including stress tolerance, regulating cellular redox balance, modulating the expression of stress-related genes, xenobiotic detoxification, thiol group protection, influencing enzyme activity, protein and nucleotide synthesis, heavy metal chelation, plant growth and senescence (Gill et al. 2013; Gietler and Nykiel 2017; Hasanuzzaman et al. 2017). Moreover, transgenic plants over-expressing GSH biosynthetic genes displayed better salinity tolerance (Bae et al. 2013; Choe et al. 2013). These multiple roles of GSH in plants indicate that the manipulation of GSH levels and metabolism has the potential to enhance stress tolerance of plants (Gill and Tuteja 2010). Recently, exogenous application of GSH has received considerable attention by the scientific community due to its involvement in the enhancement of abiotic stresses tolerance by restricting the entry of toxic ions such as mercury, cadmium, excess sodium, etc. (Ding et al. 2017; Kim et al. 2017; Zhou et al. 2018), enhancing antioxidant defences, and by altering other physio-biochemical attributes (Ding et al. 2016; Zhou et al. 2017).

Rice is the second most important staple food worldwide and the most important food in Bangladesh, where drought stress can cause 30–60% yield losses (Ouk et al. 2006; Khoury et al. 2014; BBS 2018). Among the rice cultivars in Bangladesh, BRRI dhan29 is consider as a popular mega variety due to its acreage and yield potentiality. However, BRRI dhan29 is susceptible to drought stress and the county frequently experience significant yield loss due to drought stress (Islam et al. 2017). It is well established that drought stress impaired essential mineral nutrient uptake and translocation from root to shoot due to reduced transpiration and limited transporter activity (Silva et al. 2011; Bista et al. 2018; Chen et al. 2019), however, the roles of GSH on ion accumulation and translocation remains elusive. To the best of our knowledge, this is the first report

describing the possible intriguing roles of exogenous GSH on ion homeostasis and ROS metabolism in rice plants under drought stress.

## 2 Materials and Methods

### 2.1 Plant Growth Conditions and Treatments

Rice (cv. BRR1 dhan29) seeds were sterilized with 2.5% sodium hypochlorite and 2% tween-20 solution for 15 min and were further washed four times with deionized water. The seeds were then imbibed in deionized water for 24 h. Following imbibition, the seeds were placed in petri dishes and kept for 2 days at  $28 \pm 2$  °C in the dark to allow germination. After germination, 150 germinated rice seeds were sown on floating nets in 350 mL plastic pots, containing distilled water. From the 3rd day of sowing, the rice seedlings were grown in modified hydroponic Cooper's nutrient solution (Cooper 1988) as described in Tahjib-Ul-Arif et al. (2019a) in a growth chamber (temperature of  $25 \pm 2$  °C and relative humidity of 65–70%). Used solution was changed at 3-day intervals for the duration of the experiment.

Twelve-day-old rice seedlings were pretreated with 0.2 mM GSH, in the nutrient solution, for three days. After pretreatment, 15-day-old GSH-pretreated and untreated rice seedlings were subjected to drought stress (induced by 15% polyethylene glycol 6000; PEG) for a duration of 4 days. GSH-pretreated seedlings were also grown in nutrient solution without PEG. In summary, the study comprised a total of four treatments; (1) Control (C): 0% PEG + 0 mM GSH, (2) Drought (D): 15% PEG + 0 mM GSH, (3) GSH: 0% PEG + 0.2 mM GSH and (4) D + GSH: 15% PEG + 0.2 mM GSH. Randomized complete block design with five independent replicates for each treatment was used to carry out the experiment. Different morphological and biochemical parameters of the rice seedlings were measured after 4 days of drought stress treatment.

### 2.2 Determination of Plant Growth Parameters, Relative Water Content (RWC), Pro, Photosynthetic Pigments and Carotenoid (Car) Contents

Plant growth parameters and RWC were measured according to Tahjib-Ul-Arif et al. (2018b, c). The methods of Zhang and Huang (Zhang and Huang 2013), with minor modifications, were used to measure the Pro content of rice leaves and standard curve were produced with analytical grade Pro.

Chlorophylls (Chl *a* and Chl *b*) and Car were extracted from the 2nd leaves (0.5 g) of the rice seedlings, by soaking in acetone (10 mL and 80% acetone) for 7 days inside the dark chamber. The absorbance of the acetone extracts was measured at 645, 663 and 470 nm, using a UV–Vis spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan) and Chl *a*, Chl *b* and Car contents were determined according to the method of Lichtenthaler (1987).

### 2.3 Visual Detection of Superoxide and Determination of H<sub>2</sub>O<sub>2</sub> and MDA Contents

To visualize O<sub>2</sub><sup>-</sup> in rice leaves, the 3rd leaves of rice seedlings were soaked in 0.1% nitroblue tetrazolium (NBT) reagent and stored in the dark for 12 h, according to the method of Tahjib-Ul-Arif et al. (2019a). MDA content (product of lipid peroxidation) was measured in the 3rd leaves of the seedlings according to the method of Heath and Packer (Heath and Packer 1968), using an extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

### 2.4 Determination of Activities of Antioxidant Enzymes

Three antioxidant enzymes activities were determined in extracts from the 3rd leaves of rice seedlings. Fresh leaf samples (0.05 g) were homogenized with 1 mL of 50 mM potassium-phosphate buffer (pH 8.0), using pre-chilled mortars and pestles. The homogenates were centrifuged at  $11,500 \times g$  for 10 min, and the resultant supernatants were collected to analyze the activity of CAT (EC 1.11.1.6) (Aebi 1984), APX (EC 1.11.1.11) and POX (EC: 1.11.1.7) (Nakano and Asada 1981). All procedures were performed at 0–4 °C, and all of the spectrophotometric assays were performed using a UV–Vis spectrophotometer (Shimadzu, UV-1201, Kyoto, Japan).

### 2.5 Determination of Total Soluble Sugars, Total Phenolic Compounds and Ascorbate

Total soluble sugars (TSS) were determined using the modified anthrone method as previously reported by Cihra and Brun (1978). Dried shoot samples (0.3 g of finely powdered sample) were extracted with 10 mL of 80% ethanol, and the resultant extract was filtered through Whatman filter paper (11 µm pore size) to remove particulates. 1 mL of 1:10 diluted extract was then mixed with 4 mL of 2% anthrone solution, and the mixture was then heated for 10 min and then cooled on ice. The absorbance of the solution was then measured at 620 nm using a UV–Vis spectrophotometer (Shimadzu, UV-1201, Kyoto,

Japan). The TSS contents of the samples were determined from the standard curve prepared using glucose solutions of different concentrations ( $1.0 \text{ mg mL}^{-1}$ ,  $0.8 \text{ mg mL}^{-1}$ ,  $0.6 \text{ mg mL}^{-1}$ ,  $0.4 \text{ mg mL}^{-1}$  and  $0.2 \text{ mg mL}^{-1}$ ).

The TPC was determined according to the method of Singleton and Rossi (1965), using the Folin-Ciocalteu reagent. A standard curve was constructed using gallic acid as a standard, and the results were expressed as  $\mu\text{g}$  of gallic acid equivalents (GAE) per gram of DW ( $\mu\text{g GAE g}^{-1}$  DW). The AsA content was determined according to the method of Jagota and Dani (1982). AsA was used to construct a standard curve, and the results are expressed as  $\text{nmol g}^{-1}$  FW.

### 2.6 Determination of $\text{Na}^+$ , $\text{K}^+$ , $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ Ion from Shoot Tissues

After harvesting, rice seedlings were washed rigorously with deionized water to eliminate surface contaminants. Seedlings were then oven dried at  $70^\circ\text{C}$  for 4 days, and the finely powdered plant material was digested with  $\text{HNO}_3$ :  $\text{HClO}_4$  (2:1v/v) mixture at  $220^\circ\text{C}$  for 2 h according to the method of Tahjib-UI-Arif et al. (2018b).  $\text{Na}^+$  and  $\text{K}^+$  contents were quantified by flame photometry (Jencon PFP 7, JENCONS-PLS, UK).  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  contents were measured by titration, using ethylenediaminetetraacetic acid disodium salt according to the methods of Schmid and Reilley (1957).

### 2.7 Statistical Analysis

A one-way analysis of variance was performed using Minitab 17.0. Different letters denote the statistically significant differences between treatments at  $P < 0.05$ , according to the Tukey's honest significant differences test. The heatmap and Euclidean complete linkage-based cluster analyses were performed using MetaboAnalyst 4.0 ([www.metaboanalyst.ca](http://www.metaboanalyst.ca)) and the principal component analysis (PCA) was constructed using R 3.5.3.

## 3 Results

### 3.1 GSH-Pretreatment Improved Plant Growth and Development Under Drought Stress

A significant ( $P < 0.05$ ) reduction in shoot length (SL), root length (RL), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW) and root dry weight (RDW) (38.65, 32.86, 67.61, 44.05, 43.23 and 56.06%, respectively) was found in rice seedlings in

response to drought stress as compared to unstressed control (Table 1). The pretreatment of unstressed seedlings with exogenous GSH did not influence SL, RL, SFW, RFW, SDW and RDW as compared to control plants. However, GSH-pretreated drought-stressed seedlings showed significant increases in SL, RL, SFW, RFW, SDW and RDW (42.68, 34.27, 182.50, 25.20, 30.68 and 130.17%, respectively) when compared to seedlings subjected to drought stress only (Table 1).

### 3.2 GSH-Pretreatment Protected Photosynthetic Pigments Under Drought Stress

Under drought stress, Chl *a*, Chl *b* and total Chl contents were decreased significantly (55.97, 20.61 and 49.73%, respectively) compared with control seedlings (Table 1). In contrast, GSH-pretreated drought-stressed seedlings showed a marked increase in Chl *a*, Chl *b*, and total Chl levels (83.27, 25.39 and 67.14%, respectively) as compared to seedlings under drought stress only. GSH pretreatment in well-watered rice seedling enhanced the Chl *a*, Chl *b*, and total Chl contents by 36.54, 117.37 and 50.81%, respectively, when compared with untreated control seedlings (Table 1).

### 3.3 GSH-Pretreatment Elevated RWC and TSS Whereas Declined Pro Contents Under Drought Stress

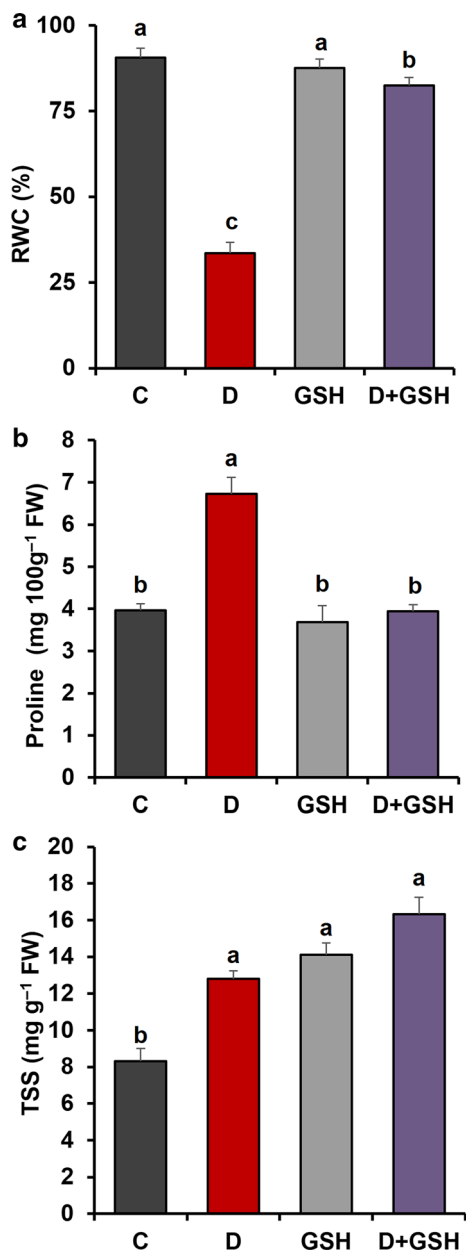
The RWC and Pro content are important parameters for assessing the physiological water status of plants. RWC and Pro showed an inverse relationship in drought-stressed rice seedlings. In response to drought stress, the RWC decreased by 62.97% whereas the Pro content showed a significant ( $P < 0.05$ ) increase of 69.69% when compared to control seedlings (Fig. 1a, b). In contrast, GSH-pretreated drought-stressed seedlings showed an increase in RWC content of 145.75% when compared to only drought-stressed seedlings, while the Pro content showed a significant decrease of 41.44% when compared to control seedlings (Fig. 1a, b). In addition, RWC and Pro in unstressed control and GSH-pretreated unstressed seedlings showed no significant changes.

Drought stress significantly ( $P < 0.05$ ) increased the shoot TSS content of 53.75% when compared to control seedlings. In contrast, GSH-pretreated seedlings displayed further increase in TSS of 27.61% when compared to drought-stressed seedlings. GSH-pretreated unstressed seedlings showed a significant escalation in TSS content of 69.83% when compared to untreated control seedlings (Fig. 1c).

**Table 1** Effects of exogenous glutathione (GSH) on growth and biomass of rice seedlings grown under PEG-induced drought stress for 4 days

Treatments	Shoot length (cm)		Root length (cm)		Fresh weight (mg)		Dry weight (mg)		Chlorophyll content (mg g <sup>-1</sup> FW)		
	Shoot length (cm)	Root length (cm)	Shoot	Root	Shoot	Root	Shoot	Root	Chl <i>a</i>	Chl <i>b</i>	Chl <i>a + b</i>
Control	13.36 ± 0.40 <sup>a</sup>	14.20 ± 0.17 <sup>a</sup>	82.33 ± 0.98 <sup>b</sup>	75.67 ± 1.44 <sup>a</sup>	15.50 ± 0.24 <sup>a</sup>	8.80 ± 0.31 <sup>a</sup>	0.143 ± 0.02 <sup>b</sup>	0.030 ± 0.009 <sup>c</sup>	0.174 ± 0.03 <sup>c</sup>		
PEG (15%)	8.25 ± 0.23 <sup>c</sup>	9.53 ± 0.21 <sup>c</sup>	26.60 ± 0.72 <sup>d</sup>	42.33 ± 1.19 <sup>c</sup>	8.80 ± 0.09 <sup>c</sup>	3.86 ± 0.39 <sup>c</sup>	0.063 ± 0.01 <sup>c</sup>	0.024 ± 0.007 <sup>d</sup>	0.087 ± 0.002 <sup>d</sup>		
GSH	13.26 ± 0.25 <sup>a</sup>	13.73 ± 0.29 <sup>a</sup>	92.3 ± 0.98 <sup>a</sup>	69.69 ± 1.19 <sup>a</sup>	15.35 ± 0.31 <sup>a</sup>	7.93 ± 0.28 <sup>b</sup>	0.196 ± 0.03 <sup>a</sup>	0.067 ± 0.008 <sup>a</sup>	0.263 ± 0.04 <sup>a</sup>		
PEG (15%) + GSH	11.70 ± 0.29 <sup>b</sup>	12.80 ± 0.37 <sup>b</sup>	75.33 ± 0.72 <sup>c</sup>	53.00 ± 0.47 <sup>b</sup>	11.50 ± 0.24 <sup>b</sup>	8.90 ± 0.33 <sup>a</sup>	0.116 ± 0.01 <sup>ab</sup>	0.030 ± 0.006 <sup>b</sup>	0.146 ± 0.01 <sup>b</sup>		

Values are means ± standard errors of five biological replicates ( $n = 5$ ). Different letters in each column indicate significant difference at  $P < 0.05$  according to Tukey's test. PEG, polyethylene glycol 6000



**Fig. 1** Effects of exogenous glutathione (GSH) on **a** relative water content (RWC); **b** proline content and **c** total soluble sugars (TSS) content of rice seedlings. Plotted data represent the average of five individual replicates for each treatment ( $n = 5$ , three leaves per replicate). Vertical bar indicates the standard error. The letter on top of each bar denotes a statistically significant difference at  $P < 0.05$  (Tukey's honest significant differences test). Control (C): 0% PEG + 0 mM GSH; Drought (D): 15% PEG + 0 mM GSH; GSH: 0% PEG + 0.2 mM GSH; D + GSH: 15% PEG + 0.2 mM GSH

### 3.4 GSH-Pretreatment Reduced ROS and MDA Contents in the Shoots and Root Tissues Under Drought Stress

Drought-stressed rice seedlings showed the highest  $O_2^-$  accumulation (observed as dark blue spots within the leaf

blade) as compared to controls. In contrast, GSH-pretreated drought-stressed seedlings showed less  $O_2^-$  accumulation as compared to seedlings without drought stress (Fig. 2a).

Drought stress led to a significant ( $P < 0.05$ ) increase in MDA and  $H_2O_2$  contents of 83.20 and 37.2% in shoots, respectively, and 47.60 and 68.05% in roots, respectively, when compared to control seedlings (Fig. 2b–e). In contrast, GSH-pretreated drought-stressed seedlings showed a decrease in MDA and  $H_2O_2$  contents (22.25 and 14.40% in the shoot and 18.98 and 31.80% in root tissues, respectively) as compared to seedlings treated with drought stress only (Fig. 2b–e). Furthermore, only GSH-pretreated seedlings showed no significant changes in MDA and  $H_2O_2$  contents when compared with untreated control seedlings.

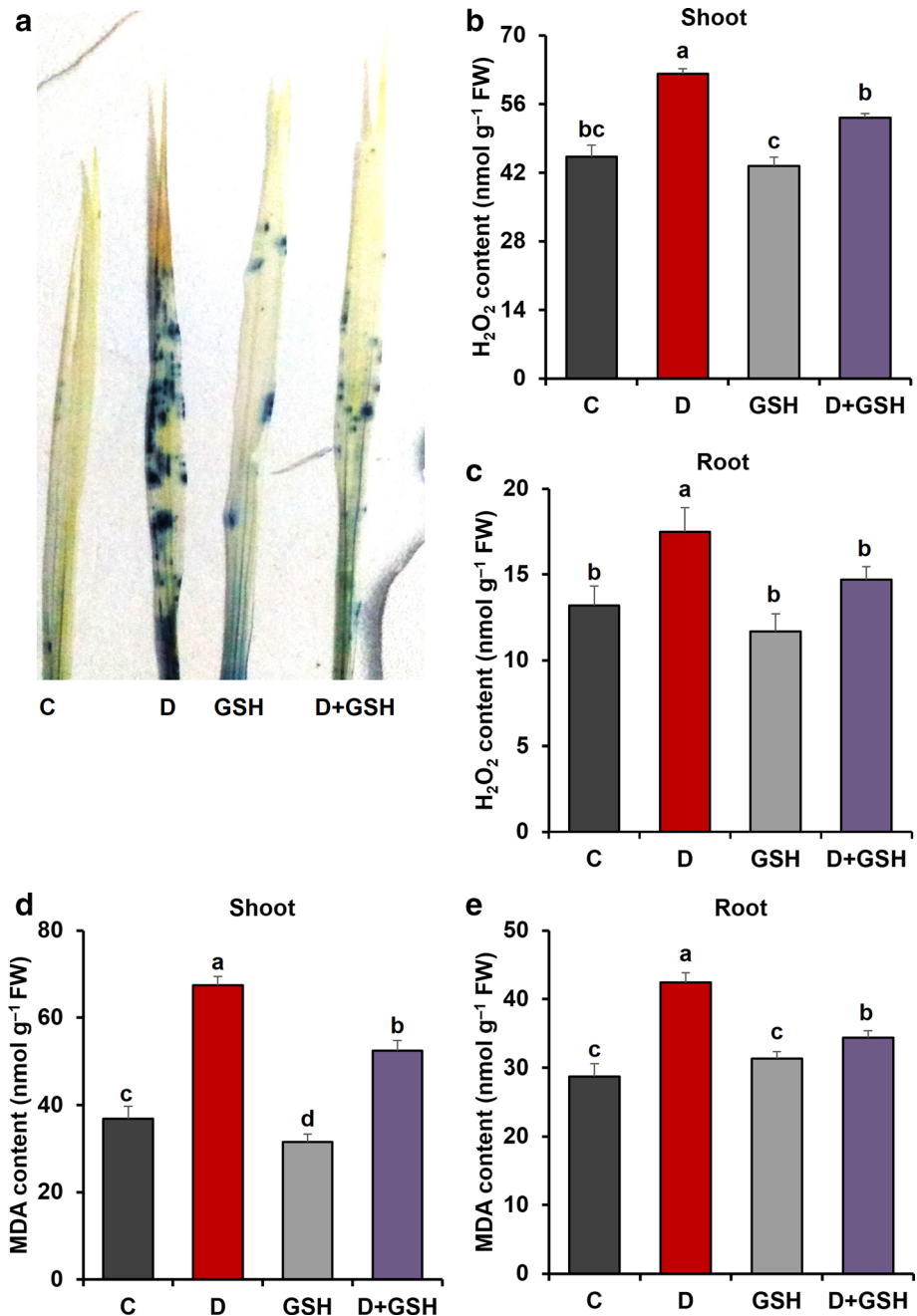
### 3.5 GSH-Pretreatment Improved Antioxidant Metabolism in Shoots and Roots of Rice Seedlings Under Drought Stress

The activities of CAT, APX and POX in the leaves and roots of rice seedlings were significantly affected by drought stress and GSH pretreatment. Drought-stressed seedlings showed a marked decline in the activities of CAT, APX and POX (30.31, 25.60 and 40.72%, respectively) in shoots, compared to untreated control seedlings. In root tissues, CAT and POX activities decreased by 60.53, and 71.21%, respectively, but APX activity increased by 109.67%, compared to untreated control seedlings (Fig. 3). In contrast, exogenous pretreatment of seedlings with GSH resulted in increases in CAT, APX and POX activities by 66.54, 63.35 and 30.60%, respectively, in shoots and by 352.70, 84.20 and 86.81%, respectively, in the roots of seedlings under drought stress, compared to the seedlings exposed to drought stress without GSH pretreatment. Moreover, GSH treatment of seedlings did not significantly alter the activities of CAT, APX and POX in the absence of drought stress (Fig. 3).

### 3.6 GSH Positively Affected Non-enzymatic Antioxidants Under Drought Stress

TPC levels showed a significant ( $P < 0.05$ ) increase of 44.17% in drought-stressed seedlings, compared to controls. GSH-pretreated unstressed or drought-stressed seedlings also showed a significant ( $P < 0.05$ ) increase in TPC contents of 39.26 and 38.65%, respectively, compared to control seedlings. However, there was no significant difference in TPC levels observed for GSH-pretreated and untreated drought-stressed seedlings (Fig. 4c). Likewise, AsA and Car contents decreased significantly ( $P < 0.05$ ) in response to drought stress by 20.76 and 54.81%,

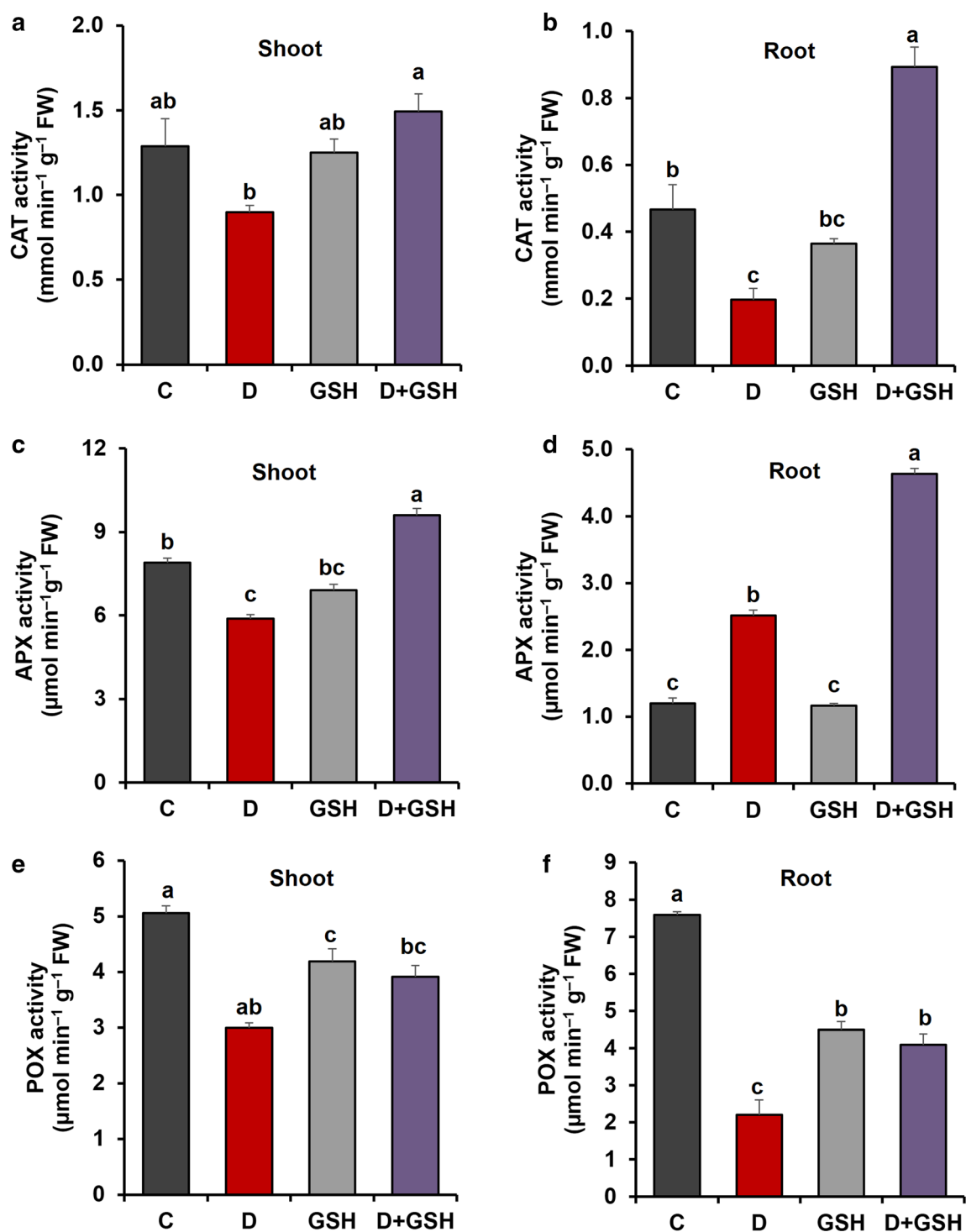
**Fig. 2** Effects of exogenous glutathione (GSH) on deposition of superoxide in leaves (a); H<sub>2</sub>O<sub>2</sub> content in shoot (b), root (c); and MDA content in shoot (d), root (e) of rice seedlings. Data represented in figures are the mean of five replicates for each treatment ( $n = 5$ , three leaves per replicate). Vertical bar indicates the standard error. Different letters represent significant differences at  $P < 0.05$  (Tukey's honest significant differences test). Control (C): 0% PEG + 0 mM GSH; Drought (D): 15% PEG + 0 mM GSH; GSH: 0.2 mM GSH; D + GSH: 15% PEG + 0.2 mM GSH



respectively, compared to control seedlings (Fig. 4a, b). In contrast, GSH-pretreated drought-stressed seedlings showed a significant ( $P < 0.05$ ) increase in AsA and Car contents of 12.41 and 152.43%, respectively, compared to the seedlings treated with drought stress only. Furthermore, GSH-pretreated seedlings in the absence of drought showed no significant change in AsA content, but significant change of 42.14% in Car content when compared to control seedlings (Fig. 4a, b).

### 3.7 GSH-Pretreatment Maintained Mineral Homeostasis of Rice Shoots Under Drought Stress

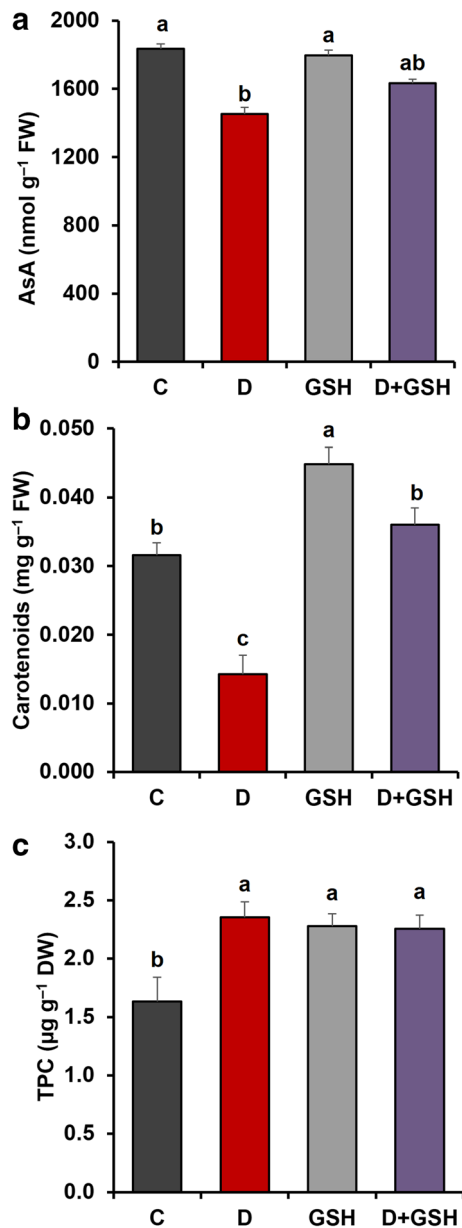
The Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> ion contents decreased by 34.91, 27.45, 19.02 and 13.00%, respectively, in drought-stressed seedlings relative to control (Table 2). However, GSH-pretreated drought-stressed seedlings showed enhance Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> ion contents of 22.92, 20.33, 56.44 and 20.48%, respectively, when compared to



**Fig. 3** Modulation of enzymatic antioxidants activity catalase (CAT) activity in shoot (a) and root (b); ascorbate peroxidase (APX) activity in shoot (c) and root (d) and peroxidase (POX) activity in shoot (e) and root (f) of rice seedlings by exogenously applied glutathione (GSH). Data represented in figures are the mean of five replicates for each treatment ( $n = 5$ , three leaves per replicate). The vertical bar

indicates the standard error. Letter on top of the bar denotes the statistically significant difference at  $P < 0.05$  (Tukey's honest significant differences test). Control (C): 0% PEG + 0 mM GSH; Drought (D): 15% PEG + 0 mM GSH; GSH: 0% PEG + 0.2 mM GSH; D + GSH: 15% PEG + 0.2 mM GSH





**Fig. 4** Effects of exogenous glutathione (GSH) on **a** ascorbic acid (AsA) content; **b** carotenoids content and **c** total phenolic compounds (TPC) content of rice seedlings. Plotted data represent the average of five replicates for each treatment ( $n = 5$ , three leaves per replicate). Vertical bar indicates the standard error. Letter on top of bar denotes the statistically significant difference at  $P < 0.05$  (Tukey's honest significant differences test). Control (C): 0% PEG + 0 mM GSH; Drought (D): 15% PEG + 0 mM GSH; GSH: 0% PEG + 0.2 mM GSH; D + GSH: 15% PEG + 0.2 mM GSH

the seedlings subjected to drought stress without GSH pretreatment. Moreover, GSH-pretreated non-stressed seedlings showed no significant differences in ion contents relative to control seedlings (Table 2).

### 3.8 Hierarchical Clustering and PCA Analysis

The morpho-physiological and biochemical data from seedlings (both drought stress and stress-free control conditions) were undergone hierarchical clustering, heat map analysis and PCA (Fig. 5). In terms of variables, three clusters (cluster-A, B and C) were formed in hierarchical clustering (Fig. 5a). The variables, POX shoot, POX root, RFW, Na<sup>+</sup>, SDW, AsA, K<sup>+</sup>, SL, RL, SFW and RWC, were clustered into cluster-A and Chl *b*, Car, Chl *a*, total Chl, RDW, CAT shoot, Mg<sup>2+</sup>, Ca<sup>2+</sup>, APX shoot, and CAT root were clustered into cluster-B. In the heat map, all the parameters of cluster-A and B displayed a significantly decreasing trend in drought-stressed rice seedlings. However, the application of exogenous GSH to drought-stressed seedlings (D + GSH) upregulated the parameters in cluster-A and B. All parameters in cluster-A and B were positively related to growth and tolerance of rice seedlings to drought stress. Thus, it can be inferred that exogenous GSH counteracted drought-induced damage. Cluster-C was comprised of H<sub>2</sub>O<sub>2</sub> shoot, H<sub>2</sub>O<sub>2</sub> root, MDA shoot, MDA root, Pro, APX root, TSS and TPC. With respect to stress-free control seedlings, these parameters exhibited increasing trends under drought stress. However, application of GSH to drought-stressed rice seedlings resulted in differential responses with respect to cluster-C parameters such as lower H<sub>2</sub>O<sub>2</sub> shoot, H<sub>2</sub>O<sub>2</sub> root, MDA shoot, MDA root, Pro values and increased values for APX activity and TSS, with no change for TPC. With respect to treatments, three clusters, cluster-X, Y and Z, were found by hierarchical clustering (Fig. 5a) and usually similar attributes clustered together. GSH application to seedlings prior to drought stress improved drought stress tolerance of rice seedlings, with 'D + GSH' being in the cluster-Y. In contrast, the only drought 'D' treatment fitted into the cluster-Z and the cluster-X consisted of the 'C', and 'GSH' treatments. It clearly shows that the application of GSH to unstressed seedlings did not have a significant effect on the different variables when compared with control seedlings. Afterward, this analysis was carried out to reveal the association among different variables with other treatment groups (Fig. 5b). The PCA biplot reveals clear segregation of the treatments to three groups (C and GSH; D; and D + GSH) and their biological replicates. The two components of PCA (PC1 and PC2) collectively explained 79.8% of data variability. The results displayed that some variables of cluster-A were intimately associated with 'C' and 'GSH' treatments, whereas other variables of cluster-A also closely associated with 'D + GSH' treatment. The cluster-B variables showed negative correlation with growth parameters and were interlinked with only 'D' treatment.

**Table 2** Effects of exogenous glutathione (GSH) on Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> contents in shoots of rice seedlings grown under PEG-induced drought stress for 4 days

Treatments	Na <sup>+</sup> content (μmol g <sup>-1</sup> DW)	K <sup>+</sup> content (μmol g <sup>-1</sup> DW)	Ca <sup>2+</sup> content (μmol g <sup>-1</sup> DW)	Mg <sup>2+</sup> content (μmol g <sup>-1</sup> DW)
Control	32.64 ± 1.85 <sup>a</sup>	194.69 ± 7.01 <sup>a</sup>	249.40 ± 8.03 <sup>b</sup>	152.59 ± 2.63 <sup>a</sup>
PEG (15%)	21.24 ± 1.22 <sup>b</sup>	142.28 ± 6.59 <sup>c</sup>	202.00 ± 10.44 <sup>c</sup>	132.76 ± 2.11 <sup>b</sup>
GSH	29.80 ± 1.26 <sup>a</sup>	188.64 ± 3.9 <sup>a</sup>	267.43 ± 8.69 <sup>b</sup>	149.13 ± 1.42 <sup>a</sup>
PEG (15%) + GSH	26.11 ± 0.92 <sup>ab</sup>	170.10 ± 5.9 <sup>b</sup>	315.58 ± 7.07 <sup>a</sup>	160.0 ± 2.89 <sup>a</sup>

Values are means ± standard errors of five biological replicates ( $n = 5$ ). Different letters in each column indicate significant difference at  $P < 0.05$  according to Tukey's test. PEG, polyethylene glycol 6000

## 4 Discussion

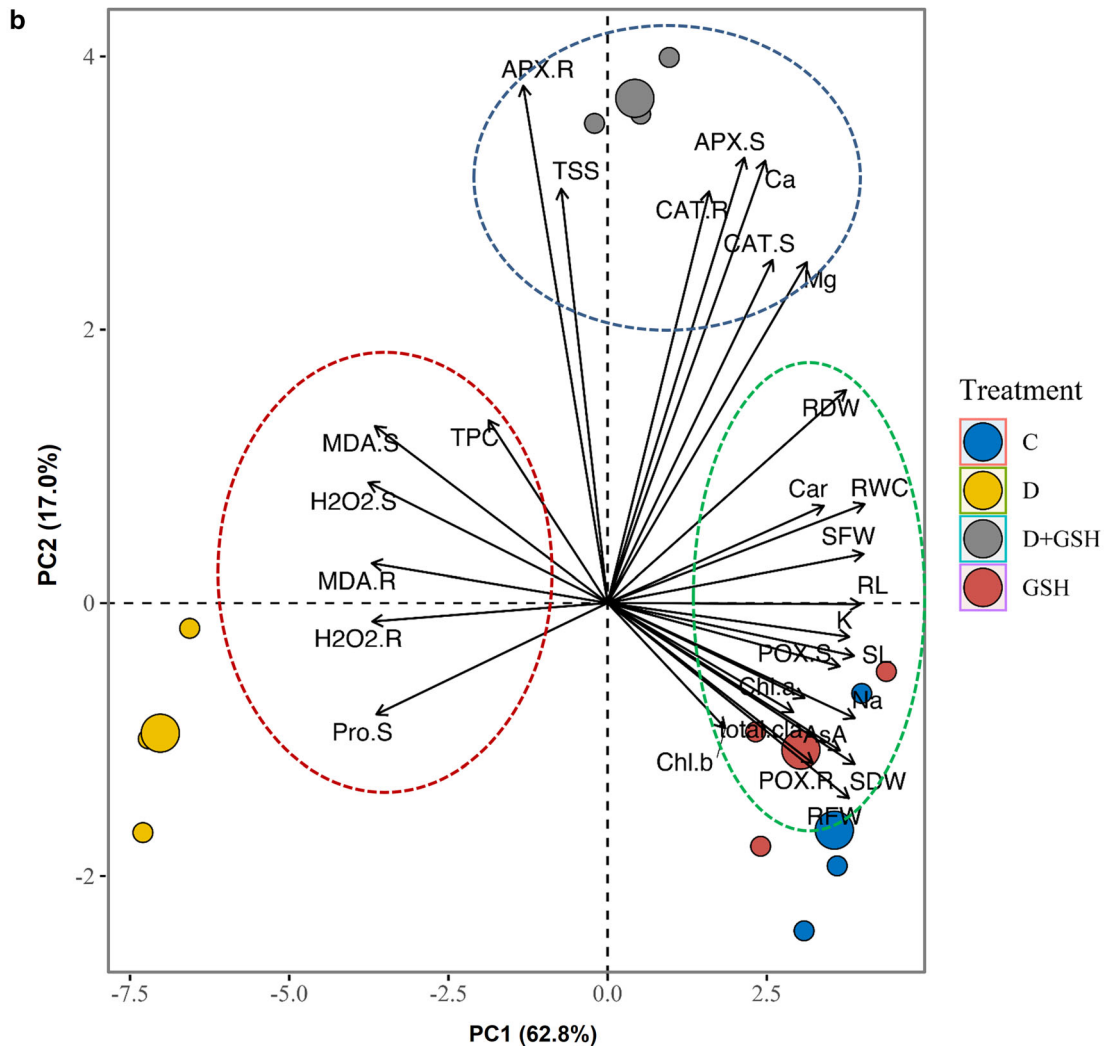
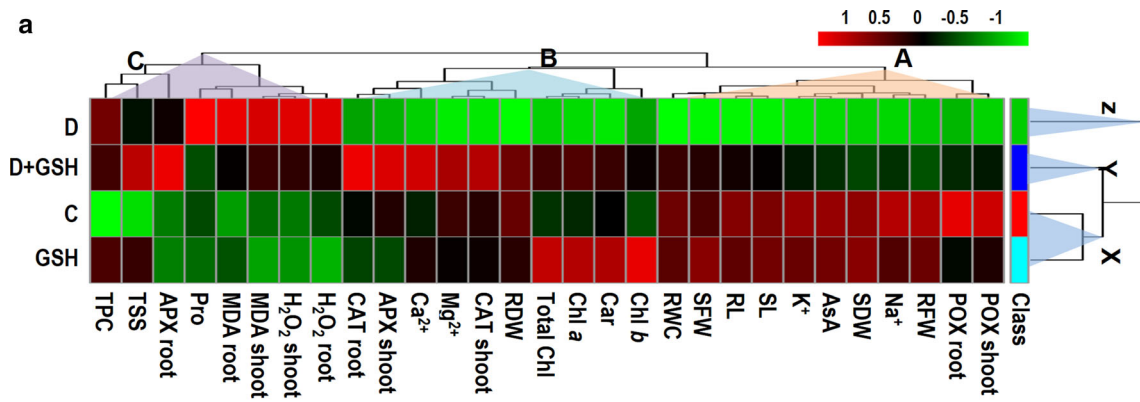
The importance of GSH for modulating physiological responses of plants under unfavorable environments has been described in a diverse plants species (Cao et al. 2017; Sabetta et al. 2017), but has not been studied so far in rice under drought stress. In the present study, GSH was found to play a critical role in enhancing drought stress tolerance by modulating physiological and biochemical traits or processes, such as root and shoot growth characteristics, photosynthetic pigments, enzymatic and non-enzymatic antioxidants, secondary metabolites and regulation of ion fluxes.

Drought stress severely inhibits plant growth and development (Salehi-Lisar and Bakhshayeshan-Agdam 2016; Fahad et al. 2017). In the present study, drought stress caused a considerable reduction in seedling growth, as reflected in reduced SL, RL, SFW, RFW, SDW and RDW (Table 1). Similar reduction in shoot and root traits was also demonstrated in rice (Sohag et al. 2020a), wheat (*Triticum durum*) (Boutraa et al. 2010) and barley (*Hordeum vulgare*) (Hellal et al. 2018). This growth inhibition might be due to lower photosynthetic pigment levels, cell dehydration, osmotic imbalance, ROS production and improper nutrient uptake (Tables 1, 2; Figs. 1a–c, 2) (Forni et al. 2017). However, GSH-pretreated drought-stressed seedlings displayed mitigation of the negative effects of drought, with improved SL, RL, SDW and RDW (Table 1). The positive influence of exogenous GSH in modulating growth and yield under drought or salinity stress has also been observed in *Arabidopsis* (Chen et al. 2012; Cheng et al. 2015), mung bean (*Vigna radiata*) (Nahar et al. 2015) and in soybean (*Glycine max*) (Akram et al. 2017). The PCA also indicated that GSH-pretreated drought-stressed rice plants showed positive relationship with growth-related parameters (Fig. 5b), suggesting protective roles of GSH in diminishing the toxic results of drought on rice seedling growth and development.

Additionally, drought-induced growth inhibition of rice plants (Table 1) might be connected with unfavorable ionic homeostasis, as water availability is directly related to

**Fig. 5** Hierarchical clustering and heatmap (a) to elucidate the treatment variable relationships in rice plants under both drought-free and drought conditions. The mean values of the various parameters obtained in this study were normalized and clustered. The color scale displays the intensity of normalized mean values of different parameters. The entire dataset was analyzed using principal component analysis (PCA) (b). The lines which is originating from the central point of biplots, display negative or positive correlations of different variables. The nearness of the lines indicates correlation strength with a particular treatment. Catalase-root (CAT.R), catalase-shoot (CAT.S), ascorbate peroxidase-root (APX.R), ascorbate peroxidase-shoot (APX.S), guaiacol peroxidase-root (POX.R), guaiacol peroxidase-shoot (POX.S) malondialdehyde-root (MDA.R), malondialdehyde-shoot (MDA.S), hydrogen peroxide-root (H<sub>2</sub>O<sub>2</sub>.R), hydrogen peroxide-shoot (H<sub>2</sub>O<sub>2</sub>. S), chlorophyll a (Chl.a), chlorophyll b (Chl.b), total chlorophyll (total chl), carotenoid (Car), ascorbate (AsA), proline (Pro.S), total soluble sugar (TSS), total phenolic contents (TPC), shoot length (SL), root length (RL), shoot dry weight (SDW), root dry weight (RDW), shoot fresh weight (SFW), root fresh weight (RFW), relative water content (RWC), sodium (Na<sup>+</sup>), potassium (K<sup>+</sup>), magnesium (Mg<sup>2+</sup>), calcium (Ca<sup>2+</sup>). Control (C): 0% PEG + 0 mM GSH; Drought (D): 15% PEG + 0 mM GSH; GSH: 0% PEG + 0.2 mM GSH; D + GSH: 15% PEG + 0.2 mM GSH

mineral uptake (Stagnari et al. 2016). Our result of PCA demonstrated a strong and negative correlations between drought treatment and uptake of minerals, such as Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> (Fig. 5b). Specifically, in this study, drought stress reduced Ca<sup>2+</sup> and K<sup>+</sup> levels in rice shoots, which in turn probably hampered growth-related features of rice plants (Table 2), because Ca<sup>2+</sup> requires in cell division, cell enlargement and cell wall formation, and K<sup>+</sup> involves in enzyme activation and photosynthesis (Hepler 2005; Silva et al. 2011). In accordance with our results, a similar decrease in essential ions in response to drought stress was reported in rice (Andrade et al. 2018) and maize (*Zea mays*) (Kaya et al. 2006). Low K<sup>+</sup> reduces the osmotic pressure of the guard cells, thereby lowering their ability to swell, and can lead to partial stomatal closure (Battie-Laclau et al. 2013). Hence, the lower K<sup>+</sup> levels observed in rice seedlings in the present study could lead to stomatal dysfunction, poor stomatal regulation of water loss and reduced photosynthesis, and subsequently, this



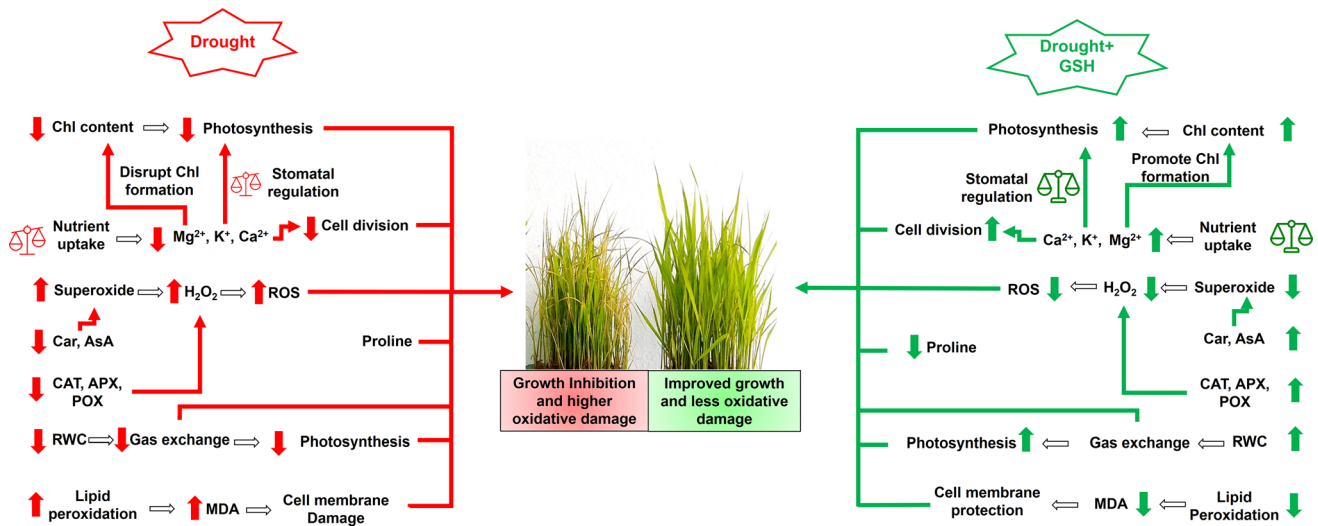
could result in poor growth of rice seedlings under drought (Table 1). A recent study suggests that optimum levels of  $\text{Na}^+$  can have a positive role in plants, as  $\text{Na}^+$  can perform some metabolic functions in plants under low  $\text{K}^+$  conditions due to the structural similarities (Maathuis 2014). Our results also showed that drought stress lowered  $\text{Na}^+$  content in rice shoots (Table 2), which was coincided with the previous studies in sunflower (*Helianthus annuus*) (Gunes et al. 2008) and tomato (*Solanum lycopersicum*) (Nahar and Gretzmacher 2002) plants under drought stress. However, pretreatment of seedlings with GSH prior to drought stress imposition enhanced  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  levels in drought-stressed rice seedlings (Table 2) and partly ameliorated the drought-induced reduction in growth of rice seedlings (Table 1). Enhancement of  $\text{K}^+$  and  $\text{Ca}^{2+}$  levels was also reported for GSH-treated salt-stressed tomato (Zhou et al. 2018) and cadmium-stressed rice (Wang et al. 2011) plants. The enhanced level of essential nutrient ions in GSH-treated drought-stressed rice seedling might be due to the modulation of genes regulating nutrient uptake and transport (Ding et al. 2017; Gheshlaghi et al. 2020).

Leaf osmotic adjustment is crucial for the survival of plants under osmotic stress which is usually regulated by the amassing compatible solutes, such as Pro and TSS (Ashraf and Foolad 2007). Thus, elevation of Pro levels is regarded as an adaptive response of drought stress (da Silva Folli-Pereira et al. 2016), and increased Pro levels were observed in the seedlings grown under drought stress only (Fig. 1b). This finding is in agreement with similar studies on rice (Dien et al. 2019). In contrast, Pro content was much lower in GSH-pretreated drought-stressed rice seedlings (Fig. 1b), which suggested that GSH might have contributed to reduce the osmotic stress. A similar finding was also reported for mung bean (Nahar et al. 2015) under salt stress. Moreover, in the present study, TSS levels in the shoots increased significantly in response to drought stress and pretreatment with GSH and subsequent imposition of seedlings to drought stress further enhanced its contents (Fig. 1c) which is in agreement with similar studies on *Haloxylon persicum* and *Haloxylon aphyllum* (Arabzadeh and Khavari-Nejad 2013) plants. In line with these findings, the RWCs of rice seedlings declined under drought stress (Fig. 1a), which is in agreement with other research findings (Kaya et al. 2006; Meher et al. 2018). However, higher RWC was observed in GSH-pretreated rice seedlings under drought stress (Fig. 1a), a similar finding was also reported for mung bean (Nahar et al. 2015). Importantly, the results of PCA revealed that TSS showed a strong positive correlation and Pro showed a negative correlation with GSH-pretreated drought-stressed plants (Fig. 5b), which let us conclude that GSH pretreatment in drought-stressed plants enhanced TSS accumulation and

maintained higher RWC, thereby reduced higher Pro accumulation in plant cells (Fig. 1).

Like osmotic stress, drought also induces oxidative stress in plants, mainly by interrupting electron flow during photosynthesis (Fahad et al. 2017). In the present study, drought stress substantially elevated  $\text{O}_2^-$ ,  $\text{H}_2\text{O}_2$  and MDA content. Similar increase in ROS levels in response to drought and other stresses in rice also reported by others (Andrade et al. 2018; Afrin et al. 2019; Wang et al. 2019; Sohag et al. 2020a, b). ROS directly attack membrane lipids and disorganize photosynthetic pigments (Gill and Tuteja 2010), which was indicated by the increased levels of MDA content and decreased levels of Chl pigments under drought stress (Fig. 2d, e; Table 1). Collectively, these findings could result in reduced plant growth and development as observed in the present study (Table 1). Particularly, our results have proven that exogenous GSH pretreatment inhibited the accumulation of ROS in plant cells (Fig. 2) and thereby maintained cell-membrane integrity and prevented Chl degradation in drought-exposed rice plants (Table 1). In agreement with our results, several recent studies were also reported the protective roles of GSH against oxidative stress in mung bean under drought stress (Nahar et al. 2015), in tomato and soybean under salinity stress (Akram et al. 2017; Zhou et al. 2017) and in cucumber (*Cucumis sativus*) under high temperature stress (Ding et al. 2016). Consequently, the PCA biplot projected that drought-stressed only rice plants had a stronger positive correlation with ROS and MDA contents in shoots and roots than GSH-pretreated drought-stressed rice plants (Fig. 5b). Besides, down-regulating the ROS-induced Chl breakdown, GSH pretreatment also upregulated the uptake of divalent cations, such as  $\text{Mg}^{2+}$  (Table 2), which might contributed to augment the Chl biosynthesis, thus maintains higher photosynthetic capacity leading to improved growth (Table 1, 2).

The severity of ROS-induced oxidative damage can be mitigated by upregulating the enzymatic and non-enzymatic antioxidants such as CAT, APX, POX, AsA, GSH, Car, TPC etc. These antioxidants exert a synergic effect to eliminate excessive drought-induced ROS accumulation and hence oxidative damage to cells (Gill and Tuteja 2010; Caverzan et al. 2016). In the present study, CAT, POX (in shoots and roots) and APX (in shoots) activity decreased, whereas the APX in roots increased in rice plants grown under drought stress (Fig. 3), which is in compliance with previous findings on rice, maize and barley plants (Sharma and Dubey 2005; Guo et al. 2006; Salekjalali and Jafari 2011; Xin et al. 2011). The non-synchronous activity of APX in root and shoot tissues of rice plants under drought stress has been reported previously (Guo et al. 2006). However, it is likely that CAT, APX and POX activities in drought-stressed seedling did not sufficient to neutralize



**Fig. 6** Schematic representation of drought-induced growth inhibition and improvement of growth by exogenously GSH pretreatment. Drought exerts its harmful effects on plants by reducing the Chl content resulting in a decreased rate of photosynthesis. It also imbalances nutrient uptake by roots influencing  $Mg^{2+}$ ,  $K^+$  and  $Ca^{2+}$  ion concentrations. Lower  $Mg^{2+}$  levels disrupt Chl formation, low  $K^+$  levels imbalance stomatal regulation and lower  $Ca^{2+}$  inhibits cell division. Water deficit increases superoxide which in turn increases  $H_2O_2$  content, contributing to excessive production of ROS. Drought induces lower levels of Car and AsA which facilitates increased superoxide levels. Lower levels of antioxidant enzymes, such as CAT, APX and POX, are also caused by drought, resulting in an increase in  $H_2O_2$ . RWC also decreases in plants under drought limiting gas exchange and photosynthesis. Lipid peroxidation is also enhanced by

drought, which damages cell membrane and promotes lipid peroxidation. In contrast, GSH pretreatment can restore the growth of drought-stressed plants and reduce oxidative damage. GSH can maintain Chl contents, help to maintain photosynthesis, and also help maintain nutrient uptake. Pretreated GSH induced increase in CAT, APX, POX, Car and AsA maintain the optimum level of ROS. GSH also increase RWC and maintained higher gas exchange and photosynthesis. GSH reduces the cell membrane damage by lowering lipid peroxidation which was confirmed through reduced MDA. Chl, Chlorophyll;  $H_2O_2$ , hydrogen peroxide; ROS, reactive oxygen species; Car, carotenoids; AsA, ascorbic acid; CAT, catalase; APX, ascorbate peroxidase; POX, guaiacol peroxidase; RWC, relative water content; MDA, malondialdehyde; and GSH, glutathione

the overproduced ROS, as also evident by the higher amounts of  $H_2O_2$  and MDA observed in drought-exposed rice plants leading to diminished seedling growth and development (Fig. 2, Table 1). In contrast, GSH pretreatment augmented the activities of these antioxidant enzymes (Fig. 3), and resulted in a lower  $H_2O_2$  and MDA accumulation under drought stress (Fig. 2b–e). Moreover, our results of PCA showed that GSH-treated drought-stressed plants had a greater positive correlations with antioxidants enzymes than those of the plants treated with drought stressed only, clearly suggesting that GSH upregulate antioxidants enzymes (Fig. 5b). A number of earlier studies have claimed that exogenous GSH application stimulates the antioxidants capacity and stress response gene expression of plants under different abiotic stresses (Chen et al. 2012; Hussain et al. 2016; Akram et al. 2017; Hasanuz-zaman et al. 2018; Pei et al. 2019).

Plant secondary metabolites including phenolics exhibits several protective mechanism against stresses (Akula and Ravishankar 2011). Our data showed that drought stress decreased the examined non-enzymatic antioxidants except TPC (Fig. 4c), thus reduced the antioxidant capacity of the rice plants and eventually plant growth hampered due to oxidative stress (Table 1; Fig. 2). It was also

evidenced in this study that exogenous GSH contributed to the removal of excess ROS by enhancing the levels of non-enzymatic antioxidants like AsA, TPC and Car (Fig. 4c). In support of this finding, GSH pretreatment showed higher levels of AsA and Car in some plant species like mung bean (Nahar et al. 2015), soybean (Seminario et al. 2017) and maize (Taïbi et al. 2016). However, in the present study, the level of TPC increased when exposed to drought stress or pretreated with GSH (Fig. 4c) and these findings are in accordance with previous reports conducted with *Hypericum pruinatum* (Caliskan et al. 2017) and garden cress (*Lepidium sativum*) (Ahmed et al. 2012) under drought and salt stress, respectively. The elevation of the TPC would increase the ROS and lipid free radicals scavenging capacity of rice plants (Gill and Tuteja 2010; Waśkiewicz et al. 2013; Das and Roychoudhury 2014).

Overall, the heatmap revealed that cluster-A and cluster-B variables showed a decreasing trend and cluster-C variables showed an increasing trend in drought-stressed seedlings, when compared with control, but displayed an inverse trend in GSH-pretreated drought-stressed seedlings, compared to that of seedlings treated with drought stress only (Fig. 5a). This provides a strong evidence that GSH pretreatment could enhance drought stress tolerance in rice

plants. Moreover, the PCA revealed that the antioxidant enzymes (CAT, APX and POD) activities and mineral contents were closely and positively associated with the 'D + GSH' treatment (Fig. 5b), which suggests that the exogenous GSH-mediated growth improvement and drought stress tolerance exclusively conferred by enhanced antioxidant activities and mineral homeostasis.

## 5 Conclusion

Based on the above findings, it can be concluded that drought stress causes severe oxidative stress in rice seedlings, as indicated by higher ROS and ROS-induced damage, as well as disruption of essential ion homeostasis and reduced seedling growth (Fig. 6). Importantly, pretreatment with GSH can help to limit the ROS-induced damage, maintain ion homeostasis and water balance, and hence results in improved growth of rice seedlings under drought through the modulation of different morpho-physiological and biochemical processes (Fig. 6). However, further studies are required to find out how GSH regulates ion homeostasis in rice seedlings under drought stress.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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