SciMedCentral

International Journal of Plant Biology & Research

Research Article

Modulation of Reactive Oxygen Species and Methylglyoxal Detoxification Systems by Exogenous Glycinebetaine and Proline Improves Drought Tolerance in Mustard (*Brassica juncea* L.)

Mohammad Anwar Hossain*[1,2], Mohammad Golam Mostofa[1], David J, Burritt[3] and Masayuki Fujita[1]

[1]*Department of Applied Biological Science, Kagawa University, Japan*
[2]*Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Bangladesh*
[3]*Department of Botany, University of Otago, New Zealand*

***Corresponding author**
Mohammad Anwar Hossain, Department of Applied Biological Science, Laboratory of Plant Stress Responses, Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan, E-mail: hossainma@gmail.com

Submitted: 07 April 2014
Accepted: 30 May 2014
Published: 02 June 2014
ISSN: 2333-6668

Keywords
- Water stress
- Oxidative stress
- Antioxidant defence
- Proline
- Glycinebetaine
- Glyoxalase system
- *Brassica juncea* L

Abstract

Drought stress severely limits crop productivity and the expansion of crop cultivation worldwide. Plants can react and adjust to water stress by shifting their cellular metabolism and invoking various defence mechanisms, and acquired stress tolerance in plants is often a result of various stress-response mechanisms that act co-ordinately or synergistically to avert cell damage and to re-establish cellular homeostasis. Metabolic adaptation via the *de-novo* synthesis of glycinebetaine or proline is often regarded as a basic strategy for the protection and survival of plants under drought stress. In the present study, we investigated the biochemical mechanisms of proline and betaine mediated drought tolerance in mustard (*Brassica juncea* L.) seedlings. Before imposition of drought stress, seedlings were fed with exogenous proline and betaine (1 mM, 24 h). Betaine or proline pre-treatment resulted in enhanced oxidative stress tolerance, as indicated by greatly reduced levels of lipid peroxidation. Endogenous hydrogen peroxide levels in proline or betaine pre-treated drought-stressed seedlings were significantly lower in comparision to seedlings exposed to drought stress without pre-treatment. A significant decrease in ascorbate peroxidase, catalase and glyoxalase II activities was found in response to drought stress, whereas dehydroascorbate reductase, glutathione peroxidase and glyoxalase I activities increased significantly. The levels of ascorbate, glutathione and the size of the glutathione disulphide pool increased significantly whereas the glutathione/glutathione disulphide ratio decreased in seedlings treated with drought stress. Importantly, drought-stressed seedlings pre-treated with betaine or proline showed significantly higher ascorbate peroxidase, glutathione reductase, catalase, glutathione *S*-transferase, and glyoxalase II activities and a higher glutathione/glutathione disulphide ratio than that of the seedlings imposed to drought stress without pre-treatment. This study proved that pre-treatment of seedlings with proline or betaine can modulate the methylglyoxal and reactive oxygen species levels and increase plant tolerance to drought-induced oxidative stress.

INTRODUCTION

The scarcity of water is a severe environmental constraint to plant productivity and the expansion of crop cultivation worldwide. One-third of the world's population resides in water-stressed regions, and with elevated CO_2 levels in the atmosphere and climatic changes predicted in the future, drought could become more frequent and severe [1]. The development of drought-resistant plants is therefore urgently needed to exploit their full genetic potential under limited soil water conditions. Thus, a deeper understanding of physiological mechanisms operating under drought conditions is a prerequisite for successful manipulation of crop plants. One of the biggest challenges to modern sustainable agricultural development is to obtain new knowledge that could allow breeding and engineering of plants with new and desired agronomical traits [2,3]. Therefore, the importance of understanding the molecular and biochemical basis of drought stress responses and tolerance is driven by both an interest in basic knowledge and the prospect that such knowledge might provide new strategies for drought stress tolerance in plants for more sustainable agricultural production.

A common consequence of exposure to drought stress is the accelerated production of reactive oxygen species (ROS) such

as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH) in the different subcellular organelles of plants [4]. Restricted entry of CO_2 in the leaves during drought stress limits CO_2 fixation and accelerates the photorespiratory pathway and finally leads to excessive H_2O_2 accumulation in the peroxisome [4]. It has been estimated that under drought stress more than 70% of total H_2O_2 accumulation is due to photorespiration [5]. Although ROS, mainly H_2O_2, can act as signals to help plants adapt to stress responses [6-9], excess ROS cause oxidative damage to plant macromolecules such as proteins, lipids and nucleic acids [8,10,11]. As a result metabolic alteration, inhibition of photosynthesis, and breakdown of cellular organization contribute to growth retardation, reduced fertility, premature senescence and even death of plants [11-13]. Therefore, the level of ROS should be judiciously regulated in plants through the coordination of ROS production and ROS scavenging systems to manage oxidative damage and simultaneously regulate signalling events [14,15], but the fundamental mechanisms are still largely unknown.

Recently, it has been demonstrated that over-accumulation of methylglyoxal (MG) in plants is a general stress response [16-18]. MG is a typical α-oxoaldehyde, which forms as a by-product of several metabolic pathways, e.g. glycolysis, lipid peroxidation and oxidative degradation of glucose and glycated proteins. It is toxic to plant cells, causing inhibition of cell proliferation, degradation of proteins and inactivation of antioxidant defence systems and as a consequence disrupts cellular functions [19-21]. Methylglyoxal accumulates in plant cells during normal physiological processes like photosynthesis; however, its levels vividly elevated under various abiotic stresses [16,18,22,23]. Saito et al. [12] reported that MG accumulated in chloroplasts during the day time from triose phoshphates, needs to be controlled by detoxification mechanisms, otherwise it will catalyse the photo reduction of O_2 to $O_2^{\bullet-}$ at photosystem I (Figure 1) and the increase in $O_2^{\bullet-}$ production during photosynthesis further aggravates oxidative damage to plant cells. MG not only directly inhibits physiological functioning, but it also inhibits it via changes in ABA synthesis in *Arabidopsis* [24,25]. Therefore, in order to survive under stressful conditions plants must up-regulate MG detoxification process to avoid cellular damage and also to keep steady state pace in different plant physiological processes.

Plants are armed with sophisticated antioxidant defence systems: both enzymatic antioxidant (multiple isoforms of Superoxide Dismutases (SOD), Ascorbate Peroxidase (APX), Monodehydroascorbate Reductase (MDHAR), Dehydroascorbate Reductase (DHAR), Glutathione Reductase (GR), Catalase (CAT), Glutathione Peroxidase (GPX), Glutathione *S*-Transferase (GST), Guaiacol Peroxidase (GPOX) and non-enzymatic Antioxidants (Ascorbate (AsA), Glutathione (GSH), tocopherol, carotenoids, flavonoids, and proline) to avoid the excessive accumulation of

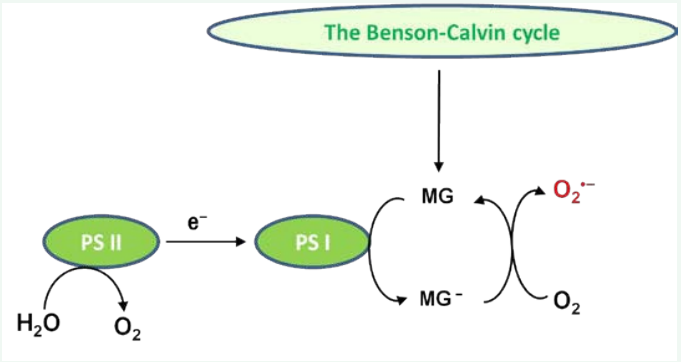

Figure 1 Methylglyoxal induced enhancement of superoxide production (O_2•−) in chloroplast (modified from Saito et al. [12].

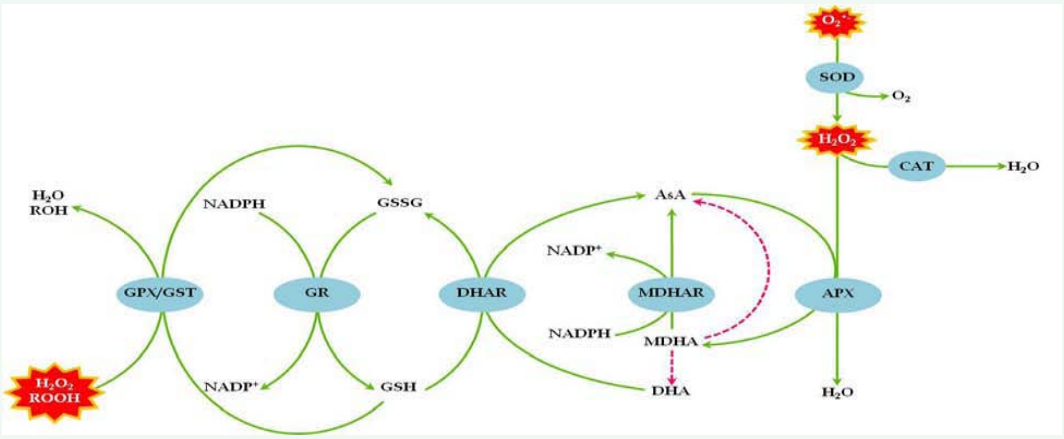

Figure 2 Reactive oxygen species detoxification systems in plants. Abbreviations are defined in the text.

ROS and to protect from oxidative damage (Figure 2), [8,11,26] in different sub-cellular organelles. The delicate balance between ROS production and scavenging that allows this duality in function to exist in plants is thought to be orchestrated by a large network of genes that tightly regulates ROS production and scavenging [6,9,27]. Additionally, the glyoxalase system play crucial role in abiotic stress tolerance by maintaining appropriate level of MG to perform its signalling functions and by regulating GSH-based ROS detoxification. A number of recent studies in plants involving stress tolerant and transgenic plants have demonstrated that both ROS and MG removal systems are equally important for stress tolerance in plants [16,18,28-30,32-37] and favourable modulation of both of these detoxification pathways rendering the plants more tolerant to various abiotic stresses.

A common physiological strategy adopted by higher plants to cope with the adverse effects of abiotic stresses, including drought stress, is the *de-novo* synthesis of large quantities of osmolytes, low-molecular-weight organic compounds, exceedingly water soluble that are non-toxic at millimolar concentration, including proline, glycinebetaine, trehalose and others [38-41]. Recent proteomic, genomic and metabolic studies have demonstrated that the function of proline is not as straightforward as initially believed. Research studies on plants, especially those on proline synthesis and catabolic gene expression have demonstrated that the proline produced under stressful conditions can act as a compatible solute in osmotic adjustment, a free radical scavenger, a metal chelator, an activator of detoxification pathways, a cell redox balancer, a cytosolic pH buffer, a source of energy, nitrogen and carbon, a stabilizer for subcellular structures and membranes including photosystem II (PS II) or act as signalling molecule [39,41-45]. Another important function of proline is that it forms a hydration shell around delicate proteins and averts their degradation under stressful conditions. Apart from its role in osmotic adjustment, betaine also involved in ROS scavenging, stabilizing macromolecules (nucleic acids, proteins, lipids) and various components of photosynthetic machinery such as PS II complexes and RuBisCO and acts as reservoir of carbon and nitrogen sources [reviewed in 46-48]. Recent microarray-based transcriptomic analyses suggest that betaine alteres the expression of many genes involved in stress responses, signal transduction, gene regulation, hormone signalling and cellular metabolisms [49,50]. Although the accumulation of proline, betaine and ROS is a common end results of biotic and abiotic insults in plants, the exact molecular and biochemical mechanisms of proline mediated plant stress tolerance with special reference to abiotic oxidative stress tolerance is still a matter of intensive research and recent plant molecular studies have demonstrated the importance of this molecule in modulating plant stress tolerance [reviewed in 41,46,47,51]. Recently, numerous studies on plants have demonstrated the possible mechanisms of proline or betaine induced oxidative stress tolerance in plants could be by enhancing reactive oxygen species detoxification system [32,33,52,53], however, there is still no evidence whether proline or betaine priming could modulate the glyoxalase system that ultimately led to drought induced oxidative stress tolerance. The prime objective of this study is therefore to investigate the effects of exogenous proline or betaine on oxidative stress tolerance induced by drought stress in the seedlings of a popular cultivar of mustard (cv. Shambal). Towards this objective, in this study a biochemical approach was employed, aiming to assess several key components of ROS and MG detoxification pathways and to explore the possible biochemical mechanisms of oxidative stress tolerance in mustard seedlings exposed to drought stress.

MATERIALS AND METHODS

Plant materials and growth conditions

Mustard (*Brassica juncea* cv. Shambal) was used as the plant material. Uniform-sized seeds were surface-sterilized with 70% ethanol then washed several times with distilled water. The seeds were then soaked with distilled water for 15 min and sown in petri dishes (9 cm in diameter) lined with 4 layers of filter paper moistened with 10 ml of distilled water for germination under dark conditions for 3 days. Seedlings were then grown using a nutrient solution (10,000-fold diluted Hyponex solution (Hyponex, Japan) in a controlled growth chamber at 25±2°C day/night temperature, at 65-0% relative humidity, with a photosynthetic photon flux density of 100 µmol photon m^{-2} s^{-1} as described previously [35].

Proline and betaine pre-treatment and drought stress treatment

After eight days of seedlings growth, they were used for proline or betaine pre-treatment. Our initial study showed that, the suitable concentration for proline and betaine pre-treatment was 1 mM. For proline and betaine pre-treatment, the root portion of the seedlings was immersed in 1 mM proline or betaine solution under light conditions for 24 h at 25°C. The controls seedlings were kept at 25°C in the light in Hyponex solution. Afterwards, the Petri dish was washed several times with de-ionized water to remove excess proline or betaine. Subsequently, proline or betaine pre-treated and non-treated seedlings were subjected to drought stress (20% PEG-6000) in Hyponex solution and grown under controlled conditions (light, 100 µmol photon m^{-2} s^{-1}; temp, 25±2°C; RH, 65–70%) for 48 h. Control plants were grown in Hyponex solution only. After 48 h of stress treatment data were taken from the leaf samples and immediately used for the analysis of different parameters.

Determination of ascorbate (AsA) and reduced glutathione (GSH) and oxidized glutathione (GSSG)

After the completion of stress treatment, mustard leaves (0.5 g fresh weight) were homogenized in 1.5 ml ice-cold acidic extraction buffer (6% metaphosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500× g for 15 min at 4°C and the supernatant were collected for analysis of ascorbate and glutathione. The AsA, GSH and GSSG content were determined following the method of Hossain et al. [32].

Enzyme extraction and activity assays

Using an extraction buffer (1 ml of 50 mM ice-cold K-phosphate buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol) the leaf samples (0.5g) were homogenized in pre-chilled mortar and pestle for extraction of enzymes. The homogenates were centrifuged at 11,500× g for 10 min and the resulted supernatants were used for determination of enzyme activity and protein content. All

procedures were performed at 0-4°C. The activities of APX, DHAR, MDHAR, GR, CAT, GST, GPX, Gly I and Gly II were determined according to the method of Hossain et al. [32].

Measurement of lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2)

The rate of lipid peroxidation was measured in leaf tissue by estimating MDA and H_2O_2 content was determined following the method of Hossain et al. [32].

Determination of protein

The protein content of each sample was determined following the method of Bradford [56] using Bovine Serum Albumin (BSA) as a protein standard.

Statistical analysis

The data obtained from three independent experiments were analyzed by one-way ANOVA using the Least Significant Difference (LSD) test at the 1% (P<0.01) probability level.

RESULTS

Effects on ascorbate (AsA) and glutathione (GSH) contents

Upon drought stress, a significant (P<0.01) increase (34%) in AsA pool was observed in relation to the control group (Figure 3A). Proline pre-treated drought-stressed seedlings showed a 9% increase in AsA whereas betaine showed a significant (P<0.01) increase (21%) as compared to the control. Both proline and betaine pre-treated seedlings showed a 18% and 9% decrease in AsA content as compared to the seedlings subjected to drought without pre-treatment.

The GSH pool significantly (P<0.01) increased (1.94-fold) in response to drought stress as compared to control group (Figure 3B). A non-significant increase in GSH content was observed in proline pre-treated drought-stressed seedlings whereas betaine pre-treated drought-stressed seedlings showed a significant (P<0.01) increase (24%) in GSH content as compared to the control group. Surprisingly, both proline and betaine pre-treated seedlings showed a 40% and 36% decrease in GSH content as compared to the seedlings subjected to drought stress without betaine or proline pre-treatment.

A robust (P<0.01) increase (4.38-fold) in GSSG content was observed in response to drought stress as compared to control (Figure 3C). Proline pre-treated drought-stressed seedlings also showed a significant (P<0.01) increase (72%) in GSSG content, whereas, a non-significant increase (42%) was observed in betaine pre-treated seedlings when compared with control.

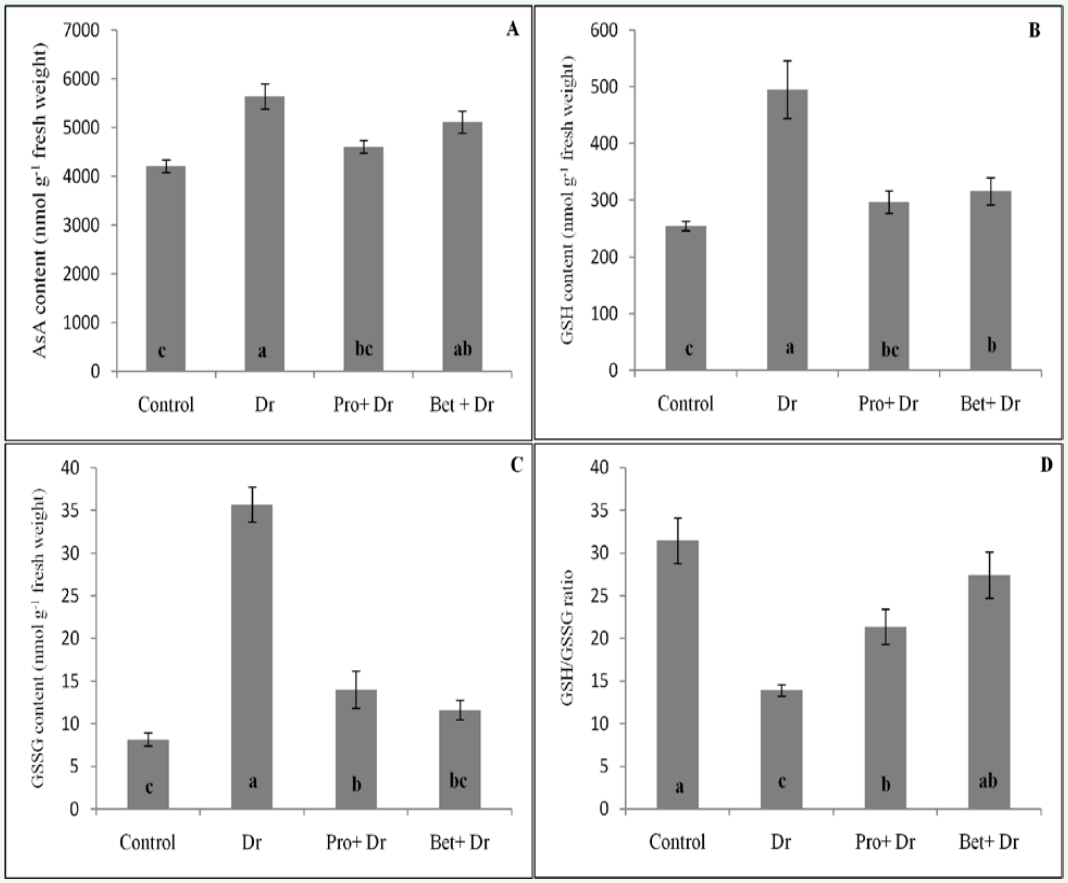

Figure 3 Effects of proline or betaine pre-treatment on ascorbate (AsA) (A), reduced glutathione (GSH) (B), oxidized glutathione (GSSG) (C) and GSH/GSSG ratio (D) in mustard seedlings under drought stress conditions. Mean (±SD) was calculated from three replicates from each treatment. Dr, Pro+Dr and Bet+Dr indicate drought, proline+drought and betaine+drought stress, respectively. Bars with different letters are significantly different at p<0.01 applying LSD test.

Compared with drought stress treatment, proline or betaine pre-treated drought-stressed seedlings maintained a significantly (P<0.01) lower level of GSSG content (61% and 68% by proline and betaine, respectively).

Relative to control, drought stress resulted in a significant (P<0.01) decrease (2.27-fold) in GSH/GSSG ratio (Figure 3D). Proline pre-treated drought-stressed seedlings also showed a significant (P<0.01) decrease (32%) in GSH/GSSG ratio as compared to control whereas a non-significant decrease (13%) was observed in betaine pre-treated seedlings. Compared with drought stress treatment, both proline and betaine pre-treated drought-stressed seedlings showed a significant (P<0.01) increase in GSH/GSSG ratio (54% and 98% by proline and betaine, respectively) as compared to the seedlings subjected to drought stress without pre-treatment.

Effects on ascorbate-glutathione (AsA-GSH) cycle enzyme activities

Drought stress resulted in a significant (P<0.01) decrease (18%) in APX activity as compared to control, whereas, drought-stressed seedlings pre-treated with proline or betaine showed a significant increase (32% and 35% by proline and betaine, respectively) in APX activity (Figure 4A). Compared with drought stress treatment, proline or betaine pre-treated drought-stressed seedlings showed significantly (P<0.01) higher APX activity (60 and 63% by proline and betaine, respectively).

As compared to the control group, drought stress led to a non-significant increase (17%) in MDHAR activity (Figure 4B). Drought-stressed seedlings pre-treated with proline or betaine also showed a non-significant increase (9% or 13% by proline and betaine, respectively) in MDHAR activity relative to control. Proline or betaine priming has no influence in MDHAR activity under drought stress conditions.

In response to drought stress, a significant (P<0.01) increase (26%) in DHAR activity was observed when compared with control (Figure 4C). Drought-stressed seedlings pretreated with proline or betaine also showed a significant (P<0.01) increase (17% and 26% proline and betaine, respectively) in DHAR activity as compared to the control. Proline or betaine supplementation has no effect on DHAR activity.

A non-significant decrease (15%) in GR activity was observed in drought-stressed seedlings as compared to control (Figure 4D). Relative to control, proline or betaine pre-treated drought-stressed seedlings showed a non-significant increase (13% and 17% by proline and betaine, respectively) in GR activity. Importantly, both betaine and proline pre-treated seedlings maintained significantly (P<0.01) higher (32% by proline and 38% by betaine, respectively) GR activity as compared to the

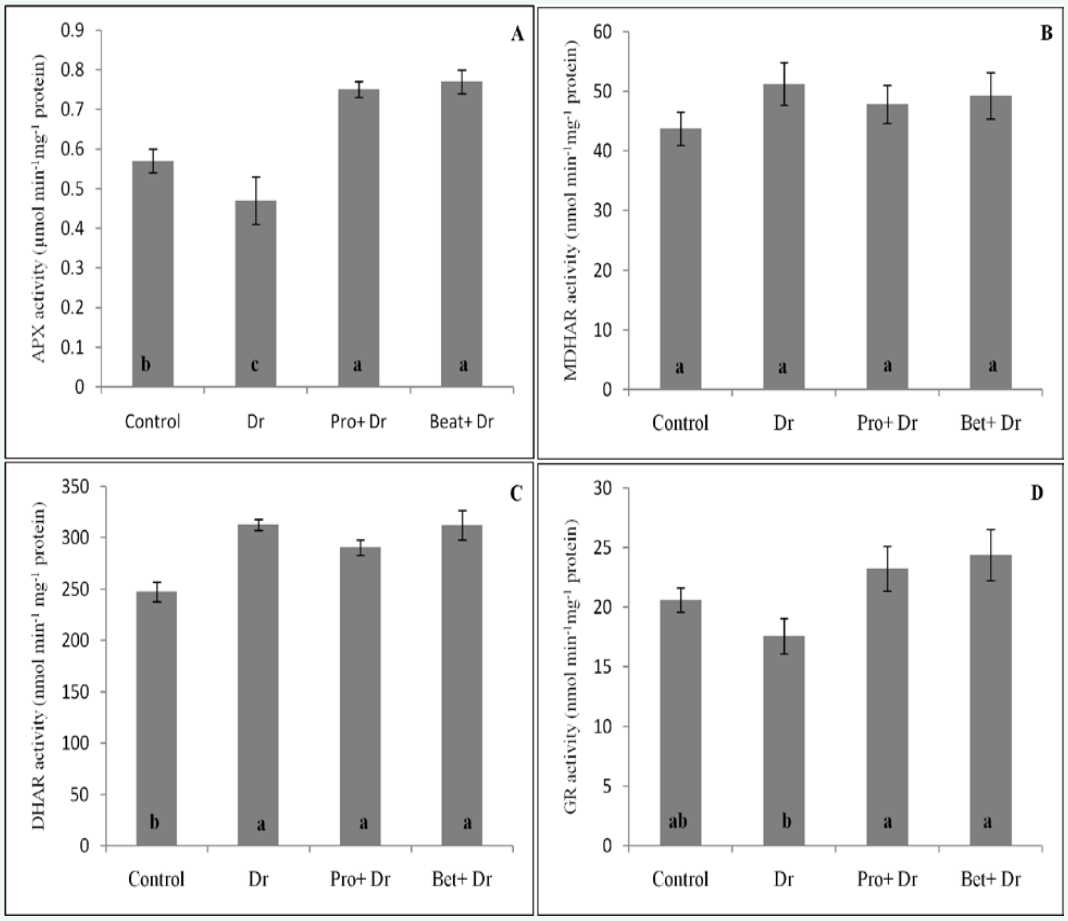

Figure 4 Effects of proline and betaine pre-treatment on the activities of APX (A), MDHAR (B), DHAR (C), and GR (D) in mustard seedlings under drought stress conditions. Other details as in fig. 3.

seedlings subjected to drought stress without pre-treatment.

Effects on CAT, GST and GPX activities

Drought stress resulted in a significant (P<0.01) decrease (23%) in CAT activity in relation to control, whereas, betaine pre-treated drought-stressed seedlings showed a significant (P<0.01) increase (27%) when compared with control (Figure 5A). Importantly, proline or betaine pre-treated drought stressed seedlings maintained a significantly (P<0.01) higher CAT activity (48% and 66% by proline and betaine, respectively) as compared to the seedlings subjected to drought stress without pre-treatment.

Drought stress resulted in a non-significant increase (13%) in GST activity whereas a significant (P<0.01) increase (39% and 67% by proline and betaine, respectively) in GST activity was observed in proline or betaine pre-treated drought-stressed seedlings as compared to control (Figure 5B). Compared with drought stress treatment, proline or betaine pre-treated drought-stressed seedlings maintained significantly (P<0.01) higher (23% and 33% by proline and betaine, respectively) GST activity.

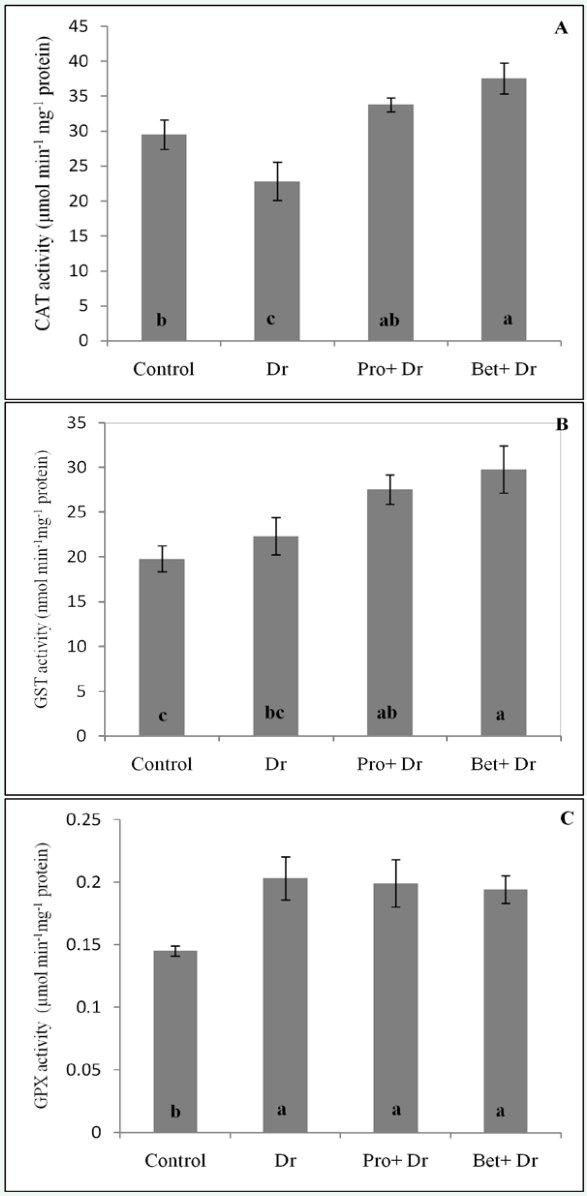

Figure 5 Effects of proline and betaine pre-treatment on the activities of CAT (A), GST (B) and GPX(C) in mustard seedlings under drought stress conditions. Other details as in figure 3.

Compared with control group, drought stress led to a significant (P<0.01) increase (40%) in GPX activity (Figure 5C). Proline or betaine pre-treated drought-stressed seedlings also showed a significant (P<0.01) increase (37% and 34% by proline and betaine, respectively) in GPX activity as compared to the control. Priming with betaine or proline had no significant variation in GPX activity as compared to drought stress.

Effects on glyoxalase system enzymes

Drought stress caused a significant (P<0.01) increase (28%) in Gly I activity as compared to control (Figure 6A). Compared with control group, proline and betaine pre-treated drought-stressed seedlings also showed a significant (P<0.01) increase in Gly I activity (32% and 36% by proline and betaine, respectively) as compared to control group. No significant variation in Gly I activity was observed in proline or betaine pre-treated and non-treated drought-stressed seedlings.

Drought stress led to a significant decrease (33%) in Gly II activity. Proline pre-treated seedlings showed a 15% decrease whereas betaine pre-treated seedlings showed a 9% decrease as compared to the control group (Figure 6B). Compared with drought stress treatment, proline or betaine pre-treated drought-stressed seedlings showed significantly (P<0.01) higher Gly II activity (28% and 33% by proline and betaine, respectively).

Effects on H_2O_2 and MDA levels

The level of H_2O_2 content increased 1.8-fold relative to the control under drought stress (Figure 7A). Drought-stressed seedling pre-treated with proline and betaine also showed a significant (P<0.01) increase in H_2O_2 content as compared to the control group. Compared with drought stressed seedlings, proline and betaine pre-treated drought-stressed seedlings maintained a significantly (P<0.01) lower level of H_2O_2 content (35% by proline and 31% by betaine, respectively).

The rate of lipid peroxidation, expressed as MDA production, significantly (P<0.01) increased (about 2-fold) relative to the control group (Figure 7B). Compared to the control group, proline pre-treatment drought-stressed seedlings showed 31% increase whereas betaine pre-treatment drought-stressed seedlings showed a 49% increase in MDA content. Compared with drought stress treatment, pre-treatment with proline and betaine maintained a significantly (P<0.01) lower level of MDA content (35% by proline and 26% by betaine, respectively).

DISCUSSION

Plant growth under drought is influenced by altered photosynthesis, respiration, translocation, ion uptake, carbohydrate and nutrient metabolism, and changes in hormone levels. Reduced mineral nutrition (uptake and transport of

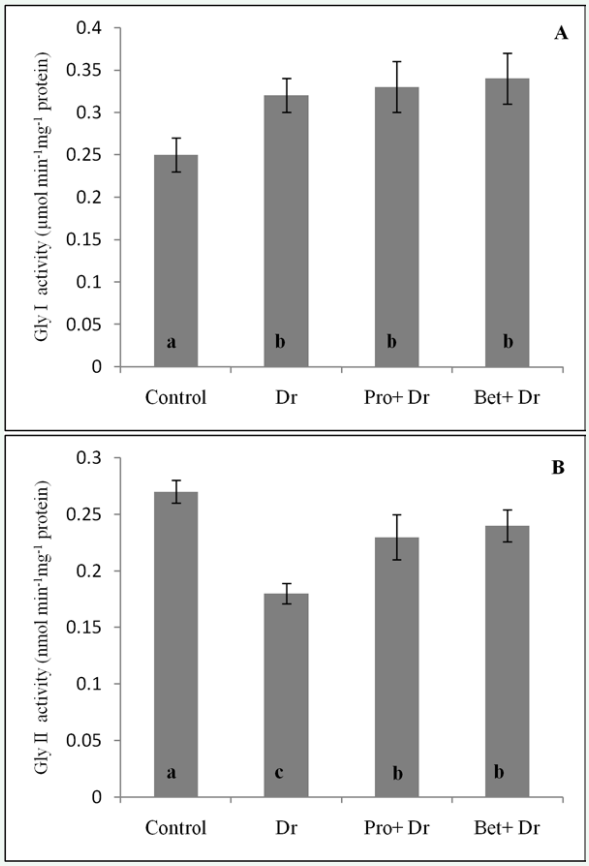

Figure 6 Effects of proline and betaine pre-treatment on the activities of Gly I (A) and Gly II (B) in mustard seedlings under drought stress conditions. Other details as in figure 3.

nutrients) and metabolism leads to a decrease in the leaf area and alteration in assimilate partitioning among the organs. The negative consequences of these physiological processes are the unrestricted production of ROS and MG. A large body of data suggests that both proline and betaine enhance abiotic stress tolerance by modulating multiple stress responsive pathways including antioxidant enzyme activity, gene and protein expression and by stabilizing photosynthetic machinery via ROS protection and protein protection [32,33,41,46,57]. The accumulation of proline and betaine in plants – either naturally, when caused by genetic transformation, or when applied exogenously – render the plants tolerant to various abiotic stresses, including drought induced oxidative stress [32,33,58-62]. To overcome this oxidative stress, it is important to maintain a stronger ROS-scavenging ability under stress conditions, especially in plant leaves where photosynthesis is dramatically impacted. Plants under abiotic stress have evolved defence systems to counteract ROS induced damage by increasing the activity of ROS scavenging enzymes. An increased capacity of the antioxidant defence system and glyoxalase system is one of the possible mechanisms responsible for oxidative stress tolerance as demonstrated by the existence of stress-resistant lines with naturally enhanced antioxidant systems [30] or the properties of transgenic plants over-expressing particular antioxidant enzymes [63-65]. In addition to direct effects on diverse plant physiological and biochemical processes, high endogenous proline levels can also act as a regulatory/signalling molecule capable of altering the transcription levels of stress-related genes. This findings added new information on the role of proline during drought conditions and more importantly, without the potential confounding effects imposed by drought stress [62]. This could be the result of an effective decrease in ROS production or the activation of effective antioxidant systems, or both. The present study hypothesized that transient pre-exposure of seedlings to proline and betaine may induce tolerance to subsequent drought stress in mustard seedlings through the activation of antioxidative and glyoxalase defence systems, in addition to multiple other influences on plant metabolism.

Ascorbate (AsA) and GSH are the two most important water-soluble non-enzymatic antioxidant important for a broad range of biological functions, including growth and development, stress tolerance and reproduction [reviewed in 66,67]. As adaptive and potential stress tolerance mechanisms plants usually up-regulate the AsA and GSH levels, as these molecules are integral components of AsA-GSH cycle, present in different cellular organelles and responsible for elimination excessive H_2O_2 levels in plants. However, both of the components have the capacity to scavenge ROS directly [66,68]. Many studies in plant have demonstrated that the levels of AsA and GSH, and the alteration of AsA/AsA+DHA and GSH/GSSG ratios positively correlate with oxidative stress tolerance [32,33,35-37,69]. Transgenic

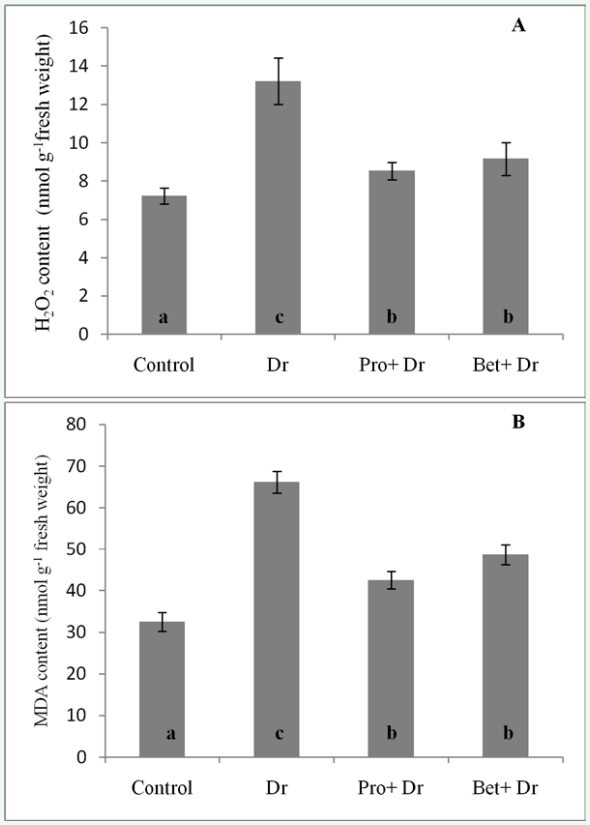

Figure 7 Effects of proline and betaine pre-treatment on H_2O_2 (A) and MDA (B) levels in mustard seedlings drought stress conditions. Other details as in figure 3.

plants over-expressing a proline biosynthetic gene under heat and drought stress conditions showed a greater GSH pool. You et al. [60] showed that genetically modified plants with greater proline biosynthesis had a greater GSH content under osmotic and drought stress conditions. Likewise transgenic plants over-expressing a betaine biosynthetic gene had higher AsA and GSH contents [70]. In our present experiments, we found that both AsA and GSH levels increased in response to drought stress, however, a greater increase was observed in the GSH pool. Increases in AsA content in response to drought stress were also observed in our previous studies [35,36] and are consistent with the findings of other research groups where increase in AsA and GSH levels were found [71,72]. The AsA content in the proline pre-treated seedlings were similar to control plants, whereas betaine significantly increased the AsA content when compared with controls. One possible explanation for the lower AsA content in the in proline and betaine pre-treated seedlings is due to the higher APX activity that utilizes AsA as a reductant or due to lower rates of AsA synthesis. In addition to its involvement with AsA-GSH cycle, GSH is also involved with other GSH related enzyme like GST, GPX and Gly I. In the present study, although GSH levels showed increases in drought stressed seedlings, we still observed severe oxidative stress in leaf tissues. Surprisingly, the GSH/GSSG ratio significantly increased in proline or betaine pre-treated seedlings. The GSH/GSSG ratio plays an important role in maintaining the redox status of cells and in the stabilization of enzymes and other proteins. A shift in its ratio leading to more formation of GSSG than GSH may decrease the concentration of –SH groups in the cell and thereby affect the redox status of the cell [73,74]. Similar to our present experimental results, Yu et al. [75] also stated that the GSH level is not the sole factor in enhancing oxidative stress tolerance. The increase in GST activity is probably the main reason for GSH loss because GST utilizes GSH to convert toxic compounds into non-toxic forms and sequesters them into the vacuole [76].

APX, CAT and GPX are the major enzymes associated with the detoxification of H_2O_2 in plant cells and also for regulating the appropriate levels of H_2O_2 to perform its signalling functions. Ascorbate peroxidase utilized AsA as a reducing equivalent during the conversion of H_2O_2 into water, whereas GSH is used by GPX. Importantly, CAT converted H_2O_2 to H_2O without any reducing equivalent and primarily associated with peroxisome where the maximum H_2O_2 produced during photorespiration, a typical situation during drought stress. The present experiment showed that the activity of APX and CAT decreased in response to drought stress whereas GPX activity showed a significant increase. The findings of the present experiments are similar to our previous studies where we observed that drought and salt stress led to a significant decrease in APX and CAT activity [33,35,36]. Such a decrease in CAT activity could indicate its inactivation by the accumulated H_2O_2 induced by drought stress and could be partly explained by photoinactivation of enzymes [77,78]. Conversely, a significant increase in APX and CAT activity was observed in proline or betaine pre-treated seedlings. The increase in CAT activity was also observed in transgenic plants over-expressing proline biosynthetic genes under heat stress conditions [79]. Nounjan et al. [80] showed that application of exogenous proline in rice seedlings enhanced the expression of CAT and APX genes. Razavizadeh and Eshanpour [81] reported that tobacco (*Nicotiana tabacum* cv. Wisconsin) plans transformed with proline biosynthetic gene showed higher CAT and APX activities under salt stress conditions. They concluded that P5CS is an inducible gene regulating the activities of CAT and APX. Khedr et al. [82] also found that exogenous proline enhances the CAT protein expression and activity. Zarei et al. [83] showed that transgenic plants over-expressing proline biosynthetic gene under drought stress conditions showed higher APX and CAT activity under drought stress conditions. Turkan et al. [84] reproved that drought tolerant genotype had higher inherent activities of SOD, CAT, APX and POX, which is correlated with proline synthesis. Wang et al. [70] also reported that CAT and APX activities increased in response to drought stress in transgenic wheat, over-expressing a betaine biosynthetic gene. Likewise, de Campos et al. [61] also showed that transgenic plants over-expressing a proline biosynthetic gene showed higher APX activities under water stress conditions. The increase in GPX activity was also observed in plants under drought stress conditions [71,85]. However, we din't find any significant increase in GPX activity in proline or betaine pre-treated drought stressed seedlings, although its level was found to increase in response to drought stress. This is probably due to higher CAT activity, which is responsible for the bulk removal of H_2O_2 produced in the chloroplast.

Recycling of metabolites directly related to stress tolerance is more important than a higher rate of synthesis under stressful conditions. Oxidation of AsA by ROS or by APX during H_2O_2 detoxification produces short-lived Monodehydroascorbate (MDHA), which can rapidly disproportionate to Dehydroascorbate (DHA). Plants can restore AsA from MDHA and DHA by using two enzymes of the AsA-GSH cyle, one is NADPH-dependent MDHAR and the other is GSH-dependent DHAR. Differential regulation of these enzymes in ascorbate recycle was observed in response to various stresses [68]. Genetically engineered plants over-expressing MDHAR and DHAR genes showed greater protection against abiotic oxidative stress and higher level of AsA content in the leaf tissues and other plant organs [64,86-88]. In the present study, we found no significant variation in MDAHR activity in proline or betaine pre-treated and non-treated drought stressed seedlings. However, DHAR activity was found to increase in response to drought stress indicating that under drought stress conditions AsA is regenerated via GSH dependent DHAR because MDHAR activity is limited due to the unavailability of NADPH under oxidative conditions [89]. Increases in DHAR activity under drought stress have been reported in a range of plant species [71,90]. Importantly, we observed no significant variation in DHAR activity in proline or betaine pre-treated drought stressed seedlings although in our previous studies we observed a significant increase in DHAR in proline or betaine pre-treated salt stressed seedlings [11]. This variation is probably due to differences in treatment methods, plant species, and the duration of stress treatment. In accordance with our present experimental results, no other studies have reported that exogenous application of proline or betaine or transgenic plants over-expressing proline or betaine biosynthetic genes showed higher DHAR and MDHAR activity under drought stress conditions.

Glutathione S-transferase plays a central role in GSH-mediated

detoxification in plant cells [71]. Glutathione *S*-transferase catalyses the binding of various xenobiotics to GSH to produce less toxic and more water-soluble conjugates [91]. Besides catalyzing the conjugation of electrophilic metabolites to GSH, GST isozymes also have peroxidase activity [92]. Furthermore, GSTs may bind to proteins that sequester flavonoids (e.g. anthocyanins) in the vacuole for protection against environmental stresses [93]. In our present study, we observed a non-significant increase in GST activity under drought stress. The present research results support our previous experimental results [35,36]. Furthermore, Liu et al. [71] and Bhardwaj and Yadav [94] also found a significant increase in GST activity under drought stress in cucumber and horsegram (*Macrotyloma uniflorum)*. Very recently, Pyngrope et al. [95] also found comparatively higher GST activity in drought tolerant plant as compared to the drought sensitive. The augmentation of GST activity under conditions of drought stress is insufficient to protect cells from drought-induced oxidative damage. Importantly, proline or betaine pre-treatment favourably modulates GST activity, suppresses the production of H_2O_2 and MDA, denoting that GST plays an important role in reducing drought-induced oxidative damage. Transgenic tobacco plants over-expressing a chloroplast localized GST gene showed higher drought tolerance due to efficient ROS detoxification in the chloroplast [96].

Glutathione reductase, one of the vital components of AsA-GSH pathway, is primarily responsible for the regeneration of GSH from GSSG using NADPH as a reducing equivalent. This enzyme plays an important role by maintaining the reduced status of GSH and AsA pools and proper GSH/GSSG ratio that is more decisive in determining plant resistance to abiotic and biotic stresses than in the actual GSH content [34,64]. The elevated level of GR might increase the ratio of $NADP^+$ to NADPH and thereby increase the availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain. Under these circumstances, the rate of electron flow to O_2 is reduced and this reduces the formation of $O_2^{\bullet-}$ and the metal catalyzed formation of •OH, through the Haber-Weiss reaction [35,97]. Importantly, higher GSSG levels in the drought stressed seedlings were attributed to a significant decrease in the GSH/GSSG ratio. A decrease in GR activity in response to drought stress was also observed in the sensitive cultivar of tomato [90]. Usually, tolerant plants tend to have higher activities of GR as compared to sensitive plants [90,98-100]. We observed a slight decrease in GR activity as well as higher GSSG content in the seedlings under drought stress conditions. Reduction in GR activity in mustard seedlings under drought stress is one of most important factors for susceptibility to drought stress. We, therefore, speculate that the inhibition or insufficient of GR was a major factor responsible for the rapid increase in the GSSG in drought-stressed seedlings, which was attributed to significant decrease in GSH/GSSG ratio. Similar to our results, decreases in GR activity under drought stress have also been reported in other sensitive cultivars [72,90]. However, proline and betaine pre-treated drought-stressed seedlings had higher GR activities. Increased GR activity in the proline and betaine pre-treated drought-stressed seedlings contributes to the maintenance of higher GSH/GSSG ratio. Similar results were also obtained with our previous studies under salt and cadmium stress tolerance [32,33] in mung bean seedlings. Transgenic plants over-expressing betaine biosynthetic gene also showed higher GR transcript under salt, cold and methyl viologen induced oxidative stress [50].

A rapid accumulation of endogenous MG has been observed in plants in response to environmental stresses and its detoxification is one of the potential mechanisms for inducing abiotic stress tolerance [11,13,17,18,22,31]. MG is toxic to plant cells, causing inhibition of cell proliferation, protein inactivation and inhibition of ROS detoxification systems and as a consequence disrupts cellular functions [19,20,27], but signalling roles for MG in inducing abiotic stress tolerance have also been reported [24,25]. The glyoxalase system is the most important MG detoxification pathway in plants. The glyoxalase system is comprised of two enzymes: Gly I and Gly II that convert MG to less toxic hydroxyacids. Glyoxalase I convert MG to S-D-Lactoylglutathione (SLG) by utilizing GSH, while Gly II converts SLG to D-lactic acid, and in this reaction GSH is regenerated. In addition to its (glyoxalase system) prime function, to detoxify highly reactive MG, the system also plays an important role in recycling trapped GSH in plant antioxidant defence system and to maintain a higher redox state [101]. Like MG, the SLG produced by Gly I was also found to be cytotoxic at high cellular concentration [102]. Plants respond to abiotic or biotic stresses by limiting over-accumulation MG levels through the upregulation of Gly I and Gly II activities and also by modulating GSH-based detoxification systems, which ultimately lower level of lipid peroxidation [22,35,103]. Recent genetic and proteomic studies have shown that the glyoxalase pathway has a profound effect on stress tolerance. The transcript abundance and activities of Gly I and Gly II are induced by various abiotic and biotic stresses [103-108]. Studies on wild-type stress tolerant studies and gain-of-function studies have shown that the antioxidative and glyoxalase defence systems are closely linked and that the glyoxalase system has a direct influence on the ROS detoxification [18,22,30,108] and plants over-expressing either Gly I or Gly II gene enhances plant abiotic stress tolerance [103,106,107,109,110]. Recently, Upadhayaya et al. [18] showed that *GalUR* gene over-expressing transgenic potato plants had greater salinity tolerance and showed stimulated activities of the antioxidant enzymes APX, DHAR, GR, GST, and GPX, and the glyoxalase system enzymes (Gly I and Gly II), as well as by enhanced GSH/GSSG ratios. Greater accumulation of AsA was found in the transgenic plants with a lower accumulation of MG levels under salt stress. Additionally, a relatively high GSH/GSSG ratio was also maintained in these transgenic plants, which could help to protect the plants from salinity induced oxidative stress. Modulation of both the ROS and MG detoxification capacity and favourable changes in the GSH and AsA redox state in the transgenic plants were thought to be the main reasons for enhanced salinity tolerance. Our results showed an increase in both GSH and GSSG levels, whereas the GSH/GSSG ratio decreased significantly. The increase in GSSG content indicates the higher oxidative load in the drought stressed seedlings. Importantly, the higher level of GSH/GSSG ratio in proline or betaine pre-treated drought stressed seedlings indicates that proline and betaine pre-treatments suppress the increase of GSSG accumulation probably due to higher GR and Gly II activites. Therefore, efficient recycling of GSH through glyoxalase system and GR activity seems to be an important determinant in plant stress tolerance. These findings

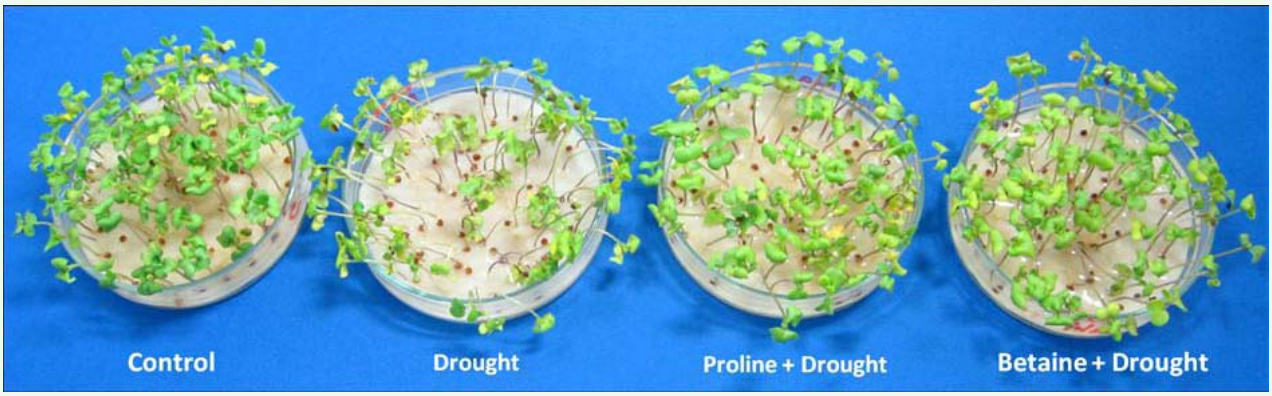

Figure 8 Differences in phenotypic appearance of control, drought-stressed, proline or betaine pre-treated drought-stressed mustard seedlings.

of the present study greatly coherent with our previous studies in different crop species under various abiotic stress situations [35-37]. The results of this study and our previous findings [32,33,35,36] affirm that simultaneous induction of antioxidative defense system and glyoxalase systems rendered the plant more tolerant to various abiotic stresses.

Accumulation of MDA, mainly produced from the ROS-induced degradation of membrane lipids, is a potential biomarker to access the severity of the stress, including drought stress [35]. Here we observed that drought stress tresulted in a sharp increase in MDA levels with a concomitant increase in H_2O_2 in leaf tissues. Similar to that observed in this study, as a result of drought stress, Filippou et al. [111] also provided a clear link between the increase of H_2O_2 and an increased MDA content in both leaves and root tissues in a model plant species, *Medicago truncatula*. Changes in membrane integrity in turn may lead to osmotic imbalances, changes in photosynthetic apparatuses and degradation of thylakoids [74]. However, the levels of MDA and H_2O_2 were significantly lower in proline and betaine pre-treated seedling as compared to the seedlings subjected to drought stress without pre-treatment. Therefore, we speculated that proline or betaine pre-treatment might contribute in alleviating the drought-induced oxidative stress through the activation of ROS and MG detoxification that ultimately helps in membrane stability. Similar to our results, Moustakus et al. [52] also found that exogenous proline pre-treatment significantly reduced the MDA and H_2O_2 levels in *Arabidopsis*. A decrease in endogenous H_2O_2 and MDA level in response to drought stress through exogenous betaine application has also been reported [53,112]. Transgenic plants over-expressing proline or betaine biosynthetic genes under drought stress conditions also showed lower lipid peroxidation [59-61,113-115]. Careful analysis of the phenotypic appearance of the seedlings showed that proline or betaine non-treated drought-stressed seedlings showed severe wilting (Figure 8), a typical symptom of susceptibility of water stress, whereas, no such wilting was observed in proline or betaine pre-treated drought-stressed seedlings.

CONCLUSION

Based on the present findings, we can conclude that burden of drought stress in *Brassica juncea* seedlings led to a severe oxidative damage as manifested by sharp increases in lipid peroxidation and H_2O_2 levels due to inappropriate induction of ROS and MG detoxification systems. Most importantly, pre-treatment of seedlings with betaine or proline modulated the activities of CAT, APX, GR, GST and Gly II and higher GSH/GSSG ratio with an associated decrease in oxidative stress parameter like MDA and H_2O_2 as compared to the seedlings subjected to drought stress without betaine or proline pre-treatment. In line with the previous findings [52,114,116], the present experimental results confirmed the beneficial effects of exogenous betaine or proline in alleviating drought induced oxidative damage. These results also allow us to conclude that co-ordinate stimulation of glyoxalase system and antioxidant defence system is an important determinant for the acquisition of drought stress tolerance and the data are of considerable value in elucidating the biochemical mechanisms of plant abiotic stress tolerance and in developing appropriate and efficient methods for crop protection against abiotic stresses. However, identification of additional key factors involved in betaine or proline induced-drought stress tolerance and the underlying signalling roles of betaine and proline warrants further research.

ACKNOWLEDGEMENT

Financial grant from Japan government (Monbuakagakusho) is gratefully acknowledged.

REFERENCES

1. Manavalan LP, Guttikonda SK, Tran LS, Nguyen HT. Physiological and molecular approaches to improve drought resistance in soybean. Plant Cell Physiol. 2009; 50: 1260-1276.
2. Le BH, Wagmaister JA, Kawashima T, Bui AQ, Harada JJ, Goldberg RB. Using genomics to study legume seed development. Plant Physiol. 2007; 144: 562-574.
3. Duque AS, de Almeida AM, da Silva AB, da Silva JM, Farinha AP, Santos D, Fevereiro P, de Sousa Araújo S. Abiotic stress responses in plants: unraveling the complexity of genes and networks to survive. In: Vahdati K, Leslie C, editor. Abiotic stress-plant responses and applications in agriculture. Croatia: INTECH-Open Access Publisher. 2013; 49-101.
4. Cruz de Carvalho MH. Drought stress and reactive oxygen species: Production, scavenging and signaling. Plant Signal Behav. 2008; 3: 156-165.
5. Noctor G, Veljovic-Jovanovic S, Driscoll S, Novitskaya L, Foyer CH. Drought and oxidative load in the leaves of C3 plants: a predominant

role for photorespiration? Ann Bot. 2002; 89: 841-850.

6. Miller G, Shulaev V, Mittler R. Reactive oxygen signaling and abiotic stress. Physiol Plant. 2008; 133: 481-489.

7. Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 2010; 33: 453-467.

8. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem. 2010; 48: 909-930.

9. Petrov VD, Van Breusegem F. Hydrogen peroxide-a central hub for information flow in plant cells. AoB Plants. 2012; 2012: pls014.

10. Jaspers P, Kangasjärvi J. Reactive oxygen species in abiotic stress signaling. Physiol Plant. 2010; 138: 405-413.

11. Hossain MA, Teixeira da Silva JA, Fujita M. Glyoxalase system and reactive oxygen species detoxification system in plant abiotic stress response and tolerance: An intimate relationship. In: Shanker AK, Venkateswarlu B, editor. Abiotic Stress/ Book 1. Croatia: INTECH-Open Access Publisher. 2011a; 235-266.

12. Saito R, Yamamoto H, Makino A, Sugimoto T, Miyake C. Methylglyoxal functions as Hill oxidant and stimulates the photoreduction of O(2) at photosystem I: a symptom of plant diabetes. Plant Cell Environ. 2011; 34: 1454-1464.

13. Krasensky J, Jonak C. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J Exp Bot. 2012; 63: 1593-1608.

14. Foyer CH, Noctor G. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell. 2005; 17: 1866-1875.

15. Rouhier N, Lemaire SD, Jacquot JP. The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annu Rev Plant Biol. 2008; 59: 143-166.

16. Hossain MA, Fujita M. Purification of glyoxalase I from onion bulbs and molecular cloning of its cDNA. Biosci Biotechnol Biochem. 2009; 73: 2007-2013.

17. Banu MN, Hoque MA, Watanabe-Sugimoto M, Islam MM, Uraji M, Matsuoka K, et al. Proline and glycinebetaine ameliorated NaCl stress via scavenging of hydrogen peroxide and methylglyoxal but not superoxide or nitric oxide in tobacco cultured cells. Biosci Biotechnol Biochem. 2010; 74: 2043-2049.

18. Upadhyaya CP, Venkatesh J, Gururani MA, Asnin L, Sharma K, Ajappala H. Transgenic potato overproducing L-ascorbic acid resisted an increase in methylglyoxal under salinity stress via maintaining higher reduced glutathione level and glyoxalase enzyme activity. Biotechnol Lett. 2011; 33: 2297-2307.

19. Martins AM, Cordeiro CA, Ponces Freire AM. In situ analysis of methylglyoxal metabolism in Saccharomyces cerevisiae. FEBS Lett. 2001; 499: 41-44.

20. Hoque MA, Uraji M, Banu MN, Mori IC, Nakamura Y, Murata Y. The effects of methylglyoxal on glutathione S-transferase from *Nicotiana tabacum*. Biosci Biotechnol Biochem. 2010; 74: 2124-2126.

21. Hoque MA, Uraji M, Torii A, Banu MN, Mori IC, Nakamura Y, et al. Methylglyoxal inhibition of cytosolic ascorbate peroxidase from Nicotiana tabacum. J Biochem Mol Toxicol. 2012; 26: 315-321.

22. Yadav SK, Singla-Pareek SL, Ray M, Reddy MK, Sopory SK. Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. Biochem Biophys Research Commun. 2005a; 337: 61-67.

23. Gómez Ojeda A, Corrales Escobosa AR, Wrobel K, Yanez Barrientos E, Wrobel K. Effect of Cd(II) and Se(IV) exposure on cellular distribution of both elements and concentration levels of glyoxal and methylglyoxal in Lepidium sativum. Metallomics. 2013; 5: 1254-1261.

24. Hoque TS, Uraji M, Ye W, Hossain MA, Nakamura Y, Murata Y. Methylglyoxal-induced stomatal closure accompanied by peroxidase-mediated ROS production in Arabidopsis. J Plant Physiol. 2012;169: 979-986.

25. Hoque TS, Uraji M, Tuya A, Nakamura Y, Murata Y. Methylglyoxal inhibits seed germination and root elongation and up-regulates transcription of stress-responsive genes in ABA-dependent pathway in Arabidopsis. Plant Biol. 2012;14:854-858.

26. Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage, and antioxidative defense mechanisms in plants under stressful conditions. J Bot. 2012; 26.

27. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. Reactive oxygen gene network of plants. Trends Plant Sci. 2004; 9: 490-498.

28. Hoque MA, Banu MNA, Nakamura Y, Shimoishi Y, Murata Y. Proline and glycinebetaine enhance antioxidant defense and methylglyoxal detoxification systems and reduce NaCl-induced damage in cultured tobacco cells. J Plant Physiol. 2008; 165: 813-824.

29. Kumar V, Yadav SK. Proline and betaine provide protection to antioxidant and methylglyoxal detoxification systems during cold stress and *Camellia sinensis* (L.) O.Kuntze. Acta Physiol Plant. 2009; 31: 261-269.

30. El-Shabrawi H, Kumar B, Kaul T, Reddy MK, Singla-Pareek SL, Sopory SK. Redox homeostasis, antioxidant defense, and methylglyoxal detoxification as markers for salt tolerance in Pokkali rice. Protoplasma. 2010; 245: 85-96.

31. Hossain MA, Hossain MZ, Fujita M. Stress-induced changes of methylglyoxal level and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene. Aust J Crop Sci. 2009; 3: 53-64.

32. Hossain MA, Hasanuzzaman M, Fujita M. Up-regulation of antioxidant and glyoxalase systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress. Physiol Mol Biol Plants. 2010; 16: 259-272.

33. Hossain MA, Hasanuzzaman M, Fujita M. Coordinate induction of antioxidant defense and glyoxalase system by exogenous proline and glycinebetaine is correlated with salt tolerance in mung bean. Front Agric China. 2011b; 5: 1-14.

34. Hossain MA, Piyatida P, Teixeira da Silva JA, Fujita M. Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. J Bot. 2012; Article ID:872875.

35. Hossain MA, Mostofa MG, Fujita M. Cross protection by cold-shock to salinity and drought stress-induced oxidative stress in mustard (*Brassica campestris* L.) seedlings. Mol Plant Breed. 2013b; 4: 50-70.

36. Hossain MA, Mostofa MG, Fujita M. Heat-shock positively modulates oxidative protection of salt and drought-stressed mustard (*Brassica campestris* L.) seedling. J Plant Sci Mol Breed. 2013b; 2:1-14.

37. Mostofa MG, Fujita M. Salicylic acid alleviates copper toxicity in rice (*Oryza sativa* L.) seedlings by up-regulating antioxidative and glyoxalase systems. Ecotoxicology. 2013; 22: 959-973.

38. Ashraf M, Foolad MR. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot. 2007; 59: 206-216.

39. Szabados L, Savouré A. Proline: a multifunctional amino acid. Trends Plant Sci. 2010; 15: 89-97.

40. Verslues PE, Sharma S. Proline metabolism and its implications for

plant-environment interaction. Arabidopsis Book. 2010; 8: e0140.

41. Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A. Role of proline under changing environments: a review. Plant Signal Behav. 2012; 7: 1456-1466.

42. Sharma SS, Dietz KJ. The relationship between metal toxicity and cellular redox imbalance. Trends Plant Sci. 2009; 14: 43-50.

43. Trovato M, Mattioli R, Costantino P. Multiple roles of proline in plant stress tolerance and development. Rendiconti Lincei. 2008; 19; 325-346.

44. Verbruggen N, Hermans C. Proline accumulation in plants: a review. Amino Acids. 2008; 35: 753-759.

45. Mattioli R, Costantino P, Trovato M. Proline accumulation in plants: not only stress. Plant Signal Behav. 2009; 4: 1016-1018.

46. Chen TH, Murata N. Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. Plant Cell Environ. 2011; 34: 1-20.

47. Giri J. Glycinebetaine and abiotic stress tolerance in plants. Plant Signal Behav. 2011; 6: 1746-1751.

48. Ahmad R, Lim CJ, Kwon SK. Glycine betaine: a versatile compound with great potential for gene pyramiding to improve crop plant performance against environmental stresses. Plant Biotechnol Rep. 2013; 7: 49-57.

49. Kathuria H, Giri J, Nataraja KN, Murata N, Udayakumar M, Tyagi AK. Glycinebetaine-induced water-stress tolerance in codA-expressing transgenic indica rice is associated with up-regulation of several stress responsive genes. Plant Biotechnol J. 2009; 7: 512-526.

50. Fan W, Zhang M, Zhang H, Zhang P. Improved tolerance to various abiotic stresses in transgenic sweet potato (*Ipomoea batatas*) expressing spinach betaine aldehyde dehydrogenase. PloS One. 2011; 7: e37344.

51. Fariduddin A, Varshney P, Yusur M, Ali A, Ahmad A. Dissecting the role of glycinebetaie in plants under abiotic stress. Plant Stress. 2013; 7: 8-17.

52. Moustakas M, Sperdouli I, Kouna T, Antonopoulou CI, Therios I. Exogenous proline induces soluble sugar accumulation and alleviates drought stress effects on photosystem II functioning of Arabidopsis thaliana leaves. Plant Growth Regul. 2011; 65: 315-325.

53. Cruz FJR, Castro GLS, Junior Silva DD, Festucci-Buselli RA, Pinheiro HA. Exogenous glycine betaine modulates ascorbate peroxidase and catalase activities and prevent lipid peroxidation in mild water-stressed Carapa guianensis plants. Photosynthetica. 2013; 51: 102-108.

54. Hossain MA, Nakano Y, Asada K. Monodehydroascorbate reductase in spinach chloroplasts and its participation in the regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiol.1984; 25: 385-395.

55. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplast. Plant Cell Physiol. 1981; 22: 867-880.

56. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.

57. Burritt DJ. Proline and the cryopreservation of plant tissues: functions and practical applications. Katkov I, editor. In: Current frontiers in cryopreservation, Croatia: INTECH-open access Publisher. 2012: 415-403.

58. Ahmad R, Kim MD, Back KH, Kim HS, Lee HS, Kwon SY, et al. Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt, and drought stresses. Plant Cell Rep. 2008; 27: 687-698.

59. Zhang K, Guo N, Lian L, Wang J, Lv S, Zhang J. Improved salt tolerance and seed cotton yield in cotton (*Gossypium hirsutum* L.) by transformation with betA gene for glycinebetaine synthesis. Euphytica. 2011; 181: 1-16.

60. You J, Hu H, Xiong L. An ornithine δ-aminotransferase gene OsOAT confers drought and oxidative stress tolerance in rice. Plant Sci. 2012; 197: 59-69.

61. de Campos MKS, de Carvalho K, de Souza FS, Marur CS, Pereira LFP, Filho JCB, et al. Drought tolerance and antioxidant enzymatic activity in transgenic 'Swingle' citrumelo plants over-accumulating proline. Environ Exp Bot. 2011; 72: 242-250.

62. de Carvalho K, de Campos MK, Domingues DS, Pereira LF, Vieira LG. The accumulation of endogenous proline induces changes in gene expression of several antioxidant enzymes in leaves of transgenic Swingle citrumelo. Mol Biol Rep. 2013; 40: 3269-3279.

63. Xu W F, Shi W M, Ueda A, Takabe T. Mechanisms of salt tolerance in transgenic Arabidopsis thaliana carrying a peroxisomal ascorbate peroxidase gene from barley. Pedosphere. 2008; 18: 486-495.

64. Wang Z, Xiao Y, Chen W, Tang K, Zhang L. Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in *Arabidopsis*. J Integr Plant Biol. 2010; 52: 400-409.

65. Wang X, Guo X, Li Q, Tang Z, Kwak S, Ma D. Studies on salt tolerance of transgenic sweet potato which harbors two genes expressing cuzn superoxide dismutase and ascorbate peroxidase with the stress-inducible SWPA 2 promoter. Plant Gene Trait. 2012; 3: 6-12.

66. Noctor G, Mhamdi A, Chaouch S, Han Y, Neukermans J, Marquez-Garcia B, et al. Glutathione in plants: an integrated overview. Plant Cell Environ. 2012; 35: 454-484.

67. Zhang Y. Biological role of ascorbate in plants. In: Zhang Y, editor: Ascorbic acid in plant-biosynthesis, regulation and enhancement. China: Springer. 2013; 7-33.

68. Gallie DR. The role of L-ascorbic acid recycling in responding to environmental stress and in promoting plant growth. J Exp Bot. 2013; 64: 433-443.

69. Wang Y, Li J, Wang J, Li Z. Exogenous H2O2 improves the chilling tolerance of manilagrass and mascarenegrass by activating the antioxidative system. Plant Growth Regul. 2012b; 6:195-204.

70. Wang GP, Hui Z, Li F, Zhao M, Zhang J, Wang W. Improvement of heat and drought photosynthetic tolerance in wheat by over accumulation of glycinebetaine. Plant Biotechnol Rep. 2010c; 4: 213-222.

71. Liu ZJ, Guo YK, Bai JG. Exogenous hydrogen peroxide changes antioxidant enzyme activity and protects ultrastructure in leaves of two cucumber ecotypes under osmotic stress. J Plant Growth Regul. 2010; 29: 171-183.

72. Chugh V, Kaur N, Grewal MS, Gupta AK. Differential antioxidative response of tolerant and sensitive maize (*Zea mays* L.) genotypes to drought stress at reproductive stage. Indian J Biochem Biophys. 2013; 50: 150-158.

73. Pukacka S, Ratajczak E. Production and scavenging of reactive oxygen species in Fagus sylvatica seeds during storage at varied temperature and humidity. J Plant Physiol. 2005; 162: 873-885.

74. Mishra P, Bhoomika K, Dubey RS. Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive Indica rice (*Oryza sativa* L.) seedlings. Protoplasma. 2013; 250: 3-19.

75. Yu CW, Murphy TM, Sung WW, Lin CH. H_2O_2 treatment induces glutathione accumulation and chilling tolerance in mung bean. Funct Plant Biol. 2002; 29: 1081-1087.

76. Dixon DP, Edwards R. Glutathione transferases. In: The Arabidopsis book. The American Society of Plant Biologists. 2010: 1-15.

77. Hertwig B, Streb P, Feierabend J. Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. Plant Physiol. 1992; 100: 1547-1553.

78. Zhang J, Kirkham MB. Drought-stress-induced changes in activities of superoxide dismutase, catalase and peroxidase in wheat species. Plant Cell Physiol. 1994; 113: 139-147.

79. Lv WT, Lin B, Zhang M, Hua XJ. Proline accumulation is inhibitory to Arabidopsis seedlings during heat stress. Plant Physiol. 2011; 156: 1921-1933.

80. Nounjan N, Nghia PT, Theerakulpisut P. Exogenous proline and trehalose promote recovery of rice seedlings from salt-stress and differentially modulate antioxidant enzymes and expression of related genes. J Plant Physiol. 2012; 169: 596-604.

81. Razavizadeh R, Ehsanpour AA. Effects of salt stress on proline content, expression of delta-1-pyrroline-5-carboxylate synthetase, and activities of catalase and ascorbate peroxidase in transgenic tobacco plants. Biotechnol Lett. 2009; 46: 63-745.

82. Khedr AH, Abbas MA, Wahid AA, Quick WP, Abogadallah GM. Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancratium maritimum* L. to salt-stress. J Exp Bot. 2003; 54: 2553-2562.

83. Zarei S, Ehsanpour AA, Abbaspour J. The role of over expression of P5CS gene on proline, catalase, ascorbate peroxidase activity and lipid peroxidation of transgenic tobacco (Nicotiana tabacum L.) plant under in vitro drought stress. J Cell Mol Res. 2012; 4: 43-49.

84. Turkan I, Bor M, Ozdemir F, Koca H. Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant P. acutifolius Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Biol. 2005; 168: 223-231.

85. Halusková L, Valentovicová K, Huttová J, Mistrík I, Tamás L. Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. Plant Physiol Biochem. 2009; 47: 1069-1074.

86. Eltayev AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Morishima I, et al. Enhanced tolerance to ozone and drought stresses in transgenic tobacco over-expressing dehydroascorbate reductase in cytosol. Physiol Plant. 2006; 127: 57-65.

87. Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, et al. Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta. 2007; 225: 1255-1264.

88. Yin L, Wang S, Eltayeb AE, Uddin MI, Yamamoto Y, Tsuji W, et al. Overexpression of dehydroascorbate reductase, but not monodehydroascorbate reductase, confers tolerance to aluminum stress in transgenic tobacco. Planta. 2010; 231: 609-621.

89. Asada K, Takahashi M. Production and scavenging of active oxygen in photosynthesis. Kyle DJ, Osmond CB, Amtzen CJ, edidors. In: Photoinhibition, Amsterdam: Elsevier Science Publication. 1987; 227-287.

90. Sánchez-Rodíguez E, Rubio-Wilhelmi M, Cervilla LM, Blasco B, Rioa JJ, Rosales MA, et al. Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. Plant Sci. 2011; 178: 30-40.

91. Edwards R, Dixon DP, Walbot V. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. 2000; 5: 193-198.

92. Foyer CH, Noctor G. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 2011; 155: 2-18.

93. Tahkokorpi M, Taulavuori K, Laine K, Taulavuori E. After effects of drought-related winter stress in previous and current year stems of Vaccinium myrtillus L. Environ Exp Bot. 2007; 6: 85-93.

94. Bhardwaj J, Yadav SK. Comparative study on biochemical parameters and antioxidant enzymes in a drought tolerant and a sensitive variety of horsegram (*Macrotyloma uniflorum*) under drought stress. Am J Plant Physiol. 2012; 7: 17-29.

95. Pyngrope S, Bhoomika K, Dubey RS. Reactive oxygen species, ascorbate-glutathione pool, and enzymes of their metabolism in drought-sensitive and tolerant indica rice (*Oryza sativa* L.) seedlings subjected to progressing levels of water deficit. Protoplasma. 2013; 250: 585-600.

96. George S, Venkataraman G, Parida A. A chloroplast-localized and auxin-induced glutathione S-transferase from phreatophyte Prosopis juliflora confer drought tolerance on tobacco. J Plant Physiol. 2010; 167: 311-318.

97. Yang Y, Han C, Liu Q, Lin B, Wang J. Effect of drought and low light on growth and enzymatic antioxidant system of Picea asperata seedlings. Acta Physiol Plant. 2008; 30: 433-440.

98. Selote DS, Khanna-Chopra R. Drought-induced spikelet sterility is associated with an inefficient antioxidant defense in rice panicles. Physiol Plant. 2004; 121: 462-471.

99. Sekmen AH, Türkan I, Takio S. Differential responses of antioxidative enzymes and lipid peroxidation to salt stress in salt-tolerant P*lantago maritima* and salt-sensitive *Plantago media*. Physiol Plant. 2007; 131: 399-411.

100. Aghaei K, Ehsanpour AA, Komatsu S. Potato responds to salt stress by increased activity of antioxidant enzymes. J Integr Plant Biol. 2009; 51: 1095-1103.

101. Creighton DJ, Migliorini M, Pourmotabbed T, Guha MK. Optimization of efficiency in the glyoxalase pathway. Biochemistry. 1988; 27: 7376-7384.

102. Thornalley PJ. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification--a role in pathogenesis and antiproliferative chemotherapy. Gen Pharmacol. 1996; 27: 565-573.

103. Singla-Pareek SL, Yadav SK, Pareek A, Reddy MK, Sopory SK. Transgenic tobacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. Plant Physiol. 2006; 140: 613-623.

104. Espartero J, Sánchez-Aguayo I, Pardo JM. Molecular characterization of glyoxalase-I from a higher plant; upregulation by stress. Plant Mol Biol. 1995; 29: 1223-1233.

105. Yadav SK, Singla-Pareek SL, Ray M, Reddy MK, Sopory SK. Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress. FEBS Letts. 2005b; 579: 6265–6271.

106. Singla-Pareek SL, Reddy MK, Sopory SK. Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. Proc Natl Acad Sci U S A. 2003; 100: 14672-14677.

107. Lin F, Xu J, Shi J, Li H, Li B. Molecular cloning and characterization of

a novel glyoxalase I gene TaGly I in wheat (*Triticum aestivum* L.). Mol Biol Rep. 2010; 37: 729-735.

108. Mustafiz A, Singh AK, Pareek A, Sopory SK, Singla-Pareek SL. Genome-wide analysis of rice and Arabidopsis identifies two glyoxalase genes that are highly expressed in abiotic stresses. Funct Integr Genomics. 2011; 11: 293-305.

109. Wu C, Ma C, Pan Y, Gong S, Zhao C, Chen S, et al. Sugar beet M14 glyoxalase I gene can enhance plant tolerance to abiotic stresses. J Plant Res. 2013; 126: 415-425.

110. Alvarez Viveros MF, Inostroza-Blancheteau C, Timmermann T, González M, Arce-Johnson P. Overexpression of GlyI and GlyII genes in transgenic tomato (*Solanum lycopersicum* Mill.) plants confers salt tolerance by decreasing oxidative stress. Mol Biol Rep. 2013; 40: 3281-3290.

111. Filippou P, Antoniou C, Fotopoulos V. Effect of drought and rewatering on the cellular status and antioxidant response of Medicago truncatula plants. Plant Signal Behav. 2011; 6: 270-277.

112. Farooq M, Basra SMA, Wahid A, Cheema ZA, Cheema MA, Khaliq A. Physiological role of exogenously applied glycine betaine to improve drought tolerance in fine grain aromatic rice (*Oryza sativa* L.). J Agron Crop Sci. 2008; 194: 325-333.

113. Molinari HBC, Marur CJ, Daros E, De Campos MKF, De Carvalho JFRP, Filho JCB, et al. Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.): osmotic adjustment, chlorophyll fluorescence and oxidative stress. Physiol Plant. 2007; 130: 218-229.

114. Ahmad R, Kim YH, Kim MD, Kwon SY, Cho K, Lee HS, et al. Simultaneous expression of choline oxidase, superoxide dismutase and ascorbate peroxidase in potato plant chloroplasts provides synergistically enhanced protection against various abiotic stresses. Physiol Plant. 2010; 138: 520-533.

115. Kumar V, Shriram V, Kavi Kishor PB, Jawali N, Shitole, MG. Enhanced proline accumulation and salt stress tolerance of transgenic indica rice by over-expressing P5CSF129A gene. Plant Biotechnol Rep. 2010; 4: 37-48.

116. Ma XL, Wang YJ, Xie SL, Wang C, Wang W. Glycinebetiane ameliorates negative effects of drought stress in tobacco. Russ J Plant Physiol. 2007; 54: 472-47.

Cite this article

Hossain MA, Mostofa MG, Burritt DJ, Fujita M (2014) Modulation of Reactive Oxygen Species and Methylglyoxal Detoxification Systems by Exogenous Glycine-betaine and Proline Improves Drought Tolerance in Mustard (Brassica juncea L.). Int J Plant Biol Res 2(2): 1014.