MITIGATION OF DROUGHT STRESS IN RAPESEED (*Brassica campestris* L.) BY EXOGENOUS APPLICATION OF OSMOLYTES

TASNIM FARHA BHUIYAN

DEPARTMENT OF AGRICULTURAL BOTANY

SHER-E-BANGLA AGRICULTURAL UNIVERSITY

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BY

TASNIM FARHA BHUIYAN

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Approved by

(Prof. Dr. Kamal Uddin Ahamed)	**(Dr. Mirza Hasanuzzaman)**
Department of Agricultural Botany	Associate Professor
Supervisor	Department of Agronomy
	Co-Supervisor

(Prof. Dr. Nasima Akhter)
Chairman
Examination Committee
Department of Agricultural Botany

DEPARTMENT OF AGRICULTURAL BOTANY
Sher-e-Bangla Agricultural University
Sher-e -Bangla Nagar, Dhaka-1207

CERTIFICATE

This is to certify that the thesis entitled ***"MITIGATION OF DROUGHT STRESS IN RAPESEED (Brassica campestris L.) BY EXOGENOUS APPLICATION OF OSMOLYTES"*** *submitted to the Department of Agricultural Botany, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE (MS) in AGRICULTURAL BOTANY****, embodies the results of a piece of bonafide research work carried out by* ***TASNIM FARHA BHUIYAN,*** *Registration No.* ***10-03828*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed during the course of this investigation has been duly acknowledged and style of this thesis has been approved and recommended for submission.

Dated:
Place: Dhaka, Bangladesh

Prof. Dr. Kamal Uddin Ahamed
Supervisor
Department of Agricultural Botany
Sher-e-Bangla Agricultural University
Dhaka-1207

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MITIGATION OF DROUGHT STRESS IN RAPESEED (*Brassica campestris* L.) BY EXOGENOUS APPLICATION OF OSMOLYTES

ABSTRACT

The experiment was conducted at the Laboratory of Plant Stress Responses, Faculty of Agriculture, Kagawa University, Japan, during June, 2016 to December, 2016 to investigate the relative protective effects of three osmolytes, proline (Pro), glycine betaine (GB) and trehalose (Tre) in mitigating the adverse effects of drought stress on rapeseed seedlings. The experiment was carried out with *Brassica campestris* L. cv. BARI Shariasha-15 and it consisted of eight treatments viz. control (well watered), Pro (0.5 mM), GB (0.5 mM), Tre (0.5 mM), Drought (20% Polyethylene glycol, D), Pro+D, GB+D and Tre+D. The experiment was laid out in a Completely Randomized Design (CRD) with three replications. In the present study, reduced plant height, fresh weight and dry weight of seedlings were observed under drought stress condition, compared to control. Leaf relative water content (RWC) and chlorophyll (chl) contents were also reduced due to drought stress with increased levels of endogenous Pro, GB and Tre. Enhanced levels of the malondialdelyde (MDA) and hydrogen peroxide (H_2O_2) were evident with the higher superoxide ($O_2^{\bullet-}$) generation under the same water deficit condition. Seedlings subjected to drought stress had significantly uplifted levels of ascorbate (AsA), reduced glutathione (GSH), glutathione disulphide (GSSG) and methylglyoxal (MG) whereas the glutathione/glutathione disulphide (GSH/GSSG) ratio was decreased. Activities of several antioxidative enzymes were increased viz. ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPX), glyoxalase I (Gly I) but glyoxalase II (Gly II) showed lesser activity in response to drought stress. However, exogenous application of Pro, GB and Tre with drought stress increased the GSH content and decreased the GSSG content and finally improved the GSH/GSSG ratio. Pre-treatment with Pro, GB and Tre reduced oxidative stress by decreasing MDA, H_2O_2 and MG contents and by upregulating the antioxidant enzymes activities. Exogenous protectants further improved the chl contents and water status of rapeseed seedlings which finally improved the growth of the plants. Among the three protectants, proline (Pro) showed the best performance in alleviating drought stress in rapeseed compared to glycine betaine (GB) and trehalose (Tre).

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LIST OF ABBREVIATIONS

AO	Ascorbate oxidase
APX	Ascorbate peroxidase
AsA	Ascorbic acid (ascorbate)
BARI	Bangladesh Agricultural Research Institute
CAT	Catalase
chl	Chlorophyll
DAE	Department of Agricultural Extension
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
GB	Glycine betaine
Gly I	Glyoxalase I
Gly II	Glyoxalase II
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulphide
GST	Glutathione *S*- transferase
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase
MG	Methylglyoxal
NADPH	Nicotinamide adenine dinucleotide phosphate
PEG	Polyethylene glycol
POD	Peroxidase
Pro	Proline
PS I	Photosystem I
PS II	Photosystem II
ROS	Reactive oxygen species
SOD	Superoxide dismutase
RuBisCo	Ribulose -1, 5- bisphosphate caroxylase or oxygenase

Tre	Trehalose
USDA	United States Department of Agriculture

Chapter I

INTRODUCTION

Rapeseed and mustard (*Brassica* sp.) belongs to the family Brassicaceae and are among the most important oilseed crops throughout the world. They have a remarkable demand for edible oil in Bangladesh. Rapeseed and mustard occupies the first position in respect of area and production among the oilseed crops grown in Bangladesh (DAE, 2016). An estimation in 2015 showed that rapeseed and mustard covered 0.581 million hectares land area with a production of 0.703 million metric tons, whereas the total area covered by oilseed crops was 0.853 million hectares and the total production was 1.003 million metric tons (DAE, 2016).

Since plants first evolution, the earth has experienced a repeatedly changing climate. As a result, in nature, plants are often exposed to diverse environmental adversities those limit crop yield by changing molecular, biochemical and physiological processes, consequently hampering the productivity (Hasanuzzaman *et al*., 2017). As plants are sessile organisms, they frequently faces a number of adverse environmental conditions known as abiotic stress including drought, salinity, temperature extremes, toxic metals etc. which negatively influence the survival, biomass production and yield of plants (Mantri *et al*., 2012; Hasanuzzaman *et al*., 2012a, 2017). Upto 50% yield reduction is the ultimate consequences of abiotic stresses (Hasanuzzaman *et al.,* 2012a).

Among the abiotic stresses, drought or water deficit condition is one of the most complex and devastating stressors because of its multifarious nature of damage to plants (Pennisi, 2008). Because of the frequent crises in both surface and ground water worldwide, drought is predicted to increase day by day (Mishra and Singh, 2010). Drought has various detrimental effects on plant growth and development which causes substantial reductions in growth rate and biomass accumulation of plants. The primary reasons for drought induced growth reduction are reduced water content and turgor loss due to imbalanced osmotic pressure (Jaleel *et al*., 2009; Din *et al*., 2011). Drought or water deficit condition inhibits ribulose-1,5-bisphosphate

carboxylase/oxygenase (RuBisCo) activity, decreases photosynthetic efficiency, respiration and stomatal conductance, and disrupt energy balance and distribution during photosynthesis (Demirevska *et al*., 2010; Rapacz *et al.*, 2010). During the photosynthesis process, insufficient energy dissipation leads to the overproduction of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) (Hasanuzzaman *et al*., 2012a) and these ROS causes oxidative damage to plant tissues. ROS are the major toxic redicals those can also potentially react and damage important biomolecules including proteins, lipids and DNA which ultimately lead to irreparable metabolic dysfunction and cell death (Vranova *et al*., 2002).

Fortunately, to counteract the harmful ROS, plants have evolved highly efficient antioxidative systems composed of both nonenzymatic and enzymatic components (Gupta *et al*., 2009; Hasanuzzaman *et al*., 2012a; Hasanuzzaman and Fujita, 2013). Efficiency of antioxidant defense system is an important strategy, which can effectively detoxify ROS. Besides ROS, methylglyoxal (MG) is another cytotoxic compound that also damages proteins, lipids and carbohydrates (Hasanuzzaman *et al*., 2011a, b, 2012b, c). However, plants also possess a detoxification system for MG (Yadav *et al*., 2005a, b) which helps to reduce adverse effect of MG under different abiotic stresses including drought (Hoque *et al*., 2007; Hasanuzzaman and Fujita, 2011).

World population continues to increase intensely. It is estimated that there will be 9 billion people on this planet in 2050 and this will require a doubling of food production. To meet this challenge, we must increase the yield potential of our food crops by reducing the yield losses caused by different kinds of biotic and abiotic stresses including drought (Tuteja *et al*., 2012). Being an agriculture-based country, Bangladesh is also struggling to be able to adapt to the changing climate, along with the challenges of a growing population for sustaining food security. In recent decades, Bangladesh has shown an increased drought frequency and intensity due to land use pattern changes and the western region of Bangladesh is at high risk of drought hazard under climate changing situation (Selvaraju *et al*., 2006). Scientists of Bangladesh has increased their concern regarding changes in precipitation, potential

evapotranspiration (PET), and drought events as well as developing drought tolerant crop varieties.

Although *Brassica* sp. is the principal oil crop in Bangladesh but its cultivation is much neglected. Moreover the yield of *Brassica* is low in Bangladesh as compared to other countries of the world. *Brassica* is extremely sensitive to drought. Experiments on drought stressed *Brassica napus* L. have shown growth reduction resulted from significantly decreased germination percentage, seedling fresh weight (FW) and dry weight (DW), vigor index etc. (Razaji *et al.,* 2014). Drought induced severe oxidative damages and antioxidant enzyme activities have also been reported in *Brassica* sp. (Alam *et al*., 2014). Thus, a well-focused approach for exploring suitable crop varieties combining the molecular, physiological, biochemical and metabolic aspects of drought tolerance is essential and one of the important tasks for the plant biologists. But this approach is time consuming and searching for other options could be effective for sustainable crop production. In recent years, exogenous protectant such as osmoregulators, plant hormones, signaling molecules, polyamines etc. were found effective for alleviating drought stress (Alam *et al.,* 2013, 2014; Hasanuzzaman *et al.,* 2014; Nahar *et al.* 2016).

Osmolytes or osmoprotectants are small, highly soluble, uncharged, and nontoxic organic molecules which help to survive organisms in extreme osmotic stresses. Organic compatible solutes like proline (Pro), glycine betaine (GB), trehalose (Tre) play significant roles under various abiotic stresses (Ashraf and Foolad, 2007; Farooq *et al*., 2010; Nawaz and Ashraf, 2010). Proline acts not only in osmotic adjustment as a compatible solute, but also efficient in scavenging ROS, chelating metal, activating detoxification pathways, balancing cells redox status, buffering cytosolic pH, storing energy (carbon and nitrogen), stabilizing subcellular membranes and structures including photosystem II (PS II), and as signaling molecule (Trovato *et al.* 2008; Verbruggen and Hermans, 2008; Sharma and Dietz, 2009; Mattioli *et al.* 2009; Szabados and Savouré, 2010; Hayat *et al*., 2012). Besides osmotic adjustment, GB is also involved in ROS scavenging, stabilizing macromolecules (nucleic acids, proteins, and lipids) and various components of photosynthetic machinery such as PS II complexes and RuBisCO and acts as reservoir of carbon and nitrogen sources (Chen and Murata, 2011; Giri, 2011; Ahmad *et al.,* 2013). Trehalose is a non-reducing

disaccharide of glucose that stabilizes biological structures and macromolecules such as proteins and membrane lipids during dehydration and other abiotic stresses (Aghdasi *et al.*, 2008; Duman *et al.*, 2010; Luo *et al.*, 2010).

Although osmolytes are naturally accumulated in plants, their natural accumulation however, is not high enough to protect plants from stress-induced damages (Subbarao *et al*., 2001; Okuma *et al*., 2002; Tamura *et al*., 2003; Tamas *et al*., 2008). Under such condition their exogenous application may help to reduce the adverse effects of various environmental stresses including drought. However, plants response is different to drought stress which varies depending on the crop varieties and the dose and duration of stress. Moreover, the role of exogenous protectants is also variable under such conditions. Although there are several studies on the effect of drought stress on *Brassica* sp. but there is hardly any study regarding the comparative role of exogenous protectants in mitigating drought stress. This study was designed to understand the phygiological mechanisms of drought stress tolerance mediated by exogenous Pro, GB and Tre on rapeseed (*Brassica campestris* L. cv. BARI Sharisha-15). Considering the above mentioned aspects, present study was undertaken with the following objectives:

i. To investigate the effect of drought on rapeseed (*Brassica campestris* L. cv. BARI Sharisha-15) plants.
ii. To investigate the protective effect of Pro, GB and Tre in mitigating drought induced damages.
iii. To find out the most effective osmoprotectant.

Chapter II

REVIEW OF LITERATURE

2.1 Rapeseed and mustard

Rapeseed and mustard are the group of plants of the genera *Brassica* and belongs to the family Brassicaceae, which are the third most important edible oilseed crops of the world after soybean and oil palm (FAOSTAT, 2015) as well as the third most important spice after salt and pepper. Among the different oilseed crops grown in Bangladesh, rapeseed and mustard occupies the first position in respect of area and production. It covered the highest cultivable land with the average maximum production among all oilseed crops grown in Bangladesh (DAE, 2016). High yielding variety and with proper management activities, yield can be as high as 1000 kg per hectare. Recent reports showed production of 12.21 Metric tons from 40.01 acres of land (USDA, 2015). Some of the many vitamins and nutrients found in rapeseed and mustard seeds are selenium and omega 3 fatty acid. The oleiferous *Brassica,* also grown as an edible or an industrial oil crop. The economic value of this crop has made its wide dispersal and it has been grown as a herb in Asia, North Africa, and Europe for thousands of years. Major producers of rapeseed and mustard include Canada, Nepal, Hungary, Great Britain, Pakistan and the United States (FAO, 2014). Brown and black seeded veriety gave higher return than their yellow counterparts. Rapeseed and mustard has a remarkable demand for edible oil in Bangladesh. With increasing growth rate of population, the demand of edible oil is increasing day by day. It is, therefore, highly accepted that the production of edible oil should be increased considerably to fulfill the demand of the country. But the production of mustard is hampered due to many reasons including abiotic stresses (Alam *et al*., 2013; Hosssain *et al.,* 2014).

Rapeseed and mustard crops are mostly affected by drought due to the fact that they are mainly grown in arid and semiarid areas. However, different research organizations including Bangladesh Agricultural Resrarch Institute (BARI) as well as researchers are working hard to develop and release drought tolerant mustard and rapeseed varieties to reduce drought induced drastic yield reduction (BARI, 2015)

2.2 Abiotic stress

Between 2009 and 2050, world population is expected to increase by over a third, or 2.3 billion people (Hasanuzzaman *et al*., 2014). So, it's a major task of 21st century agriculture to produce more food to feed the ever increasing population and adapt to climate change. However, there exists a huge gap between the productivity and food demand of the growing population. The reason behind the lower productivity of crop plants in most of the cases, are the abiotic stresses.

In natural environmental condition, plants are frequently exposed to various abiotic stresses due to the unpredictable nature of the environment and global climate change (Mittler and Blumwald, 2010). The negative impact that is exerted by the environment on the living organism may be collectively termed as abiotic stress (Hasanuzzaman *et al.*, 2012a). The major abiotic stresses include salinity, drought, extreme temperature, flooding, toxic metal/metalloids, ozone, UV radiation, high light, etc.

Abiotic stresses are the greatest constraint to crop production worldwide which can causes upto 50% of yield reduction (Hasanuzzaman *et al*., 2012a). Abiotic stresses greatly affect plant growth and metabolism and ultimately disturbs plant life cycle (Bray *et al*., 2000; Ahmad and Prasad, 2012a, b). Majority of world's arable lands are exposed to these abiotic stresses and has a negative impact on global crop production. If the stresses continues for an extended period of time or becomes high it may lead to an irreparable metabolic damage to cells, reducing growth, and in severe cases, results in plant death (Hasanuzzaman *et al*., 2012a, b). Plants can respond and adapt to various stress condition by altering their cellular metabolism and regulating various defense mechanisms (Ghosh *et al.*, 2011). Survival of plants under this stressful condition also depend on their abilities to perceive the stimulus, generate and transmit a signal, and initiate various physiological and biochemical changes (Tanou *et al*., 2009; El-Shabrawi *et al*., 2010).

Molecular studies showed that plants under stress condition are associated with the production of deleterious chemical entities called ROS, such as, $^1O_2$, $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$ etc. (Choudhury *et al.*, 2013). The production of ROS greatly varies depending on the degree and duration of stress and types of crop. ROS are highly reactive and may cause cellular damage through oxidation of lipids, proteins, and nucleic acids (Apel

and Hirt, 2004). Plants have well-developed enzymatic and non-enzymatic scavenging pathways or detoxification systems to counter the deleterious effects of ROS that include the enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione *S*-transferase (GST), glutathione peroxidase (GPX) and peroxidases (POX) as well as non-enzymatic compounds such as ascorbate (AsA), glutathione (GSH), carotenoids and tocopherols (Hasanuzzaman *et al.*, 2012a, b). Collins *et al.* (2008) reported that the tolerance to abiotic stress is multigenic and quantitative in nature and thus a massive challenge exists to understand the key molecular mechanisms for advanced selective breeding purposes. Similarly, Patakas (2012) reported that the understanding abiotic stress responses in plants is difficult due to the complexity, interrelationship, and variability of mechanisms and molecules involved a fact that consist their evaluation an important and challenging topic in plant research.

2.3 Drought stress

Drought is a meteorological term and is commonly defined as a period without significant rainfall. Generally drought stress occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation. Drought conditions can worsen after prolonged periods of no rainfall, especially in areas where the water supply is short. Due to drought and desertification each year 12 million hectares of cultivable land are lost, where 20 million tons of grain could have been grown (FAO, 2014). Since 1900, more than 11 million people have died as a consequence of drought and among them more than 2 billion have been affected by drought, more than any other physical hazard (FAO, 2015). In Bangladesh a strong drought can cause greater than 40% damage to broadcast Aus, significant destruction to the T. Aman crop in Kharif season in 2.32 million ha and about 1.2 million ha of land during Robi season. Past droughts have naturally affected about 53% of the population and 47% of the country (Dey *et al.*, 2011).

Drought is one of the most destructive environmental stresses that affects the growth and development of plants. Thus, limitation of crop production by drought stress has been recognized as more severe than any other environmental stress in the world

(Cattivelli *et al.,* 2008; Mishra and Singh, 2010). Drought stress severely limits crop productivity and the expansion of crop cultivation worldwide. The effects of drought stress are expected to increase with the climate change and a growing water crisis (Harb *et al.,* 2010). However, the adverse effects of drought stress on growth and development of crop plants are multidimentional in nature (Hasanuzzaman *et al*., 2012 a, b).

The primary effect of drought stress is largely a reduction in plant growth, which depends on cell division, cell enlargement, cell differentiation, and involves genetic, physiological, ecological, and morphological events, and their complex interactions. These events are seriously inhibited by drought stress, which adversely affects a variety of vital physiological and biochemical processes in plants, including stomatal conductance, membrane electron transport, carbon dioxide (CO_2) diffusion, carboxylation efficiency, water-use efficiency (WUE), respiration, transpiration, water loss, photosynthesis, and membrane functions. Disruption of these key functions limits growth and developmental processes, and leads to reductions in final crop yield. Available literature revealed that plants develop different mechanisms like reduced growth, metabolic alteration, accumulation of compatible solutes, activation of the antioxidant defense system, and suppression of energy consuming pathways to cope with a water limited environment (Nahar *et al*., 2016)

A principal sign of drought stress at the molecular level is the accelerated production of ROS such as $^1O_2$, $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$ to levels that are often beyond the plant's scavenging capacity. This causes oxidative stress that damages cells and cellular components, disrupts the physiological and biochemical life processes, and even leads to plant death (Li *et al.,* 2010; Faize *et al.,* 2011; Hasanuzzaman and Fujita, 2011). The excess production of ROS is common in drought and results from impaired electron transport processes in the chloroplasts and mitochondria. Photorespiration is one of the major causes of ROS production under drought stress which accounts for more than 70% of the total H_2O_2 produced. Plants have endogenous mechanisms for adapting to ROS production and are thought to respond to drought stress by strengthening these defense mechanisms. Therefore, enhancement of the functions of the naturally occurring antioxidant components (enzymatic and non-enzymatic) may be one strategy for reducing or preventing oxidative damage (Hasanuzzaman *et al.,*

2014) and improving the drought resistance of plants. The mechanism of drought tolerance includes ion homeostasis, biosynthesis of osmolytes, scavenging of harmful radicals, water circulation and coordination of a long distance response system (Reddy *et al.,* 2004).

2.4 Effects of drought stress on *Brassica* and other plants

Drought stress has adverse effects on growth, physiology and yield of various crop plants. *Brassica* sp. is also very much sensitive to drought stress. Different crop species respond differently to drought depending on the intensity of stress and types of crops. Several research studies have been conducted to investigate the effects of drought stress on plants.

Brassica campestris (Sarson), *B.* carinata (Ethopian mustard), *B. juncea* (Brown mustard), and *B. napus* (oilseed rape) were assessed after subjected them to repeated drought cycles for 24 days. It was found that biomass production and water status significantly decreased in all four sp. under drought stress. Responses to drought stress were different in all four species. *B. carinata* produced significantly lower fresh and dry biomasses and had less water content, wax on leaf surface, and total protein content in shoots and roots. By contrast *B. napus* produced relatively greater fresh and dry biomass and had higher water content, chl content and protein content in shoots and roots (Ashraf, 1990).

Kaya *et al*. (2006) studied the germination of the sunflower *(Helianthus annuus* L.) using distilled water as control and under osmotic potentials of −0.3, −0.6, −0.9 and −1.2 MPa which were imposed by polyethylene glycol (PEG 6000). The germination percentage under −0.3, −0.6 and −0.9 MPa PEG treatment was 98.6%, 96% and 26.7% respectively. None of the seeds was able to germinate at −1.2MPa of PEG. The drought stress was not only accompanied with inhibition of germination but also accompanied with increase of abnormal germination and seedling. With decrease in osmotic potential the mean germination time (MGT) increased that caused delayed seedling emergence.

Effects of drought on yield and yield components of 14 Indian mustard genotypes were studied by Chauhan and Tyagi (2007). They reported that plant height, primary branches, secondary branches per plant, 1000-seed weight and seed yield were

reduced under non irrigated drought condition. PSR-20 and JMMWR-941 were among the top genotypes those showed relatively low drought severity index for one or more characteristics, such as primary branches per plant, secondary branches per plant, harvest index and seed husk ratio.

Nouri-Ganbalani *et al.* (2009) conducted an experiment with winter and intermediate wheat genotypes and evaluated under normal irrigation and drought stress condition. Drought stress decreased the average grain yield of all wheat genotypes by 50%. The yield reduction was related to the reduction of the number of fertile tillers, peduncle length, number of grains per spike, weight of grains per spike and 1000-grain weight.

Tomato plants were subjected to drought stress at different stages of growth. Germination percentages were 99.36, 89.66, 91.67, 90; and emergence percentages were 91.5, 77.5, 78.5, 81.0, respectively, under the treatments of control, early drought stress (when first truss has set the fruits), middle stress (when fruits in first truss were fully matured and started changing their color), and late drought stress (when fruits on first truss were ripened fully) (Pervez *et al*., 2009).

An experiment was carried out to study the effect of drought stress on physiological growth indices of three cultivars (Zarfam, Okapi and Licord) of winter rapeseed (*B. napus*). The results showed that total dry matter (TDM), leaf area index (LAI), relative growth rate (RGR) and crop growth rate (CGR) were significantly different among the rapeseed cultivars, whereas, drought stress had effects of practical significance on TDM, LAI, RGR and CGR. The results also showed that the highest TDM, LAI, RGR and CGR were obtained from the cultivar, Zarfam under no-drought condition. The findings firmly established that the drought stress sorely reduces physiological growth indices of winter rapeseed cultivars under conditions of drought stress (Moaveni *et al*., 2010).

Ardestani *et al*., (2011) conducted an experiment with the aim of evaluating agronomic traits related to two rapeseed varieties (Zarfam and Opera) subjected to drought stress condition. Results revealed that, with increase in stress intensity, seed yield and rest of tested traits drastically decreased. Regarding seed yield Zarfam showed 18.49% superiority rather than Opera. Plots with irrigation condition of I1

(control) showed 36.89% enhancement of seed yield as compared to units with I3 condition (severe stress).

An experiment was conducted by Rad and Zandi (2012) to evaluate the spring rapeseed (*B. napus*) cultivars subjected to drought stress. Water deficit stress, caused reduction in plant height, branch plant^{-1}, siliqua plant^{-1}, seed siliqua^{-1}, 1000-seed weight, seed yield, biological yield, oil content, and oil yield while it did not affect harvest index significantly. Correlation coefficient analysis revealed that number of siliquae per plant had the highest correlation with seed yield as compared with other yield components.

Drought tolerance of canola cultivars viz., Shirale, Oscar, Con-II, Rainbow and 19H was investigated after exposure to drought stress at various growth stages in a pot experiment. Water stress was imposed at flowering and pod filling growth stages. Data of various physiological (leaf chl *a* & *b*, Pro and protein contents) and agronomic attributes (number of pods/plant, seeds/pods, grain yield/plant) was recorded. The data revealed significant differences among the various canola genotypes for leaf chl *a, b* and Pro accumulation. The chl *a* & *b* content of all the *napus* genotypes declined due to drought stress at both the growth stages (Mahmood *et al.,* 2012).

Thameur *et al*. (2012) demonstrated that drought stress of 50% field capacity reduced the yield of barley. The yield of cultivar Switir was 40% and the yield of Tlalit was 34%, compared with the yield of those cultivars under adequately irrigated control (100% field capacity).

Seeds of different varieties of wheat were imposed to drought (induced by PEG with osmotic potentials of 0, -4/0, -8/0, and -2/1 mp). Germination rate (GR), germination index (GI), mean of emergence time (MET), final germination percentage (FGP), and germination rate index (GRI) were negatively affected (Jahanbin *et al*., 2012).

Water stress declines photosynthetic pigment contents. This is another reason for decreasing photosynthesis under drought stress. Reduced photosynthetic pigment content including chl *a*, chl *b*, total chl, and carotenoid under drought stress has been

reported in several plant species, such as *Avena* sp., *Triticum* sp., and *Gossypium* sp. (Pandey *et al*., 2012).

Similar results were investigated in canola (Din *et al*., 2011), in *Albizia lebbeck* and *Cassia siamea* seedlings (Saraswathi and Paliwal, 2011), in black gram (Pratap and Sharma, 2010), in water lettuce (Singh and Pandey, 2011), in bean *(Phaseolus vulgaris)* (Abass and Mohamed, 2011).

Alam *et al*. (2013) conducted an experiment with *Brassica* sp. He found that drought stress reduced tissue water content, leaf RWC and total chl content in *Brassica* sp.

Rice (*Oryza sativa* L. cv. Hashmi) plants were subjected to drought stress at different growth stages: mid tillering, panicle initiation and 50% flowering stage. Drought stress showed adverse effects on number of fertile tillers, number of panicle per unit area, number of filled and unfilled grains and plant height had a significant effect on grain yield, but did not show significance effect on 1000-grain weight (Sabetfar *et al.,* 2013).

Different levels of drought stresses (−4, −6, −8 and −12 bar) were applied in rapeseed (*B. napus*) and its performance was compared with control seedlings of rapeseed grown without drought stress. With the increase of drought stress germination percentage, seedling FW, seedling DW, shoot length, root length, and vigor index significantly decreased whereas proline content increased as compared to control (Razaji *et al.,* 2014).

Hossain *et al*. (2014) carried out an experiment with mustard (*Brassica juncea* L.) under drought stress. He revealed that drought stress resulted in enhanced oxidative stress as indicated by increased levels of lipid peroxidation and H_2O_2. It was also found that 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

An experiment was carried out by Aliakbari *et al.* (2014) in order to assess drought tolerance in fifteen rapeseed (*Brassica napus*) cultivars. Highly significant differences among the rapeseed cultivars for yield was observed in normal and stress conditions as well as all the drought tolerance indices. Karun cultivar had the maximum seed yield in both conditions.

Growth and yield responses of three canola (*B. napus*) cultivars (CON-II, CON-III and Dunkeld) to drought stress were investigated. Results revealed that drought stress significantly reduced the plant height, number of branches plant^{-1}, biological yield, number of siliqua plant^{-1}, number of grains siliqua^{-1} and grain yield and there was significant differences among canola cultivars, in terms of drought stress tolerance (Huq *et al*., 2014).

In alfalfa (*Medicago sativa* L.), PEG induced drought stress significantly hampered its germination and growth. Germination was delayed by drought stress, and energy and power of germination were reduced due to drought stress. On the other hand growth parameters including length of leaves, FW and DW from roots and leaves decreased under drought stress (Castroluna *et al*., 2014).

Zirgoli and Kahrizi (2015) evaluated the drought stress tolerance in rapeseed (*B. napus*) varieties. It was found that drought stress had significant effect on plant height, stem diameter, lateral shoot number, biological yield, flowering duration, days to maturity, pod per plant, seed per pod, 1000 seed weight, seed yield and harvest index. Seed yield average reduced by 29.18% due to drought stress. Meanwhile, pod per plant reduced more than other traits (32.54%). Cultivars were significantly different for all traits except stem diameter, flowering duration, days to maturity.

Koh *et al*. (2015) examined the proteome changes of canola (*B. napus*) under drought stress over a 14-day period. They identified 1976 proteins expressed during drought stress. Among them, 417 proteins showed significant changes in abundance, and 136, 244, 286, and 213 proteins were differentially expressed in the third, seventh, 10th, and 14th day of stress, respectively. Functional analysis indicated that the number of proteins associated with metabolism, protein folding and degradation, and signaling

decreased, while those related to energy (photosynthesis), protein synthesis, and stress and defense altered in response to drought stress.

Shekari *et al*. (2015) conducted an experiment to investigate the effect of draught stress on water relations, stomatal density, chlorophyll content and yield of rapeseed. The results showed that the lowest relative water content and leaf water potential were obtained at 30% AWC (Available water content) and silique development stage. Meanwhile, the highest water use efficiency (WUE) was observed during flower bud and silique development stages and 70% AWC. Chl contents, Seed protein and seed oil contents were adversely affected by stress level and stress application times. Severe water deficit led to the decrease of chl *a*, *b*, total chl, seed protein, oil content and yield.

Majidi *et al.* (2015) studied the response of drought in *Brassica* sp. under normal, moderate and severe stress environments and found that moderate and intense stress caused reduction in seed yield and the most studied traits. Moderate drought stress significantly increased the ratio of chl *a* to chl *b* while severe stress decreased it. Positive correlation was also found between proline content and drought susceptibility index under both stress conditions.

2.5 Oxidative stress in plants under drought stress

Drought stress may lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation, exposing chloroplasts to excessive excitation energy, which in turn can increase the generation of ROS and induce oxidative stress (Mittler, 2002; de Carvalho, 2008). The excess production of ROS during drought stress results from impaired electron transport processes in the chloroplasts and mitochondria (Smirnoff, 1993). Down-regulation of PSII results in a disproportion between the generation and utilization of electrons, resulting in changes in quantum yield. These changes in the photochemistry of chloroplasts in the leaves of drought-stressed plants result in the dissipation of excess light energy in the PSII core and antenna, thus generating free radicals like $O_2^{\bullet -}$, $^1O_2$, H_2O_2 and $OH^{\bullet}$, which are potentially dangerous under drought stress (Li *et al.,* 2010a; Faize *et al.,* 2011). In fact, under drought stress, ROS production is enhanced in different ways. However, it

is quite complicated to assess the part of ROS generated by the Mehler reaction to that generated by photorespiration. Photorespiration is one of the major causes of ROS production under drought stress; more than 70% of total H_2O_2 is produced due to photorespiration (Noctor *et al.*, 2002b). ROS accumulation and oxidative stress increase under drought stress (Li *et al.*, 2010a; Faize *et al*., 2011; Sorkheha *et al*., 2011) and drought-induced oxidative stress significantly increases lipid peroxidation (Pandey *et al.*, 2010; Hasanuzzaman and Fujita, 2011) . Numerous research findings describe the ROS generation and their successive damage effects under drought or water deficit stress.

Drought stress weakens antioxidant system (reduced activities of SOD, APX and CAT and contents ascorbate, AsA) in *Triticum aestivum* L. which exhibited high H_2O_2 and oxidized ascorbate levels and leading to enhanced membrane damage during severe drought stress, indicated by the accumulation of MDA. Between two cultivars of wheat, 'Sids' (drought susceptible) exhibited more oxidative stress, compared to 'Veery' (drought resistant) (Al-Ghamdi, 2009).

Many studies observed that reproductive organ development is extremely sensitive to drought stress across different crop species when ROS cause severe damages to reproductive organ (Lalonde *et al.*, 1997; Liu *et al*., 2006). During the anther development in rice, CAT, APX, DHAR enzymes were suppressed severely during the meiosis stage which enhanced ROS production (Nguyen *et al.*, 2009).

Ma *et al.* (2011) observed the changes of antioxidant system and oxidative state of leaves of 2-yearold potted apple (*Malus domestica* Borkh.) plants under drought stress. During severe drought stress, activities of the enzymes (APX, MDHAR, DHAR, GR) and redox state of AsA and GSH decreased to a great extent for which the the generation of ROS is evident which resulted in increased levels of malondialdehyde (MDA) or lipid peroxidation.

Deeba *et al.* (2012) studied the effects of drought stress in cotton genotype RAHS 187 for changes in physiology, biochemistry and proteome. The gas-exchange parameters of net photosynthesis, stomatal conductance and transpiration decreased gradually with the increase of drought intensity. The fluorescence parameters of effective

quantum yield of PSII, and electron transport rates also showed a declining trend. These impaired events were distinguished for creating oxidative stress which was indicated by increased generation of H_2O_2 and MDA levels.

Uzilday *et al.* (2012) compared the differences between antioxidant responses to drought in C3 (*Cleome spinosa*) and C4 (*C. gynandra*). Parallel to results of MDA, H_2O_2 content was also remarkably increased in *Cleome spinosa* as compared to *C. gynandra* under drought stress because in *C. spinosa*, antioxidant defence system was insufficient to suppress the increasing ROS production under stress condition. On the other hand, in *C. gynandra*, although its induction was lower as compared to *C. spinosa*, antioxidant system was able to cope with ROS formation under drought stress.

Drought (–0.2 MPa and –0.4 MPa) stress effects were observed in the antioxidant system and oxidative damage of in melon seedlings. Significant rise of H_2O_2 level and MDA content was directly correlated to the changes of antioxidant components of melon seedlings. However, oxidative stress increased with the increase in severity of drought stress and the melon cultivar Galia is more tolerant than Kırkağac (Kavas *et al.*, 2013).

2.6 Antioxidant defense mechanism

Plants antioxidant defense system primarily constitutes of some antioxidant enzymes and non-enzymatic antioxidant components. Antioxidant enzymes include SOD, CAT, APX, GR, MDHAR, DHAR, GPX, GST and POD. Non-enzymatic antioxidants may include AsA, GSH, carotinoids, phenolic compounds, alkaloids, non-protein amino acids and α-tocopherols (Gill and Tuteja, 2010; Hasanuzzaman *et al.*, 2012c).

Plant adaptation and improved tolerance to drought stress have been documented to correlate with higher antioxidative capacity in different studies. Under stress condition including drought to quench the higher or excess amount of ROS produced by stress, plants improve or uplift their antioxidant system by increasing the amount or activity of components of antioxidant system. Ascorbate is an important antioxidant that reacts with numbers of ROS such as H_2O_2, $O_2^{\bullet-}$ and $^1O_2$ (Smirnoff, 2005). Ascorbate is utilized by APX to reduce H_2O_2. In this reaction H_2O and MDHA

generates. Then MDHA (monodehydroascorbate) by MDHAR activity is converted into DHA and AsA where NADPH (nicotinamide adenine dinucleotide phosphate) is electron doner (Gapper and Dolan, 2006). Glutathione directly scavenge ROS (Noctor and Foyer, 1998), it helps in regeration of other antioxidants in AsA-GSH (Ascorbate glutathione) cycle (Foyer and Halliwell, 1976). Tocopherol is mainly responsible to reduce $^1O_2$ and $OH^{\bullet}$ (lipid peroxyl radicals, LOO•) in thylakoid membranes and plays vital roles in protecting photosynthetic membrane (Maeda *et al.,* 2005). Among the antioxidant enzymes SOD encompasses the frontline defense against ROS by catalyzing the removal of $O_2^{\bullet-}$ by forming H_2O_2 and O_2. Catalase (CAT) helps to convert H_2O_2 into H_2O and O_2 chiefly in peroxisomes and glyoxysomes (Sanchez-Casas and Klesseg, 1994; Agarwal *et al*., 2009). Catlase is very powerful enzyme having the highest turnover rate. It can reduce around six million molecules of H_2O_2 (Gill and Tuteja, 2010). Four enzymes APX, MDHAR, DHAR and GR comprise of the AsA-GSH cycle with other non-enzymatic antioxidant components. In AsA-GSH cycle, APX converts H_2O_2 to H_2O and MDHA which can be further converted into AsA in ways (Chen *et al.,* 2003). Glutathione reductase is a potential enzyme of the AsA-GSH cycle that helps in ROS detoxification. Activity of GR catalyses the NADPH-dependent reduction of disulphide bond of GSSG and recycles back GSH and is thus important for maintaining the GSH pool. Glutathione peroxidase reduces H_2O_2 by using GSH. The GPX also functions as an oxidative signal transducer (Miao *et al.,* 2006). Glutathione *S*-transferase catalyzes the conjugation of electrophilic xenobiotic substrates with GSH and produces less toxic and more water-soluble conjugates. Various abiotic stresses including drought induce GST activity in plants and confers stress tolerance to plants (Dixon *et al*., 2010). Numerous research studies proved that the enhanced antioxidant system confer abiotic stress tolerance including the drought stress tolerance.

Drought stress (induced by 20% PEG) significantly increased GSH and glutathione disulfide (GSSG) content; decreased AsA content MDHAR and GR activity; modulated DHAR, GST, GPX, CAT activities and resulted in significant increases of H_2O_2 and MDA levels in *Brassica napus* seedlings. But exogenous selenium pretreatment alleviated oxidative stress and enhanced drought stress tolerance by improving AsA and GSH content, GSH/GSSG ratio, increasing the activities of APX, DHAR, MDHAR, GR, GST, GPX, CAT (Hasanuzzaman and Fujita , 2011).

The GSH redox pool was higher in acclimated wheat plants, compared than that of drought non-acclimated plants which improved the drought tolerance in acclimated plants (Selote and Khanna-Chopra, 2006).

Enhanced activities of SOD and APX, MDHAR, DHAR and GR provided antioxidative defense system under drought-induced oxidative stress in rice (Sharma and Dubey, 2005). Under drought stress the activities of GPX, APX and CAT in the roots and shoots of drought tolerant *Zea mays* L. var. 704 plants were higher, compared to sensitive var. 301(Mohammadkhani and Heidari, 2007).

Mohammadkhani and Heidari (2007) observed a positive and strong correlation between antioxidant enzymes and drought stress while investigating the responses of *Zea mays* L. var. 704 (drought-tolerant) and var. 301 (drought-sensitive).

Comparing the activities of APX, DAHR, MDHAR and GR and AsA content of different species, it is evident that among all the cultivars, the tolerant cultivar Zarina had the highest content of AsA and the highest activities of those antioxidant enzymes which made Zarina to be tolerant to drought induced oxidative damage (Sánchez-Rodríguez *et al.,* 2010).

Performance of wheat seedlings were investigated under drought. One set of seedlings were acclimated and other was not acclimated before exposing drought stress. Non-acclimated wheat seedlings showed reduced activities of antioxidant enzymes including APX, MDHAR, DHAR, GR and the reduced AsA-GSH redox balance which were responsible for excessive accumulation of H_2O_2 and lipid peroxidation, greater water loss. In contrary, drought acclimation enhanced AsA–GSH cycle components and activities of SOD, CAT, POD in wheat seedlings which efficiently removed oxidative damage effects from those seedlings (Selote and Khanna-Chopra, 2010).

Shehab *et al*. (2010) reported an increase in the activity of various antioxidant defense enzymes (SOD, APX, GR and CAT) in rice, representing protective activity to counteract the oxidative injury caused by drought.

Selote and Khanna-Chopra (2010) demonstrated that drought acclimation induces oxidative stress tolerance of wheat seedlings, attributed to a well-coordinated induction of the ROS detoxification system.

In a study with ten cultivars of oilseed rape (*B. napus*) Abedi and Pakniyat (2010) reported that the oilseed rape variety with the highest level of enzyme activity under both optimum and limited irrigation regimes (drought) was considered to be the most tolerant cultivar while the varieties with the lowest enzymes activities were considered to be sensitive to drought stress.

The importance of well coordinated antioxidant defense in inducing drought tolerance was also observed in a study with rapeseed seedlings (*B. napus* cv. BINA sharisha 3) by using exogenous Se (Hasanuzzaman and Fujita, 2011). Se-pretreated (25 m M Na_2SeO_4, 48h) seedlings exposed to drought stress showed a rise in AsA and GSH content, maintained a high GSH/GSSG ratio, and evidenced increased activities of APX, DHAR, MDHAR, GR, GST, GPX and CAT accompanied by lower levels of H_2O_2 and MDA and thus gave tolerance to drought-induced oxidative stress

Filippou *et al.* (2011) described that CAT has a primary role in H_2O_2 detoxification in *Medicago* plants. Activity of CAT was significantly induced in leaves after imposing water stress. *Lycopersicon esculentum* was subjected to drought stress (50% field capacity) for 22 days. Drought stress significantly increased the H_2O_2 and MDA level in different cultivars of *Linum esculentum*.

Canola seedlings exposed to drought (5, 10, and 15% PEG) stress showed oxidative stress/lipid peroxidation. The both cultivars of canola Hyola 308 and SLM046 showed oxidative stress with more adverse effects on Hyola 308. With the increased of water stress SOD, POD, CAT and APX antioxidant enzyme activities of both shoots and roots of those cultivars increased but 14 activity of these antioxidants in SLM046 cultivar was obviously higher than in Hyola 308 cultivar. These results showed a higher water stress tolerance for SLM046 cultivar (Mirzaee *et al*., 2013).

2.7 Glyoxalase system and its roles under drought stress

Methylglyoxal is a cytotoxic compound for plant cells highly produced during abiotic stresses including drought stress that can cause oxidative stress and can damage the protein, lipids, carbohydrates (Yadav *et al*., 2005a, b; Singla-Pareek *et al*., 2006; Singla-Pareek *et al*., 2008; Hasanuzzaman *et al*., 2011a, b; Hasanuzzaman *et al*., 2012b, c). To detoxify the toxic effect of MG, plants posses glyoxalse system consisting of two enzymes: Glyoxalase I (Gly I) and glyoxalase II (Gly II) (Yadav *et al*., 2005a, b). The Gly I converts MG to s-D-lactoylglutathione by utlizing GSH. Gly II converts s-D-lactoylglutathione to D-lactic acid. At the end of these reactions, GSH is recycled backed. Upregulation or overexpression of Gly I and Gly II helps to alleviate the MG under different abiotic stresses including drought (Hoque *et al*., 2007; Hasanuzzaman and Fujita, 2011).

Drought stress resulted in high rise of MG content in rice seedlings (Yadav *et al*., 2007). Similar rise of MG level was also observed in *Brassica* seedlings (Saxena *et al*., 2005). It was also found that drought stress enhanced gly II transcript expression in *Brassica* and in rice which conferred drought stress tolerance in rice. These studies have clearly implicated a role of the glyoxalase pathway in plants during stress exposure (Saxena *et al*., 2005; Yadav *et al*., 2007).

Overexpression of the glyoxalase pathway in transgenic tobacco and rice plants has been found to reduce ROS and MG under drought stress conditions by maintaining glutathione homeostasis and antioxidant enzyme, glyoxalase enzyme levels. A sharp increase in Gly I activity (1.82-fold) was observed in response to drought (1.27-fold) stress. A sharp increase in Gly I activity due to drought suggested that Gly I expression might be under stringent regulation of photoreceptors. There is also evidence that in addition to glyoxalase, other pathways, such as the aldose reductase pathway, may also be involved in MG detoxification in plants. The role of MG and the glyoxalase pathway in signal transduction are also important under drought stress conditions (Yadav *et al*., 2008).

The regulatory role of selenium in drought stress affected *Brassica napus* was investigated. Drought stress increased H_2O_2 and MDA content which are indicators for oxidative stress. The components of glyoxalase system were also altered by

drought stress. However, Se-pretreated seedlings exposed to drought stress showed a rise in GSH content and enhanced activities of Gly I and Gly II enzymes which were supposed to reduced drought induced MG and the subsequent oxidative damages (Hasanuzzaman and Fujita, 2011).

Alam *et al*. (2013) observed the performance of *Brassica juncea* L. cv. BARI-Sharisha 11 under drought (induced by 10 and 20% polyethyleneglycol) stress. They studied different physiological attributes including the MG detoxification system under drought stress and combination of drought with salicylic acid. The glyoxalase system components; Gly I and Gly II activities decreased due to drought and GSH content increased slightly. Spraying seedlings with 50 μM salicylic acid enhanced the glyoxalse system components (Gly I and Gly II activities, and GSH content), reduced oxidative stress and improved physiological parameters and thus drought tolerance was ensured.

2.8 Osmoprotectants

Osmoprotectants or compatible solutes are small, electrically neutral, non-toxic molecules at molar concentrations and highly soluble organic compounds those act as osmolytes and help organisms survive extreme osmotic stress (Nahar *et al*., 2016a). Osmoprotectants comprise of i) α-amino acids such as Pro and ectoine, ii) ammonium compounds such as GB, *b*-alanine betaine, dimethyl sulfoniopropionate (DMSP), choline and iii) polyols, sugars and sugar alcohols such as Tre, sorbitol and mannitol etc (Nahar *et al*., 2016a). Naturally, plants can accumulate osmoprotectants at the levels ranging 5–50 μmol g^{-1} FW (Rhodes and Hanson, 1993; Bohnert *et al.*, 1995). Osmoprotectants are typically confined to cytosol, chloroplasts, and some other cytoplasmic compartments occupying 20% or less of the volume of mature cells and these organic compatible solutes play important role under different abiotic stress conditions (Ashraf and Foolad, 2007; Farooq *et al*., 2010; Nawaz and Ashraf, 2010). These osmoprotectants perform vital functions in osmotic adjustment, stabilizing proteins and membranes. Most of the reports primarily emphasize the osmoregulatory roles. The other mechanisms of osmolytes are biological membrane protection, detoxification of toxic compounds such as ROS and MG, alleviation of ionic toxicity, protection of photosynthetic and mitochondrial structure, and metabolism (Alam *et al*., 2014). Moreover, the signaling role of osmolytes has also been designated as a

vital stress-protective mechanism. The amino acid Pro plays a highly beneficial role in plants exposed to various stress conditions as an osmolyte Pro acts not only in osmotic adjustment as a compatible solute, but also in scavenging ROS, chelating metal, activating detoxification pathways, balancing cells redox status, buffering cytosolic pH, storing energy (carbon and nitrogen), stabilizing subcellular membranes and structures including photosystem II (PS II) and as signaling molecule (Trovato *et al.*, 2008; Verbruggen and Hermans, 2008; Sharma and Dietz, 2009; Mattioli *et al*., 2009; Szabados and Savouré, 2010; Hayat *et al*., 2012). Besides osmotic adjustment, GB is 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 (Chen and Murata, 2011; Giri, 2011; Ahmad *et al.*, 2013). Various beneficial effects were observed in different physiological parameters. Increased relative growth rate, chl content, N content, DW and biomass plant^{-1} were observed with Tre supplementation. Tre application also improved Pro accumulation, K^+ accumulation and K^+/Na^+ ratio. In addition, Tre has functions in stabilizing the biomolecules and structures like membrane lipids, proteins (Aghdasi *et al*., 2008; Duman *et al*., 2010; Luo *et al*., 2010). Reports exist describing the beneficial roles of exogenously applied osmoprotectants under drought stress. Researches in metabolomic, polyomic, transcriptomic, and transgenic levels explored that plants overexpressing osmolyte biosynthesis or metabolic genes showed enhanced stress tolerance (Rontein *et al*. 2002; Chen and Murata, 2002, 2008; Ashraf and Foolad, 2007; Peñuelas *et al*., 2013).

2.9 Role of proline, glycine betaine and trehalose in mitigating drought stress

2.9.1 Role of proline in drought stress tolerance

Drought-induced accumulation of osmolytes has been reported in different plant species. Different research findings demonstrated that exogenous Pro, GB and Tre can mitigate the devastating damages caused by drought and also enhance drought stresss tolerance.

Water stress reduced growth and photosynthetic capacity (chl *a* and *b* decrease) of 2 maize cultivars 2 maize cultivars, viz., EV-1098 and AGAITI 2002. However, exogenous application of Pro counteracted the adverse effects of water stress on growth of both maize cultivars. Photosynthetic rate of water stressed plants of both maize cultivars was also enhanced due to foliar applied Pro which was positively associated with sub-stomatal CO_2 (*C*i,) and stomatal conductance (*g*s) as well as photosynthetic pigments (Ali *et al*., 2007).

Responses of two *Z. mays* cultivars EV-1098 and Agaiti 2002, under drought stress was studied where the protective effects of exogenous Pro was investigated. Water stress reduced the concentration of all four mineral nutrients in the shoots and roots of both maize cultivars. However, exogenous application of Pro counteracted the adverse effects of water stress on nutrient uptake because it promoted the uptake of K^+, Ca^{2+}, N and P in both maize cultivars (Ali *et al*., 2008).

An experiment was conducted to assess the effect of exogenous application of Pro as a presowing seed treatment on morpho-physiological and yield attributes of spring wheat (*T. aestivum*) under water deficit conditions. Proline when used as pre sowing seed treatment under drought stressed seedlings showed improved shoot and root fresh and dry weights, shoot length and grain yield under both non-stress and stress conditions (Kamran *et al.*, 2009).

Similarly, strawberry grown in osmotic stress, Gerdakaneh *et al*. (2010) reported that exogenously applied Pro in the callus culture medium increased the growth rate of calluses and their internal level of free Pro, however, the maximum increase was observed with 10 mM Pro level.

Aggarwal *et al*. (2010) reported that exogenous application of Pro (50 m*M*) resulted in an increase in its internal levels and reduced the water stress injury with an increase in enzymatic (SOD, POD, CAT) and non-zymatic antioxidants, especially the components of ascorbate glutathione cycle.

Furthermore, Ahmed *et al*. (2010) reported the stress inducible effects of exogenous application of proline in olive plants. Exogenous application of Pro improved the

antioxidant enzyme activities of stress plants. They also reported that exogenous proline application also increased thephotosynthetic rate of stressed plants that was associated with plant growth and water status.

While working on reducing the adverse effects of drought stress on yield characteristics of different vegetables, Caronia *et al.* (2010) reported that foliar-applied Pro resulted in stress tolerance in terms of nitrogen absorption and fruit weight gain. Of foliar-applied levels, 400 mg L^{-1} was found to be most effective in increasing the total solid contents of fruits.

Moustakas *et al.* (2011) investigated the effects of exogenous applied Pro, on PS II photochemistry of drought stressed (DS) 4-week old *Arabidopsis thaliana* plants. The maximum quantum yield of PSII photochemistry (F_v/F_m) in DS plants decreased significantly to 77% of that of the control with increased free Pro and total soluble sugars (SS) in response to DS. Exogenous foliar application of Pro by spraying, led to a remarkable increase in the accumulation of Pro and surprisingly also of SS. DS plants sprayed with Pro showed a tolerance to photoinhibition lead to conclude that Pro appears to be involved in the protection of chloroplast structures by quenching ROS.

Drought stress significantly affected the chemical composition of maize seed but foliar application of Pro ameliorated the adverse effects of drought on seed chemical composition. Increased seed sugar, oil, protein, moisture, fiber and ash, and oil oleic and linoleic acid contents were found in *Z. mays* when treated with exogenous Pro. Increased antioxidant contents such as phenolics, carotenoids, flavonoids, and tocopherols Enhanced oil DPPH (1,1-diphenyl-2- picrylhydrazyl) free radical scavenging activity were also evident (Ali *et al*., 2013)

Hossain *et al*. (2014) observed the effect of drought in mustard (*B. juncea*) seedlings and the role of Pro and GB in mitigating oxidative damages caused by drought. Exogenous Pro and GB ameliorated the oxidative stress by improving the antioxidant system. Besides reducing the MDA and H_2O_2 contents, Pro and GB also significantly increase the ascorbate peroxidase, glutathione reductase, catalase, glutathione *S*-transferase, and glyoxalase II activities and a higher glutathione/glutathione

disulphide ratio which can modulate the methylglyoxal and reactive oxygen species levels and increase plant tolerance to drought-induced oxidative stress.

Molla *et al*. (2014) investigated the role of Pro and GB in drought stressed lentil (*Lens culinaris* L.) plants. Exogenous application of 15 mM GB or Pro with drought stress resulted in an increase in GSH content, maintenance of high activities of GST and Gly I as compared to the control with a concomitant decrease in GSSG content and H_2O_2 level suggesting important role in protecting cell from the toxic effects of ROS and MG detoxification system. These findings suggest that both Pro and GB provided a protective role in drought induced oxidative stress by reducing H_2O_2 levels and by increasing the antioxidant defense systems, where Pro exhibited better protection in plant under drought stress.

Osman (2015) reported that drought stress reduced the growth and yield of pea plant. Long-term drought was more effective to reduce growth and yield than drought at flowering stage. GB increased the yield and its soluble protein concentration more than proline. Proline recorded the maximum increase in non-enzymatic antioxidant defense system under drought. Application of GB or Pro enhanced the activity of SOD, APX and catalase in leaves under drought, while in seeds they increased SOD activity under long-term drought stress. Thus both osmolytes found to improve drought stress tolerance.

A field experiment was conducted to investigate the improvement of water stress tolerance in wheat by exogenous application of Pro in Wheat cv. BARI Gom-24. ater stress caused significant reductions in growth and yield. Water stress also decreased N, P, K and S uptake by wheat. On the other hand, exogenous application of Pro resulted in a significant increase in growth, yield components, and grain and straw yields and also associated with increased uptake of N, P, K and S. The study showed that interaction effects of exogenous Pro and water stress were significant in aspects of higher growth and yields and increased uptake of N, P, K and S in BARI Gom-24. Foliar application of 50 mM Pro was found to be more effective in improving water stress tolerance (Kibria *et al*., 2015).

2.9.2 Role of glycine betaine in drought stress tolerance

In some studies role Of GB in ameliorating the adverse drought effects and developing plant drought stress tolerance have been revealed.

Sakamoto and Murata (2002) reported that foliar-applied GB in plants under water deficit conditions enhanced the biomass production by maintaining leaf osmotic adjustment, leaf water status and increased photosynthesis, primarily due to enhanced stomatal opening and increased rubisco activity.

Ma *et al*. (2006) demonstrated that GB-treated wheat plants maintained a higher net photosynthetic rate during drought stress than non-GB treated plants. Exogenous GB can preserve the photochemical activity of PS II, for GB-treated plants maintain higher maximal photochemistry efficiency of PS II (Fv/Fm) and recover more rapidly from photoinhibition. In addition, GB-treated plants can maintain higher antioxidative enzyme activities and suffer less oxidative stress.

In a field trial, Bardhan *et al*. (2007) reported that exogenous application of different levels of GB (10 and 20 mg L^{-1}) as foliar spray in different cultivars of chickpea, significantly increased the final grain yield that was associated with the improved biomass production under water deficit conditions.

In a further study Ma *et al.* (2007) reported that GB treated Tobacco plants maintained leaf water status apparently due to the improved osmotic adjustment. GB application enhanced the photosynthesis in water-deficit experiencing plants, mostly due to a greater stomatal conductance and carboxylation efficiency of CO_2 assimilation. PS II activity in GB-treated plants was higher, as suggested by higher actual efficiency of PS II. GB increased anti-oxidative enzyme activities under water deficit conition. All these effects resulted in an improved shoot biomass and height. Therefore, foliar GB application at the rapid growth stage favors plant growth in drought-stressed plants, mainly by improving water status and increasing PS II activity.

Farooq *et al.* (2008) studied the role of GB to improve drought tolerance in rice (*Oryza sativa* L.) cultivar Super-basmati. Drought stress greatly reduced the rice growth while GB application improved it both under well-watered and drought conditions. Drought tolerance in rice was strongly related to the maintenance of tissue water potential and antioxidant system, which improved the integrity of cellular membranes and enabled the plant to maintain high photosynthesis.

In sunflower, the effect of exogenous GB was studied to investigate its role in improving plant performances under water deficit condition. Three levels (0, 50, and 100 mM) of GB were applied as seed treatments or as foliar application at the vegetative or reproductive growth stage. Water stress significantly decreased leaf water contents and osmotic and turgor potentials. Seed treatment of GB was not effective in alleviating drought damage, whereas, foliar application of GB at the vegetative or reproductive growth stage increased leaf water content and turgor potentials under water stress. Among the doses, foliar spray with 100 mM GB was more effective (Iqbal *et al.,* 2008).

Sunflower plant was exposed to water stress at their vegetative and flowering stage. Water stress significantly decreased the head diameter, number of achenes, 1000-achene weight, achene yield, and oil yield. Drought stress increased the free leaf Pro and achene oil contents. Exogenous GB (100 mM) resulted in further increase of free Pro level and significantly improved these yield attributes by ameliorating the harmful effects of water stress. However, GB showed more prominent effects when it was applied on the flowering stage (Hussain *et al*., 2008).

The ameliorative effect of pre-sowing seed treatment with GB in wheat was studied. Drought stress caused reduction in shoot fresh and dry biomass, shoot length, leaf area per plant, grain yield, net CO_2 assimilation and transpiration rates, and stomatal conditions. However, exogenous application of GB as a pre-sowing seed treatment increased shoot fresh biomass and leaf area per plant while its effect was non-significant on net CO_2 assimilation rate, stomatal conductance and water use efficiency, and shoot and root N, K^+, Ca^{2+} and P (Mahmood *et al*., 2009)

Ali and Ashraf (2011) found that GB could ameliorate the inhibitory effects of shortage of water on maize seed and seed oil composition and oil antioxidant potential. Foliar-applied GB significantly increased the contents of seed sugar, oil, protein, moisture, fiber, ash, GB contents and micro and macro-nutrients of both maize cultivars under well irrigated and water deficit conditions. Furthermore, exogenous application of GB increased oleic and linoleinic acid contents of the oil. All different lipophilic compounds estimated in the seed oil increased due to foliar applied GB. GB also increased seed oil antioxidant activity appraised in terms of oil DPPH free radical scavenging activity.

In *Zea mays* L. the role of exogenous GB was investigated in drought stress tolerance. The gas exchange and chlorophyll concentration were substantially declined in both maize cultivars under water stressed conditions. However, this reduction was less in Dongdan-60 than ND-95. Nonetheless, GB-treated plants considerably maintained higher gas exchange rate and chl concentration during drought stress than non-GB treated plants. The GB-induced improvement in gas exchange and chl synthesis under water stress ultimately resulted in improved growth and yield in both maize cultivars (Anjum *et al*., 2011)

Furthermore, Iqbal *et al*. (2011) reported that foliar-applied GB to sunflower plants at different growth stages enhanced the endogenous levels of GB, soluble proteins and total soluble sugars in drought stressed plants that increased the biomass production. In contrast to the earlier reports, there are also reports in the literature which show no effects of exogenously applied GB.

Xin *et al*. (2011) while working with two maize (*Z. mays*) cultivars i.e. drought-tolerant Shaandan 9 (S9) and -sensitive Shaandan 911 (S911) under long-term mild drought stress (LMDS) found that long-term mild drought stress was found to decrease dry matter (DM), grain yield (GY) and leaf relative water content (RWC), but to increase MDA accumulation in leaves of both cultivars. Dry matter, GR, RWC and these antioxidative enzymes activities were greater but MDA concentration was lower for S9 than those for S911 under LMDS. Additionally, exogenous GB application increased DM, GR, RWC and antioxidant enzymes activities measured,

but reduced MDA accumulation in both cultivars under LMDS unlike well-watered control, which exhibited no such obvious effect with GB

An experiment was conducted to investigate the morpho-physiological improving effects of exogenous GB on tomato (*L. esculentum*) cv. PS under drought stress conditions. Drought stress reduces the yield and production of tomato. Foliar application of GB (5 and 10 mM) Increased shoot height, root length, leaf number, leaf area, shoot FW, total shoot DW, RWC, and stress tolerance index, number of flowers, fruit number, and weight of fruit and recommended to alleviate effects of drought condition (Rezaei *et al*., 2012).

Raza *et al.* (2012) reported that application of GB effectively alleviated adverse effects of drought stress in wheat plant. Drought stress was imposed at the tillering, flower initiation, and milking stages, and GB supplementation improved the rate of transpiration and photosynthesis and uptake of P and Ca, whereas GB reduced N, K, and Na uptake.

Corn plants were subjected to drought stress by reducing irrigation intervals. Drought stress signifcantly reduced growth, yield components, and yield of corn. In the contrary, application of exogenous GB (50, 100, and 150 ppm) in drought-affected corn plants altered the plant physiology so that its growth, yield components, and yield were improved significantly. Among different concentrations, 150 ppm concentration while spraying before flowering had great positive effects (Miri and Armin, 2013).

Application of 100 mM GB also rendered drought tolerance in rice plant in terms of various physiological parameters; GB improved maximum quantum yield of PS II and total chl and photon yield of PS II, net photosynthetic rate, plant height, and yield traits such as panicle length and weight, fertility percentage, and hundred-grain weight (Cha-um *et al.*, 2013).

Raza *et al*. (2014) again reported that different doses of GB (50, 100, and 150 mM) were beneficial for improving drought tolerance of wheat plant when the stress was imposed at the milking stage. Application of GB significantly improved plant water

potential and increased plant height, spike length, the number of spikelets spike^{-1}, the number of grains spike^{-1}, and grain yield. Among different doses, 100 mM GB contributed best results.

2.9.3 Role of trehalose in drought stress tolerance

In an experiment, Shen and Welbaum (2004) observed the stimulating and inhibitory effects of exogenously applied varying levels (0, 15, 50, 100 and 200 mM) of Tre on seedling growth of sweet alyssum, tomato and muskmelon. They reported that exogenous application of Tre increased the shoot and root length of tomato plants while the opposite was true for sweet alyssum and muskmelon.

Llorente *et al*. (2007) reported that root-applied 1 mM of Tre increased the proliferation rate and decreased the root development with some anatomical changes in roots.

Ali and Ashraf (2011) investigated the ameliorative effects of foliar-applied Tre on water stress-affected maize seedlings. Water stress significantly reduced the plant biomass production, photosynthetic attributes, and water relation parameters. Water stress generated oxidative stress and altered the activities of antioxidant enzymes and levels of nonenzymatic antioxidant components. Foliar applied Tre (30 mM) significantly increased plant biomass production and improved photosynthetic attributes and plant-water relation parameters including leaf water potential, solute potential, turgor potential, and RWC. Trehalose application relieves plants from oxidative damage by enhancing the activities of some key antioxidant enzymes (POD and CAT) and levels of nonenzymatic compounds (tocopherols and phenolics).

Drought stress significantly reduced the seed oil but increased oleic acid and linolenic acid contents of the oil with a concomitant decrease in linoleic acid content, which resulted in an increased oil oleic/linoleic ratio in both maize cultivars. Whereas, oil phenolic content and oil antioxidant activity decreased. Exogenously applied Tre positively influenced seed composition of both maize cultivars under non-stress and water stress conditions. Exogenous application of Tre further

increased the oil oleic and linolenic acid contents with a subsequent decrease in linoleic acid. Furthermore, exogenous application of Tre increased the oil antioxidant activity for free radical scavenging activity with an increase in oil tocopherols, total flavonoids and total phenolics contents (Ali *et al*., 2012).

During studying with wheat callus under drought stress, Ma *et al.* (2013) observed severe oxidative damage and reduced callus growth. Exogenous Tre (50 mM) application in drought medium efficiently altered oxidative stress. Trehalose was efficient to increase the activity of SOD and GR; Tre also increased the levels of AsA and GSH which were involved in the reduction of ROS and subsequent oxidative damage. The overall effect of Tre was better in callus development under drought stress.

Different species of *Brassica* such as *B. napus*, *B. campestris*, and *B. juncea* were subjected to drought stress (15% PEG) for 48 h. Drought-affected *Brassica* seedlings showed growth reduction, reduced photosynthetic pigment, elevated Pro, oxidative stress, and MG toxicity. But exogenous Tre (5 mM) application significantly reduced Oxidative stress and MG toxicity. Methylglyoxal toxicity generated from drought stress was also reduced by exogenous Tre, where Tre reduced MG content by improving glyoxalase system components. Trehalose also rendered increased photosynthetic pigment level, regulated endogenous Pro, increased leaf RWC, and improved overall growth of plant (Alam *et al*., 2014).

The impact of Tre on water stress-induced changes in the enzymatic and non-enzymatic antioxidative defense system within roots (edible part) of *Raphanus sativus* L. (radish) plants. It was observed that water deficiency significantly reduced the root FW, while increased the accumulation of carotenoids, AsA, MDA, GB, and TSP contents coupled with an increase inthe activities of CAT, POD and SOD enzymes in the roots of radish. Both modes of exogenous application of Tre were effective in reducing MDA contents, while improving root FW, and contents of AsA, total phenolics, GB, total tocopherols and TSP as well asthe activities of SOD, CAT and POD (Shafiq *et al.*, 2015)

Aldesuquy and Ghanem (2015) investigated the effect of exogenous Tre in water stressed two wheat cultivars (Sahel-1, drought tolerant) and (Gemmieza-7, drought sensitive. Drought significantly increased the activity of ascorbic acid oxidase (AAO), POD and phenylalanine ammonia lyase (PAL) activities and induced non-significant reduction in polyphenol oxidase (PPO) activity in flag leaves of both wheat cultivars during grain-filling in comparing with well watered plants. Among cultivars, tolerant one showed higher enzymes activity than the sensitive one. Application of Tre markedly increased AAO, POD and PAL activities and non-significant decrease in PPO activity in flag leaf of water stressed wheat plants. Generally, Tre treatment appeared to be the most effective treatment in counteracting the negative effects of water stress and Sahel-1 appeared to induce better results than Gemmieza-7 and proved to be more tolerant.

Effect of water stress and the foliar application of Tre (25 and 50 mM) in two Radish (*Raphanus sativus* L.) cultivars were investigated by Akram *et al.* (2015). Under drought stress conditions, shoot fresh and dry weights, shoot and root lengths, photosynthetic rate (A), transpiration rate (E), stomatal conductance (g_s), internal CO_2 concentration (C_i), relative water contents (RWC), shoot K^+ and P and shoot and root Ca^{2+} decreased, while water-use efficiency (WUE) and relative membrane permeability (RMP) increased in both radish cultivars. Exogenous application of Tre improved shoot fresh and dry weights, chl *a* content and accumulation of P in shoot, but decreased RMP in both cultivars.

Chapter III

MATERIALS AND METHODS

3.1 Location

The experiment was conducted in the Laboratory of Plant Stress Responses, Kagawa University, Kagawa, Japan, during the period from June, 2016 to December, 2016.

3.2 Plant material

BARI Sharisha-15 (*Brassica campestris* L.) was used as a plant material in the experiment. Seeds were collected from BARI, Joydebpur, Gazipur, Bangladesh.

3.3 Experimental condition

The seedlings were grown under the controlled condition (light, 350 μmol photon m^{-2} s^{-1}; temperature, 25±2°C; RH, 65–70%).

3.4 Stress imposition

The whole experiment was conducted in controlled laboratory condition. Drought stress was artificially induced by using 20% PEG (polyethylene glycol).

3.5 Protectants

Proline (Pro), gycine betaine (GB), and trehalose (Tre) were used as the protectants with 0.5 mM concentration. All chemicals were purchased from Wako, Japan.

3.6 Treatments

The experiment consisted of eight treatments:

1) Control (well watered condition)
2) 0.5 mM proline (Pro)
3) 0.5 mM glycine betaine (GB)
4) 0.5 mM trehalose (Tre)
5) Drought (D, 20% PEG)
6) Pro+D
7) GB+D

8) Tre+D

3.7 Design and layout of the experiment

The experiment was laid out in a Completely Randomized Design (CRD) with three replications.

3.8 Growing condition and treatment procedure of the experiment

Rapeseed (*Brassica campestris* L. cv. BARI Sharisha-15) seeds of uniform size were selected and surface-sterilized with 70% ethanol for 5 minutes and then washed thoroughly with sterilized distilled water. Then the seeds were soaked in distilled water for 10 minutes and then sown in petri plates (9 cm) lined with 6 layers of filter paper moistened with 10 ml of distilled water and placed in dark in favour of germination for three days. Each Petri dish contained about 60 germinated seedlings. Germinated seedlings were then allowed to grow in semi-hydroponic medium under controlled condition (light, 350 μmol photon m^{-2} s^{-1}; temperature, 25±2°C; RH, 65–70%). 5000-fold diluted Hyponex solution (Hyponex, Japan) was used as nutrient which was applied every day as necessary. After six days of seedlings growth, six sets of seedlings were used for Pro, GB and Tre pre-treatment (two sets for each osmolyte). For Pro, GB and Tre pre-treatment the root portion of the seedlings were immersed in 0.5 mM proline, 0.5 mM GB and 0.5 mM Tre solution and allowed to grow for 48 h. Afterwards, the prtreated Petri dishes were washed several times with deionized water to remove excess Pro, GB or Tre. Further, these pretreated eight-day-old seedlings were grown without stress or exposed to drought (induced by 20% PEG in Hyponex solution) stress both individually or in combination. One set of eight-day-old seedlings were exposed to drought stress (20% PEG, polyethyleneglycol) for 48 h alone. Control plants were grown in Hyponex solution only. After 48 h of stress treatment, at ten days, data were taken from the leaf samples and immediately used for the analysis of different parameters. The experiment was repeated three times under the same conditions.

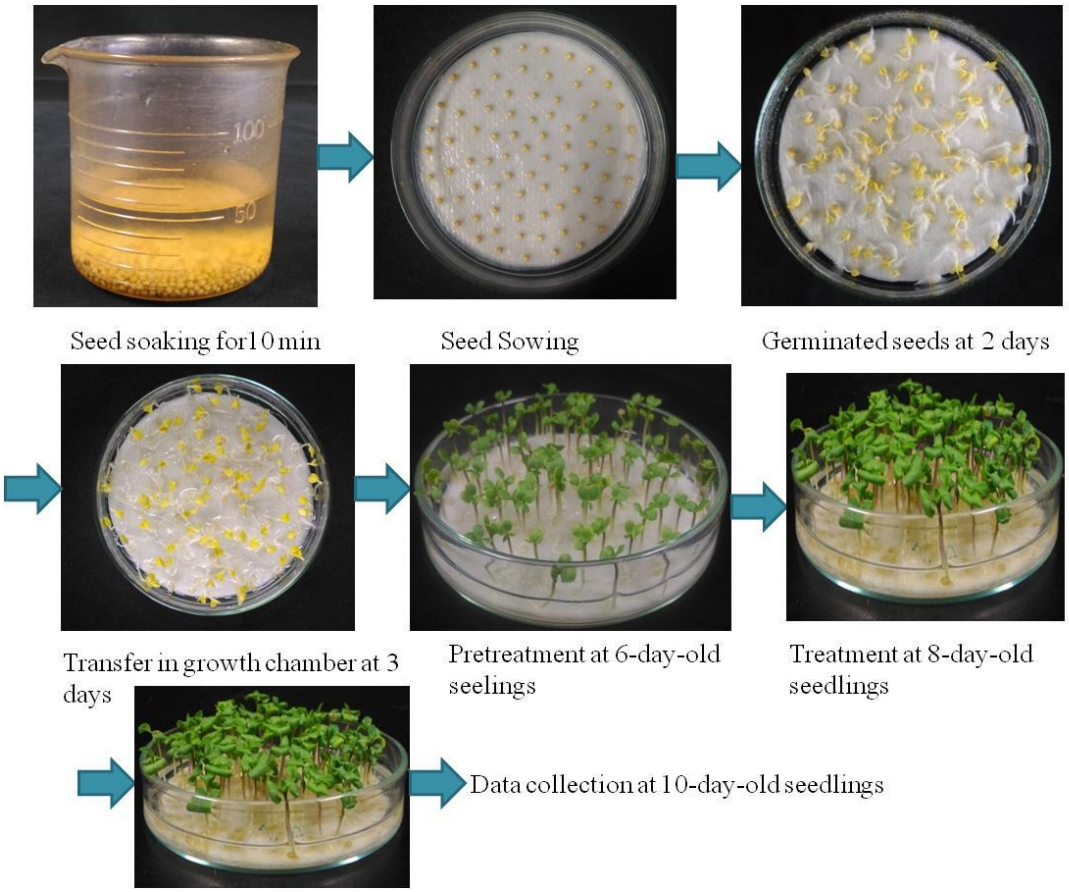

Figure 1: Different steps and treatment procedure of the experiment

3.9 Collection of data

3.9.1 Crop growth parameters

- Plant height
- Fresh weight plant^{-1}
- Dry matter weight plant^{-1}

3.9.2 Physiological parameters:

- Relative water content (RWC)
- Photosynthetic pigments (chl *a*, chl *b* and total chl contents)

3.9.3 Oxidative stress indicators:

- Lipid peroxidation
- H_2O_2 content
- Proline content
- Glcine betaine content
- Trehalose content
- Methylglyoxal content
- Glyoxalase enzymes (Gly I and Gly II)
- Ascorbic acid content
- Glutathione content
- Activities of antioxidant enzymes (CAT, APX, MDHAR, DHAR, GR, GPX, Histochemical detection of H_2O_2 and $O_2^{\bullet-}$

3.10 Procedure of assaying growth, physiology parameters, oxidative stress indicators, antioxidant system, MG content and glyoxalase system

3.10.1 Measurement of plant height

The height of the rapeseed seedlings was recorded after the duration of treatment was completed; beginning from the root tip to up to the top of the leaf was counted as height of the plant. The average height of five plants was considered as the height of the plant for each treatment

3.10.2 Measurement of fresh weight and dry weight of seedlings

Ten randomly selected fresh seedlings from each treatment were weighed, recorded and considered as FW. Dry weight was determined after drying the seedlings at 80°C in oven (ADVANTEK, SP-450, Japan) for 48 h.

3.10.3 Measurement of relative water content

Relative water content (RWC) was measured according to Barrs and Weatherly (1962). Leaf discs from randomly chosen plants were taken from the fully developed leaves. Discs were weighed as FW and then immediately floated on distilled water in

a petri dish for 8 h in the dark. Turgid weights (TW) of leaves were obtained after removing excess surface water with paper towels. Dry weights (DW) of leaves were measured after drying at 80 °C for 48 h. Then, RWC was calculated using the following formula

RWC (%) =[(FW-DW)/ (TW-DW)] ×100

3.10.4 Determination of chlorophyll content

Chlorophyll (chl) content was determined by taking fresh leaf samples (0.5 g) from randomly selected seedlings. The samples were homogenized with 10 ml of acetone (80% v/v) using pre-cooled pestle and mortar and the homogenate was centrifuged at 2000×g for 10 min. The absorbance of the supernatants was measured with a UV-visible spectrophotometer at 663 and 645 nm for chl *a*, chl *b* contents, respectively. Total chl (*a*+*b*) was calculated using chl *a* and *b* values. Chlorophyll contents were calculated using the equations proposed by Arnon (1949).

3.10.5 Determination of proline content

Free Pro in leaf tissues was measured following the protocol of Bates *et al*. (1973). Fresh leaf tissue (0.5 g) was homogenized well in 10 ml of 3% sulfo-salicylic acid on ice. The homogenate was centrifuged at 11,500 × *g* for 15 min. Two ml of the filtrate was mixed with 2 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) and 2 ml of glacial acetic acid. The mixture was placed at 100 °C in water bath for 1 h, then transferred in to test tube and kept in ice to be cooled, after a while when it was cooled, 2 ml of toluene was added and mixed thoroughly by vortex mixture. After sometime by transferring the upper aqueous layer, the optical density of the chromophore containing toluene was read spectrophotometrically at 520 nm using toluene as a blank. The amount of Pro was calculated using a standard curve.

3.10.6 Determination of glycine betaine content

Glycine betaine content in the leaves was determined following the method described by Greive and Grattan (1983) with some modifications. Dry leaf sample of 0.1 g was homogenized and extracted with 4 ml deionized water by shaking for 24 h at 25 °C in

a mechanical shaker. The sample was filtered with Whatman No. 1 in a test tube and kept in Ice. 0.25ml filtrate is taken in centrifuge tube and 0.25ml of 2N H_2SO_4 was added (Plant sample filtrate: 2N H_2SO_4) = (1:1). This 0.5 ml mixture in centrifuge tube was kept in ice and cooled for 1 h and 0.2 ml cold Iodine-potassium iodide (I_2–KI) reagent was mixed in each sample vortexed gently. The tubes were than stored at 4°C in a refrigerator for 16 h. After 16 h the tubes were centrifuged at 13500 × *g* for 15 minutes at 0 °C. The supernatant was aspired carefully as fast as possible. The precipitate (Periodide crystals) then dissolved in 9 ml of 1, 2-dichloroethane (reagent grade) and vigorously mixed for dissolving the crystals completely in the solvent forming a pale gold colored solution. The absorbance was measured after 2-2.5 hrs at 365 nm. The amount of GB in the tested samples was done using a standard curve. GB content was expressed the on the dry-weight-basis and GB concentration was calculated as micromoles per gram DW using a standard curve.

3.10.7 Determination of trehalose content

Trehalose content in the second leaves was determined following the method described by Li *et al.* (2014) with some modifications. The leaves (0.5 g) were homogenized in 5 mL of 80 % (v/v) hot ethanol and centrifuged at 11,500 × g for 20 min. The supernatants were dried at 80 °C for 3 hrs followed by resuspension in 5 mL distilled water. The solution (100 µL) was mixed with 150 µL 0.2 N H_2SO_4 and boiled at 100 °C for 10 min to hydrolyze any sucrose or glucose-1-phosphate, etc., and then chilled on ice. NaOH (0.6 N, 150 µL) was added to the above mixture and boiled for 10 min to destroy reducing sugars, and then chilled again. To the above mixture, 2.0 mL of anthrone reagent (0.05 g anthrone per 100 ml of 72% H_2SO_4) was added and boiled for 10 min to develop a color, and then chilled again. The absorbance was recorded at 630 nm, and trehalose concentration was calculated as micromoles per gram FW using a standard curve developed with commercial Tre.

3.10.8 Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating malondealdehyde (MDA) content according to Heath and Packer (1968) with slight modification by Hasanuzzaman *et al.* (2012b). Leaf samples (0.5 g) were homogenized in 3 mL 5% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 11,500 × g

for 15 min. The supernatant (1 mL) was mixed with 4 mL of thiobarbituric acid (TBA) reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at 95 °C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged again at 11,500 × g for 10 min. The absorbance of the colored supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. MDA content was calculated by using extinction coefficient 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW.

3.10.9 Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu *et al.* (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf samples with 3 ml of 50 mM potassium-phosphate (K-P) buffer (pH 6.5) at 4 °C. The homogenate was centrifuged at 11,500 × *g* for 15 min. Three ml of supernatant was mixed with 1 ml of 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v) and kept in room temperature for 10 min. After that the mixture was again centrifuged at 11,500 × *g* for 12 min. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content using extinction coefficient 0.28 μM^{-1} cm^{-1} and expressed as nmol g^{-1} fresh weight.

3.10.10 Histochemical detection of H_2O_2 and $O_2^{\bullet-}$

Localization of $O_2^{\bullet-}$ in leaf was detected following Chen *et al.* (2010) with slight modification. Leaves were stained in 0.1% 3-diaminobenzidine (DAB) and 0.1% nitrobluetetrazolium chloride (NBT) solution for 24 h under a dark condition for H_2O_2 and $O_2^{\bullet-}$ detection, respectively. Incubated leaves were then blenched by immersing in boiling ethanol. After that, brown spots appeared resulting from the reaction of DAB with H_2O_2 and dark blue spots appeared resulting from the reaction of NBT with $O_2^{\bullet-}$ (Thordal-Christensen *et al*., 1997). Photographs were then taken by placing the leaves on glass.

3.10.11 Extraction and measurement of ascorbate and glutathione

Rapeseed leaves (0.5 g FW) were homogenized in 3 ml ice-cold acidic extraction buffer (5% meta-phosphoric 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 was collected for analysis of ascorbate and glutathione.

Ascorbate content was determined following the method of Huang *et al*. (2005) with some modifications as described by Hasanuzzaman *et al*. (2011a). AsA was assayed spectrophotometrically at 265 nm in 100 mM K-P buffer (pH 7.0) with 0.5 units of ascorbate oxidase (AO). A specific standard curve of AsA was used for quantification. The GSH pool was assayed according to a previously described method (Yu *et al*. 2003) with modifications as described by Paradiso *et al.* (2008). Aliquots (0.2 mL) of supernatant were neutralized with 0.3 mL of 0.5 M K-P buffer (pH 7.0). Based on enzymatic recycling, GSH is oxidized by 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GR, and GSH content was evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. Oxidized glutathione (GSSG) was determined after removing GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used. The content of GSH was calculated by subtracting GSSG from total GSH.

3.10.12 Measurement of methylglyoxal level

Methylglyoxal was measured following the method of Wild *et al.* (2012). Leaves were homogenized in 5% perchloric acid and centrifuged at 4 °C for 10 min at 11,000 × g. The supernatant was decolorized by adding charcoal. The decolorized supernatant was neutralized by adding a saturated solution of sodium carbonate at room temperature. The neutralized supernatant was used to estimate MG by adding sodium dihydrogen phosphate and N-acetyl-L-cysteine to a final volume of 1 mL. Formation of the product N-α-acetyl-S-(1-hydroxy-2-oxoprop-1-yl) cysteine was recorded after 10 min at a wavelength of 288 nm, and the MG content was calculated using a standard curve of known concentration.

3.10.13 Determination of protein

The protein concentration of each sample was determined following the method of Bradford (1976) using BSA as a protein standard.

3.10.14 Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM

β-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500 × *g* for 15 min and the supernatants were used for determination of enzyme activity. All procedures were performed at 0−4 ºC.

CAT (EC: 1.11.1.6) activity was assayed following the method of Hasanuzzaman *et al.* (2012b) by monitoring the decrease in absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution in a final volume of 700 µL. The reaction was initiated with the enzyme extract and activity was calculated using extinction coefficient 39.4 M^{-1} cm^{-1}

APX (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 700 µl. The reaction was started by the addition of H_2O_2 and the activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 $mM^{-1}cm^{-1.}$

MDHAR (EC: 1.6.5.4) activity was determined by the method of Hossain *et al.* (1984). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO and enzyme solution in a final volume of 700 µl. The reaction was started by the addition of AO. The activity was calculated from the change in absorbance at 340 nm for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

DHAR (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 265 nm for 1 min using extinction coefficient of 14 $mM^{-1}cm^{-1}$.

GR (EC: 1.6.4.2) activity was measured by the method of Hasanuzzaman *et al.* (2011b). The reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 ml. The

reaction was initiated with GSSG and the decrease in absorbance at 340 nm was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

GPX (EC: 1.11.1.9) activity was assayed using the method of Elia *et al.* (2003). The reaction mixture consisted of 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 (as a substrate), and 20 µL of sample solution. The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using extinction coefficient 6.62 mM^{-1} cm^{-1}.

Glyoxalase I (EC: 4.4.1.5) assay was carried out according to Hasanuzzaman *et al*. (2011a). Briefly, the assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH and 3.5 mM MG in a final volume of 700 µ l. The reaction was started by the addition of MG and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 mM^{-1} $cm^{-1.}$

Glyoxalase II (Gly II; EC: 3.1.2.6) activity was determined according to the method of Principato *et al*. (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.2), 0.2 mM DTNB and 1 mM *S*-D-lactoylglutathione (SLG) in a final volume of 1 ml. The reaction was started by the addition of SLG and the activity was calculated using the extinction coefficient of 13.6 $mM^{-1}cm^{-1}$

3.11 Statistical analysis

The data obtained for different parameters were statistically analyzed following computer based software XLSTAT 2016 (AddinSoft, 2016) and mean separation was done by LSD at 5% level of significance.

Chapter IV

RESULTS AND DISCUSSION

4.1 Plant height

Significant variation in plant height was observed among stressed and non stressed seedlings. Upon drought exposure, the plant height decreased in rapeseed seedlings by 20%, compared to control (Table 1). Pro, GB and Tre pre-treatment alone had no significant effect on plant height of rapeseed seedlings. But after supplementation of Pro, GB and Tre with drought stress, the increase in plant height was observed and it was about 11, 8 and 7% respectively, compared to drought stress alone. Proline showed the best performance in improving plant height under drought stress than the other two osmolytes (Table 1).

4.2 Fresh weight and dry weight of seedlings

Drought stress resulted in a significant reduction in the fresh weight of the rapeseed seedlings. Upon drought exposure fresh weights were reduced by 18%, compared to control seedlings (Table 1). Similarly, dry weights of seedlings were also reduced by 13% when exposed to drought stress (Table 1). Pre-treatment with Pro, GB and Tre with drought stress improved the fresh weight and dry weight of seedlings.

Drought stress has diversified adverse effects on plant growth, physiological and metabolic processes (Hasanuzzaman *et al*., 2014). Drought affects plant-water relations, reduces water contents of leaf and plant, causes osmotic stress, inhibits cell expansion and cell division as well as growth of plants as a whole (Kirkham, 2005; Mahmood *et al.*, 2012; Alam *et al*., 2013). Osmoprotectants like Pro, GB and Tre efficiently maintain osmotic balance and stabilize proteins and membranes under drought and other stress conditions and improve overall growth (Nahar *et al.*, 2016a). In present study, drought stress reduced plant height, fresh and dry weight of rapeseed seedlings (Table 1). Reduction of plant biomass and growth under drought stress are very common as studied by many researches (Alam *et al*., 2013; Mahmood *et al.*,

2012). However, growth reduction was restored by exogenous Pro, GB and Tre supplementation under drought stress as evidenced by improved plant height, fresh weight and dry weight of seedlings. Exogenous osmolytes induced improved growth might have been due to an active role of this osmolyte in plant osmotic adjustment which in turn might have enhanced water uptake and hence improved growth of crop plants. Among the three osmolytes, Pro showed the best performance in improving growth under drought stress. Other studies also revealed Pro, GB and Tre as effective restorer of plant water content and growth under water deficit condition in *Brssica* sp. (Hossain *et al*., 2014; Alam *et al*., 2014).

4.3 Relative water content (RWC)

Leaf relative water content of rapeseed seedlings decreased significantly with the imposition of drought stress. In the media containing 20% of PEG, RWC decreased by 23%, compared to control (Table 1). Non-stressed seedlings which were pretreated with Pro, GB and Tre did not show any differences in RWC compared to control. The drought-stressed seedlings pre-treated with Pro, GB and Tre showed 22, 19 and 7% increase in RWC, respectively, compared to the drought stressed seedlings alone. A maximum increase in RWC was observed due to exogenous application of Pro and GB as compared to that of Tre (Table 1)

Drought stress has a great impact on plant–water relations that is attributed to osmotic stress and reduced relative water content in the plant parts (Kirkham 2005) However, water content restoration was observed by exogenous Pro, GB and Tre supplementation under drought stress which resulted in the improved leaf RWC as well as growth. Exogenous osmolytes actively imparted in the osmotic regulation which ultimately resulted in the improved water status of the seedlings. Considering the restoration of leaf RWC of seedlings induced by Pro, GB and Tre under drought stress, the performance of Pro was the best (Table 1). Similar findings were also documented previously in *Brssica* sp. with Pro, GB and Tre addition under drought stress (Ali 2011; Alam *et al.*, 2014; Hossain *et al*., 2014)

Table 1. Plant height, FW, DW and RWC (%) of rapeseed seedlings induced by Pro, GB and Tre under drought stress condition.

Treatments	Plant height (cm)	FW (g seedling^{-1})	DW (g seedling^{-1})	RWC (%)
Control	2.65a	0.43a	0.051ab	90.9a
Pro	2.55ab	0.45a	0.0506bc	90.4a
GB	2.47bc	0.42ab	0.0496c	89.8a
Tre	2.54ab	0.44a	0.0514a	91.7a
Drought (D)	2.12e	0.35c	0.0433c	69.5d
Pro+D	2.36cd	0.38bc	0.0482c	84.8b
GB+D	2.29d	0.36c	0.0465c	82.6b
Tre+D	2.28de	0.36c	0.0478c	74.2c

Means (±SD) were calculated from three replicates for each treatment. Values in a column with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.4 Proline content

Proline content increased noticeably in rapeseed seedlings upon exposure to drought stress. Proline content increased by 10-fold compared to the unstressed control. Addition of exogenous Pro, GB and Tre in combination with drought resulted in Pro levels that were 26, 33 and 28% lower respectively, than the levels found in seedlings exposed to drought stress alone (Figure 2).

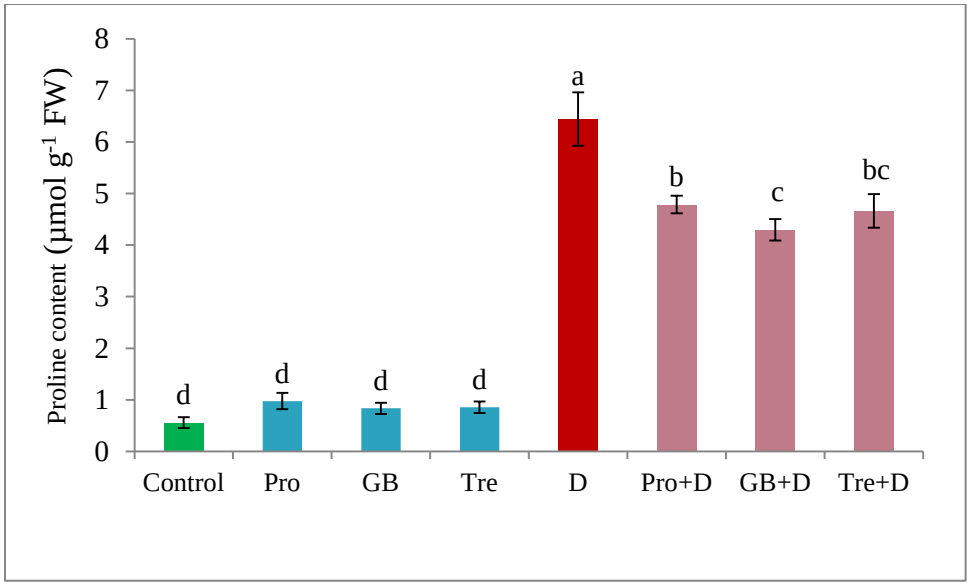

Figure 2. Effect of exogenous Pro, GB and Tre on Proline content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

Proline plays very important role under abiotic stress conditions including drought stress. Proline has vital roles in osmotic adjustment, stress signal transduction and it also acts as an antioxidant. Increase of Pro level under physiological stresses including drought stress condition were documented previously (Bates *et al*., 1973; Nahar *et al*., 2013). Similarly profound increases of Pro levels under drought stresses were observed in rapeseed seedlings. Proline, GB and Tre addition with drought stress reduced Pro levels in rapeseed seedlings (Figure 2). Prevention of extra Pro biosynthesis due to exogenous Pro, GB and Tre addition under drought stress suggests that Pro, GB and Tre prevented rapeseed seedlings from adverse effects of drought stress by other means so that the studied rapeseed seedlings did not need to increase the Pro levels further. These results are corroborated to previous studies (Ali and Ashraf, 2011; Nounjan *et al*., 2012; Nahar *et al*., 2013; Alam *et al*., 2014).

4.5 Glycine betaine content

For understanding the endogenous content of GB and its contribution to the drought stress tolerance in rapeseed seedlings, GB content was estimated. The endogenous GB activity was significantly increased by 192% in drought-stressed *B. campestris* over control. The Pro, GB and Tre pre-treated drought-stressed seedlings restored GB activities, compared to the seedlings exposed to drought stress without pre-treatment. Interestingly, highest endogenous GB activity was found in the exogenous GB pretreated plants by 12% as compared to stressed group alone (Figure 3)

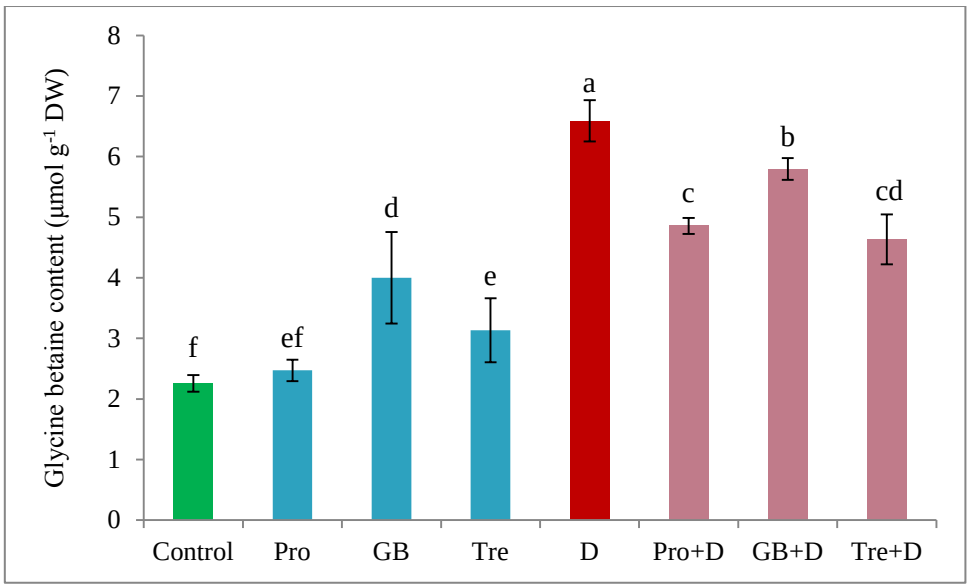

Figure 3. Effect of exogenous Pro, GB and Tre on glycine betaine (GB) content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

Glycine betaine is one of the quaternary ammonium compounds (QACs) which functions as an osmolyte donate adaptation capacity for the cultivated plants under drought (Nahar *et al*., 2016a). In the present study, leaves GB content increased under drought stress. Similarly, in a previous study with two varieties of *Phaseolos vulgaris* (sensitive to salinity) and *Sesbania aculeate* (resistant to salinity), the GB amount has significantly increased in plant leaves with increasing the salinity (Ashraf and Bashir, 2003). In the present study exogenous Pro and Tre application in stressed seedlings

showed increased endogenous GB level but highest GB level was observed in GB-pretreated non-stressed and stressed seedlings (Figure 3) indicating that GB rapidly accumulated in the rapeseed plants. It was reported that GB is created through re-synthesis (*de novo*) during the water stress (Grieve and Grattan, 1983). This osmolyte is mainly accumulated in chloroplasts and it has an important role in protecting the thylakoids membrane of chloroplasts, thus it may effect on photosynthesis (Rogers *et al*., 2009).

4.6 Trehalose content

Trehalose level was estimated to observe the influence of exogenous Tre on the endogenous content of Tre and its contribution to the enhanced tolerance of the rapeseed plants to drought stress**.** Drought exposure significantly increased the endogenous levels of Tre in the *B. campestris* seedlings by 59% compared to the control group. Pre-treatment with Pro led to non significant increase, GB resulted in 12%, on the other hand Tre pretreatment significantly increased the levels of endogenous Tre by 31%, respectively, compared to the control group. Relative to drought stress seedlings alone, Tre pre-treated drought stressed seedlings showed further significant increasement in endogenous Tre contents by 23% (Figure 4).

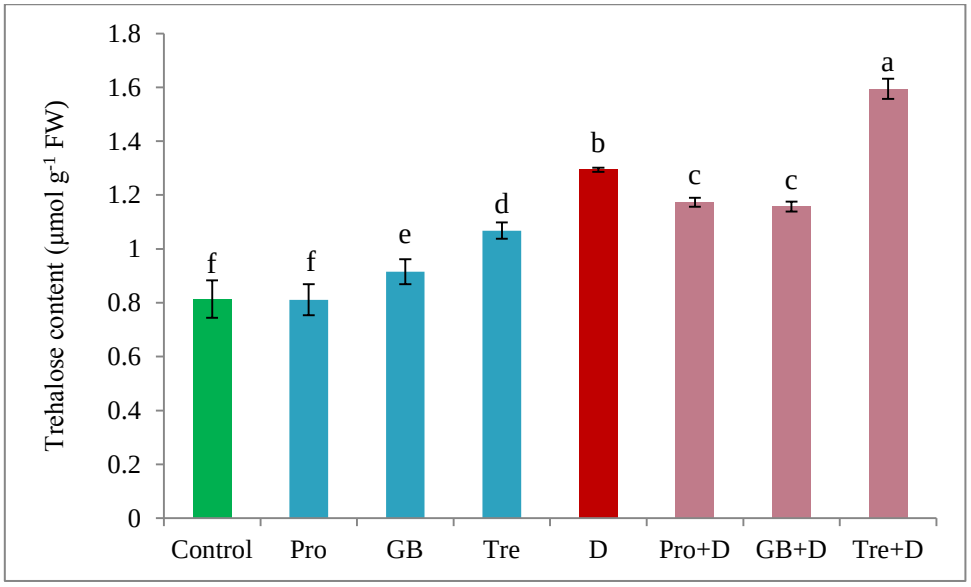

Figure 4. Effect of exogenous Pro, GB and Tre on trehalose (Tre) content of rapeseed seedlings under drought stress.
Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

Trehalose as an osmoprotectant maintains cellular osmotic balance. It stabilizes dehydrated enzymes, proteins and lipid membranes efficiently (Alam *et al*., 2014). It protects biological structures from damage at desiccation (Garg *et al.,* 2002). Furthermore, Tre has been shown to efficiently stabilize dehydrated enzymes, proteins and lipid membranes, as well as protect biological structures from damage during desiccation by replacing water. Although Tre accumulates in negligible amounts in crop plants, it has been considered a quantitatively important compatible solute and stress protectant (Fernandez *et al.,* 2010). In the present study, non-stressed and stressed rapeseed seedlings pretreated with Tre increased the endogenous level of Tre (Figure 4), which indicated that Tre was readily absorbed by the roots and easily transported to the aerial parts. Present results corroborate previous findings (Luo *et al.,* 2010; Ali and Ashraf, 2011; Ma *et al*., 2013; Nounjan *et al*., 2012; Mostofa *et al.,* 2014). Exogenous Pro and GB pre-treated drought stressed plants also showed increased endogenous Tre level to some extent which also indicated their regulatory role to give protection against drought stress. Present experiment also suggested that external application of Tre could be an alternative approach to modify the level of endogenous Tre, and thus potentially strengthen plant capacity to withstand the deleterious effects of drought stress. From this point of view, it can be assumed that additional protection against drought stress in the rapeseed seedlings was attributed to the increase in the endogenous level of Tre.

4.7 Chlorophyll contents

Chlorophyll *a* and chl *b* contents significantly decreased in rapeseed seedlings under drought stress by 55 and 49%, compared to control which contributed to the reduction in total chl (*a*+*b*) content by 50%. As compared to drought treatment alone, the drought-stressed seedlings pre-treated with Pro, GB and Tre showed increased total chl (*a*+*b*) contents. Pro, GB pretreated seedlings showed significantly increased total chl (*a*+*b*) contents by 64 and 53%, respectively, whereas contents of total chl (*a*+*b*) in Tre pretreated seedlings showed no significant changes. Of three exogenously applied osmolytes highest increase in chlorophyll *a*, *b* and total chl (*a*+*b*) contents in rapeseed seedlings were observed due to exogenous pretreatment with Pro and GB as compared to Tre (Figure 5 A, B, C)

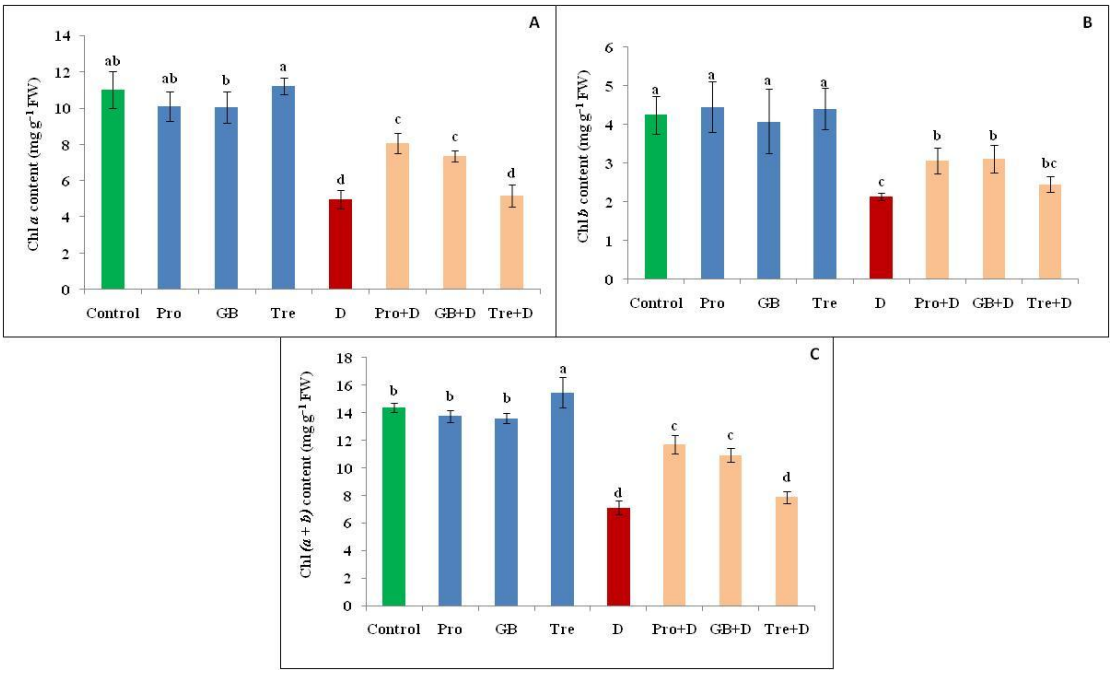

Figure 5. Effect of exogenous Pro, GB and Tre on (A) Chlorophyll (chl) *a* (B) chl *b*, (C) and total chl (*a*+*b*) content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

Water deficit conditions caused a marked suppression in plant photosynthetic efficiency, which has been found to be mainly due to closing of stomata, which limits CO_2 diffusion into the leaf, or due to inhibition in rubisco, a non-stomatal factor (Demirevska *et al*., 2010; Rapacz *et al*., 2010). Water stress also causes a decline in photosynthetic pigments contents including chl, carotenoid, anthocynanin, etc. in various types of crop plants which is due to the oxidation of pigments, impaired pigment biosynthesis and so on (Garcia-Plazaola *et al*., 2003; Abbaspour *et al*., 2011; Anjum *et al*., 2011; Saraswathi and Paliwal, 2011; Pandey *et al*., 2012). Findings of the present study are also evident by reduced chl *a*, chl *b* and total chl *(a+b)* under drought stress (Figure 5A, B, C). Interaction of Pro, GB and Tre with drought stress improved the photosynthetic pigment levels in studied species which might be due to the higher biosynthesis of these pigments. Present findings are corroborated with the results of previous studies on other stresses including drought stress (Hoque *et al*., 2007; Hasanuzzaman *et al.*, 2014; Alam *et al.*, 2014). Exogenous osmolyte application was found to be effective in mitigating the harmful effects of water deficit conditions on photosynthetic capacity of plants possibly due to their protective effect

on biological membranes responsible for quenching light energy and on enzymes involved in chloroplastic metabolism as already reported (Hasegawa *et al*., 2000; Zeid, 2009). Recent evidence suggests that improved photosynthetic performance can be achieved by increased stomatal conductance while avoiding dehydration through transpiration (Raza *et al*., 2006; 2007; Arfan *et al*., 2007).

4.8 Levels of lipid peroxidation (MDA content)

A sharp increase in MDA content (the product of lipid peroxidation) was observed under drought stress. The seedlings with 20% PEG caused 82% increase in MDA content compared to control (Figure 6). On the other hand, Pro, GB and Tre supplemented drought-stressed seedlings showed significantly lower MDA content compared to drought exposed seedlings without Pro, GB and Tre (32, 29 and 11% lower compared to the seedlings exposed to 20% PEG only). Furthermore, this reduction in leaf MDA content was found more in plants those were pretreated with Pro as compared with that with GB or Tre.

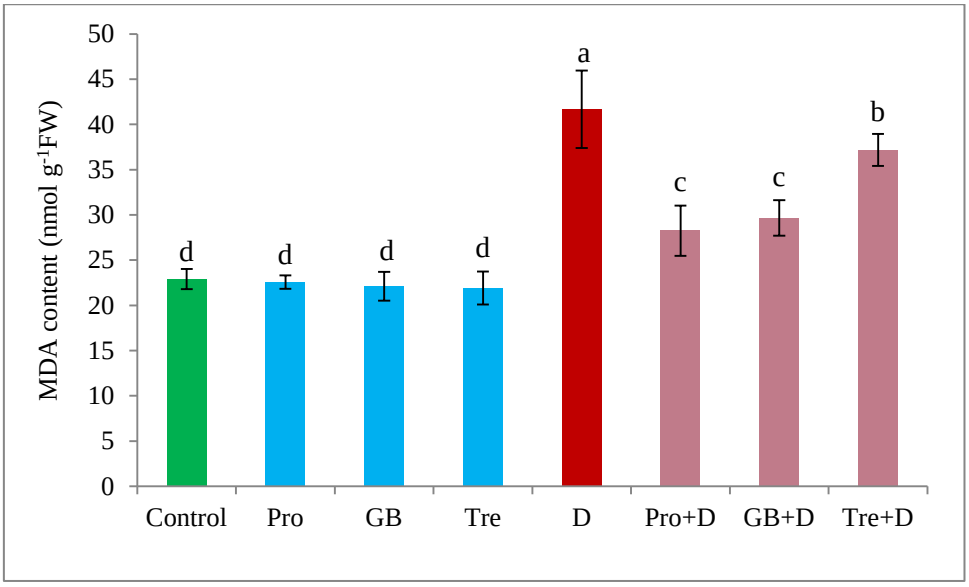

Figure 6. Effect of exogenous Pro, GB and Tre on MDA content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.9 Levels of H_2O_2

Compared to control, the levels of H_2O_2 increased by 131% with 20% PEG. However, pretreatment with Pro, GB and Tre maintained same level of H_2O_2 as control (Figure 7). Importantly, a significant reduction of H_2O_2 by 29, 17 and 10% were observed in Pro, GB and Tre supplemented drought-stressed seedlings compared to the seedlings expose to drought only.

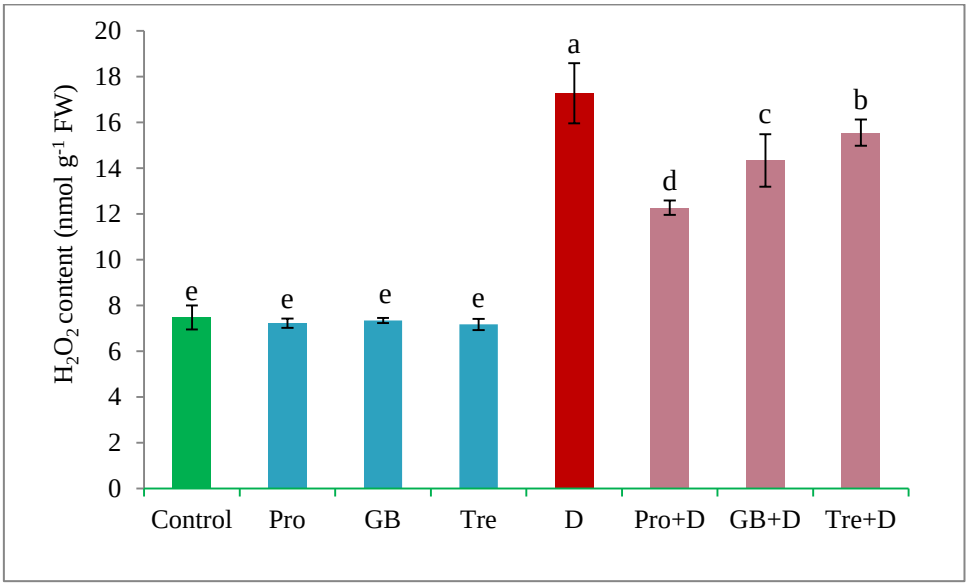

Figure 7. Effect of exogenous Pro, GB and Tre on H_2O_2 content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

Accumulation of MDA, mainly produced from the ROS induced degradation of membrane lipids, is a potential oxidative stress marker (Garg and Manchanda, 2009) Hydrogen peroxide is a toxic compound which is injurious to the cell and excessive accumulation of H_2O_2 is one of the indicators of oxidative stress (Hasanuzzaman *et al*., 2011). Like all other abiotic stresses, drought stress resulted in a sharp increase in MDA levels with a concomitant increase in H_2O_2 which is a clear evidence of oxidative stress. Same result was also observed in our present study where rapeseed seedlings exhibited enhanced generation of these products (Figure 6, 7). These increases are in consistent with other studies (Hasanuzzaman and Fujita, 2011). Exogenous application of Pro, GB and Tre reduced the oxidative damage as

evidenced by significantly decreased levels of MDA and H_2O_2 (Figure 6, 7). This reduction in MDA contents could also have been due to the putative role of osmolytes in alleviating the stress-induced deleterious effects on the structure of cell membranes and activities of different enzymes (Ashraf and Harris, 2004; Paul *et al*., 2008; Fernandez *et al*., 2010) as well as reducing the generation of highly destructive free radicals (Paul *et al*., 2008; Fernandez *et al*., 2010). Therefore, it was speculated that Pro, GB and Tre pre-treatment might contribute in alleviating the drought-induced oxidative stress through the activation of ROS detoxification that ultimately helps in membrane stability. Protective effects of Pro, GB and Tre in reducing oxidative damage were also proved in other studies in different plant species (Moustakas *et al*., 2011; Hasanuzaman *et al.,* 2014; Hossain *et al.,* 2014, Alam *et al.,* 2014; Molla *et al*., 2014). However, in the present study, exogenous Pro-treated seedlings showed better protection against oxidative stress than other two osmolytes.

4.10 Histochemical detection of H_2O_2 and $O_2^{\bullet-}$ generation

Leaves from each treatment were subjected to staining by DAB and NBT to see the generation of ROS. DAB staining resulted in brown patches of H_2O_2 and NBT staining resulted in dark blue spots of $O_2^{\bullet-}$. Drought stress increased H_2O_2 and $O_2^{\bullet-}$ generation in the leaves compared with control. Applying Pro, GB and Tre to the drought-stressed plants decreased the spots on the leaves produced by H_2O_2 and $O_2^{\bullet-}$, indicating a reduction in ROS generation compared with drought stress alone (Figure 8). Among the three protectants Pro treated leaves showed the lowest mark on leaves produced by H_2O_2 and $O_2^{\bullet-}$.

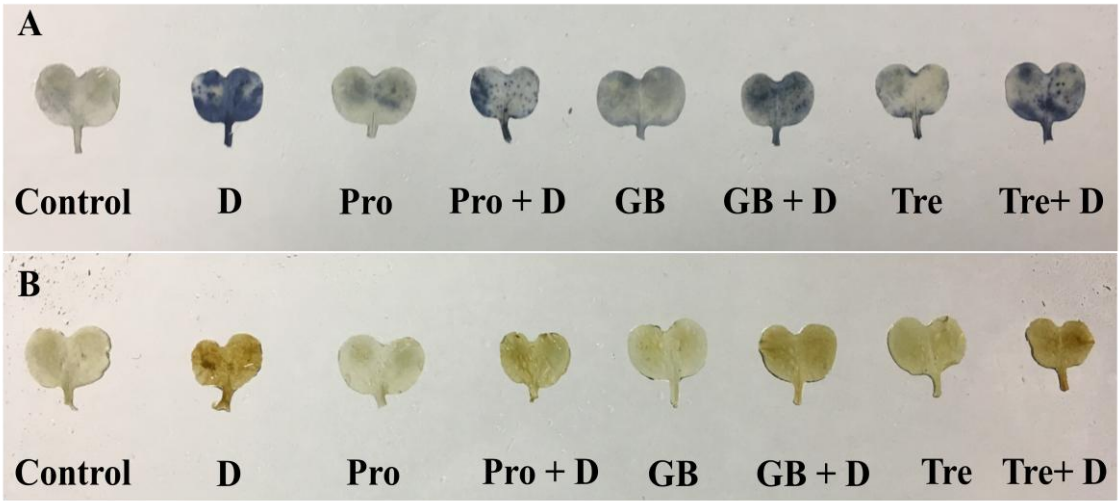

Figure 8. Histochemical detection of (A) superoxide ($O_2^{\bullet-}$) and (B) hydrogen peroxide (H_2O_2) in the leaves of rapeseed seedlings under drought stress.

Reactive oxygen species destroy cell biomolecules causing lipid peroxidation and fatty acid oxidation (Hasanuzzaman *et al,*. 2012a; Nahar *et al*., 2016). Drought-induced oxidative stress in the present study increased the oxidative stress that is reflected in the overproduction of H_2O_2, $O_2^{\bullet-}$, which is also clear from the results of histochemical detection in the leaves and their contents at the cellular level. Similar findings were reported in other studies on different stresses (Nahar *et al*., 2016; Rahman *et al*., 2016).

4.11 Detoxification of MG through enhanced glyoxalase system

4.11.1 Methylglyoxal content

MG contents increase significantly under drought stress. Compared to control MG content under drought stress increased by 96%. The MG content after exogenous Pro, GB and Tre application decreased by 34, 27 and 18%, respectively, as compared to drought stress alone (Figure 9).

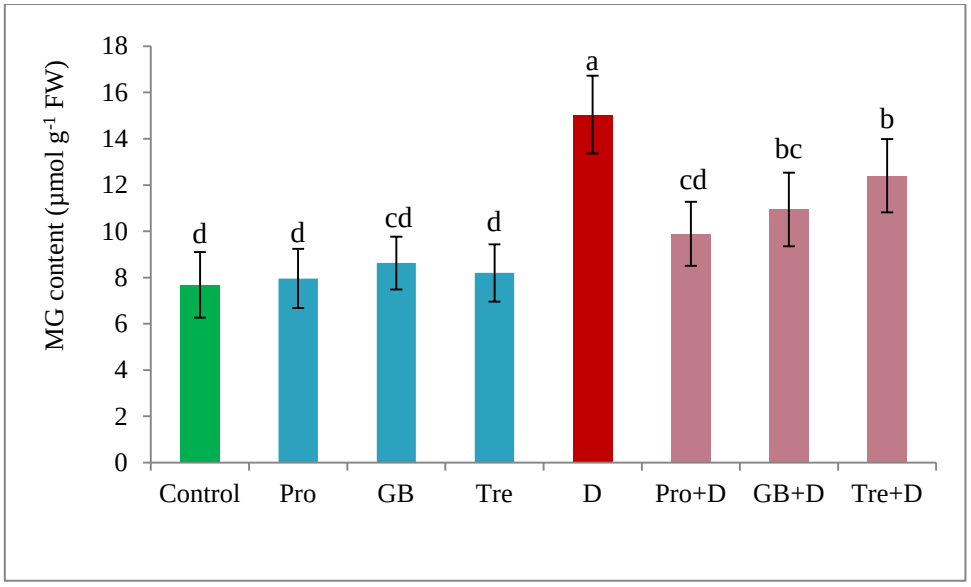

Figure 9. Effect of exogenous Pro, GB and Tre on MG content of of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.11.2 Glyoxalase I activity

Drought stress caused a non-significant increase in Gly I activity by 5% as compared to control. With the combination of Pro pre-treatment and drought stress, Gly I activity increased by 13% but no significant variation in Gly I activity was observed in GB and Tre pre-treated drought-stressed seedlins (Figure 10).

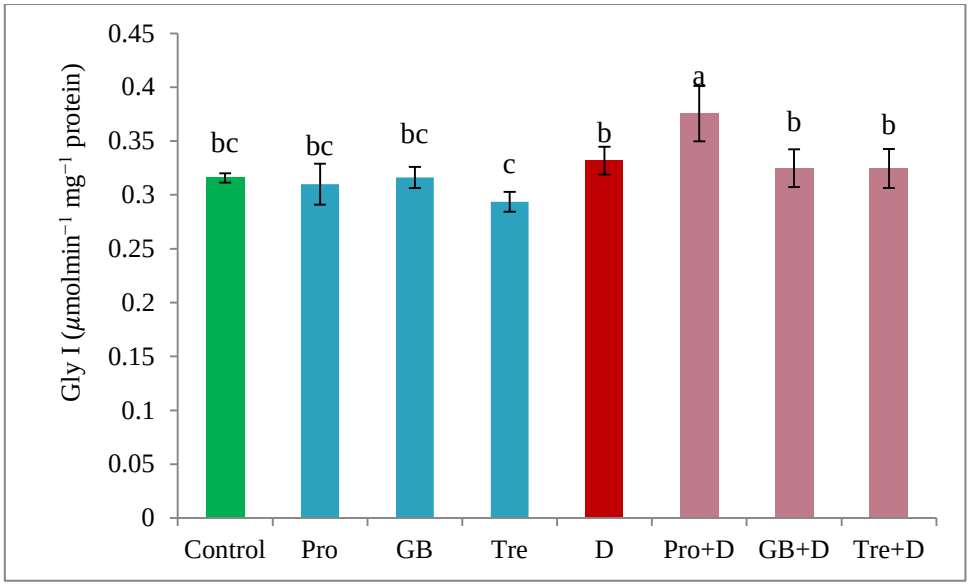

Figure 10. Effect of exogenous Pro, GB and Tre on Gly I activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.11.3 Glyoxalase II activity

Drought stress led to a significant decrease by 29% in Gly II activity over control. Compared with drought stress treatment alone, Pro, GB and Tre pre-treated drought stressed seedlings showed significantly higher Gly II activity by 53, 33 and 46%, respectively (Figure 11).

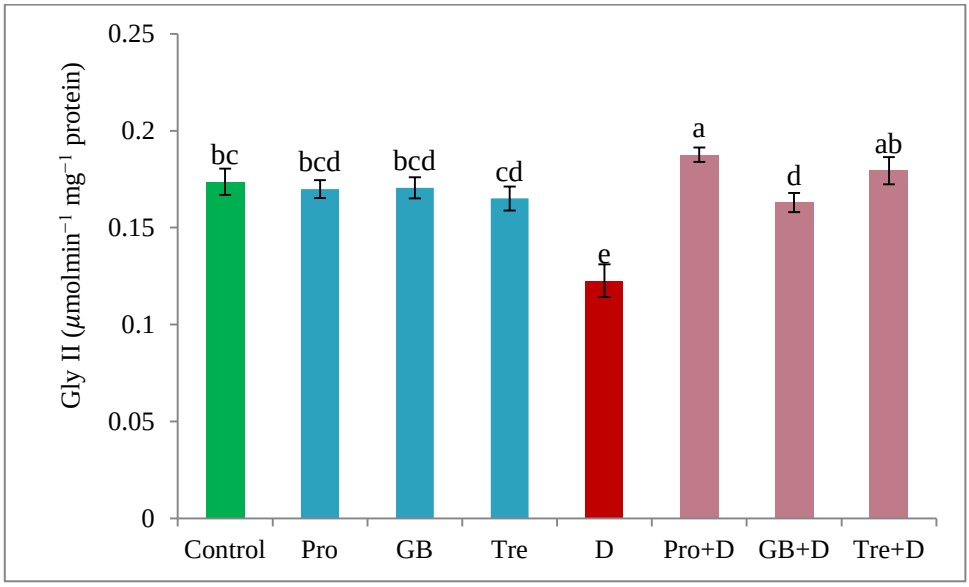

Figure 11. Effect of exogenous Pro, GB and Tre on Gly II activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

Methylglyoxal is a cytotoxic compound, normally present in a lower amount in plant cells, but increases several fold under stress conditions depending on stress intensity and duration (Yadav *et al*., 2005, 2008; Rahman *et al*. 2016; Hasanuzzaman *et al*., 2017). The glyoxalase system is the most important MG detoxification pathway in plants (Yadav *et al*., 2005, 2008). The glyoxalase system consists of two vital enzymes, Gly I and Gly II, which can detoxify MG in a two-step reaction. In the first step, Gly I uses one molecule of reduced GSH to convert MG to *S*-D-lactoylglutathione (SLG). The second step converts SLG to D-lactate and produces reduced GSH. Thus the GSH is recycled back (Yadav *et al.,* 2005; Mustafiz *et al.* , 2010). In the present study, drought stress showed increased Gly I activities and decreased Gly II activities (Figure 10, 11). Under drought stress along with the alteration of Glyoxalage enzymes, MG level also increased significantly (Figure 9). Increases in MG levels under abiotic stresses (Yadav *et al*., 2005a; Singla-Pareek *et al.,* 2006; Banu *et al*., 2010; El-Shabrawi *et al*., 2010; Turóczy *et al*., 2011) and altered Gly I and Gly II activities were also documented under drought stresses in previous studies (Alam *et al*., 2013, Hossain *et al.,* 2014). Comparing the plant species performance under drought condition and Pro, GB and Tre combined drought stress condition, it is observed that exogenous Pro, GB and Tre application increased the Gly I activity and Gly II activity in rapeseed seedlings. But results of this experiment showed higher Gly I and Gly II activities induced by Pro, GB and Tre that might be helped to reduce the MG induced damages effects under drought stress which are in agreement with the previous studies (Hasanuzzaman and Fujita, 2011; Alam *et al.,* 2013).

4.12 Contents of ascorbate and glutathione

4.12.1 Ascorbate content

Drought stress increased the endogenous AsA levels significantly by 10% in rapeseed seedlings compared to untreated control. Proline, GB and Tre pre-treated seedlings showed a non-significant decrease in AsA content compared to the control. Whereas, Pro, GB and Tre pre-treated drought stressed seedlings showed 3% increase and 2 and 4% decrease in AsA content, respectively, as compared to the seedlings subjected to drought without pretreatment (Figure 12).

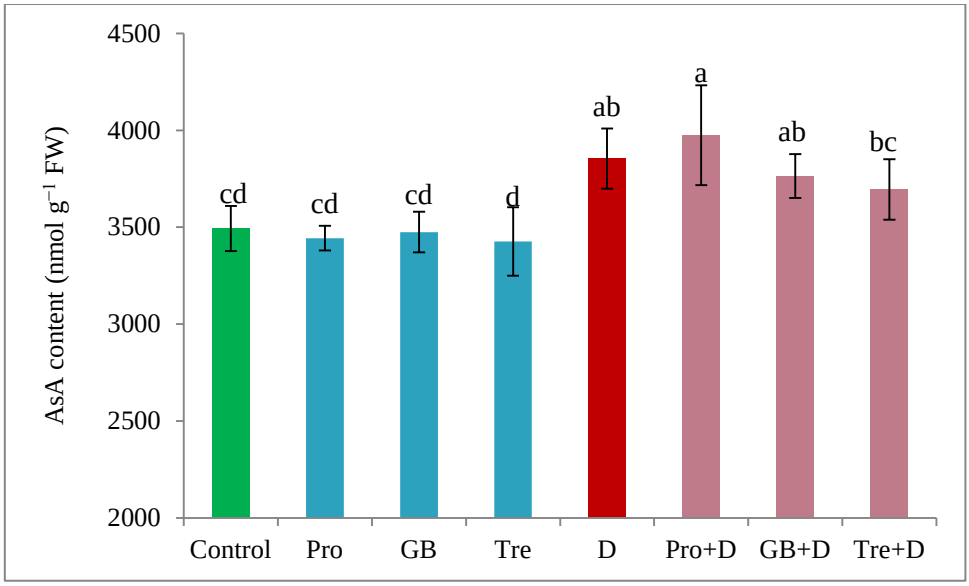

Figure 12. Effect of exogenous Pro, GB and Tre on AsA content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.12.2 Reduced glutathione content

The GSH content was enhanced by 72% in drought-stressed seedlings compared to unstressed control. Addition of exogenous Pro in combination with drought stress further increased GSH level by 9% but addition of exogenous GB and Tre in combination with drought stress decreased GSH level by 7 and 10%, respectively, compared to non-pretreated drought stressed seedlings (Figure 13).

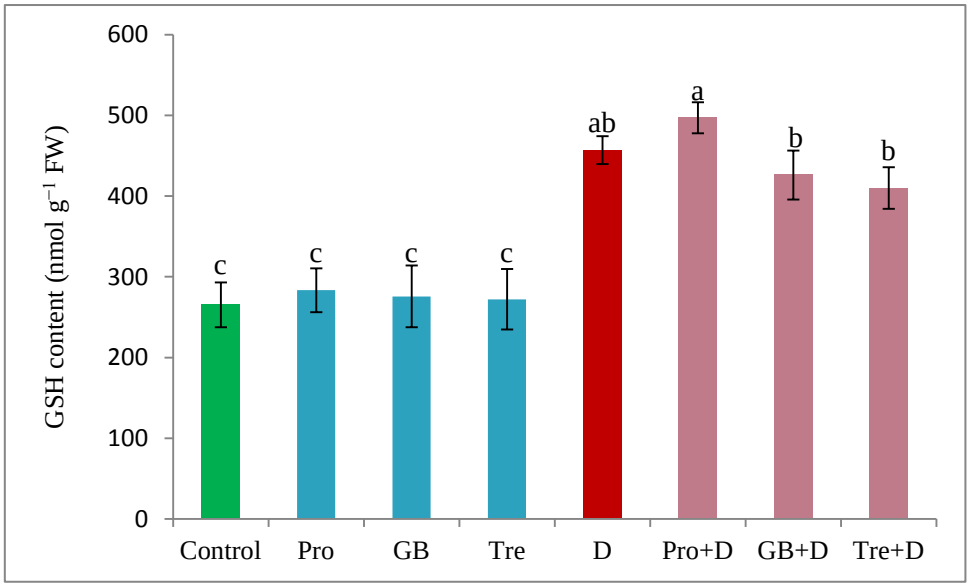

Figure 13. Effect of exogenous Pro, GB and Tre on GSH content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.12.3 Glutathione disulfide content

GSSG content markedly increased under drought and it was observed that compared to control, the GSSG contents were 178% higher under drought stress. In contrary, the seedlings which were supplemented with Pro, GB and Tre showed lower GSSG levels similar to control. But addition of Pro, GB and Tre in combination with drought stress resulted in decreased GSSG content by 47, 43 and 35%, respectively, compared to drought stress alone (Figure 14).

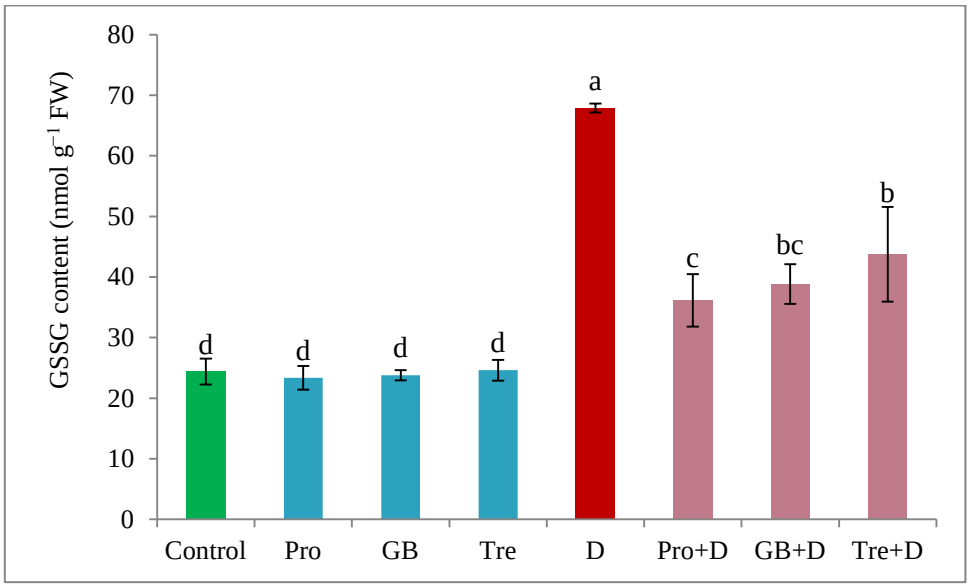

Figure 14. Effect of exogenous Pro, GB and Tre on GSSG content of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.12.4 Glutathione and glutathione disulfide ratio (GSH/GSSG)

The ratio of GSH/GSSG showed a decreasing trend and it significantly decreased by 38% than control upon exposure to drought stress. However, Pro, GB and Tre supplemented drought stressed seedlings showed significantly improved GSH/GSSG ratio by 106, 63 and 41% higher, respectively, compared to drought stress alone (Figure 15).

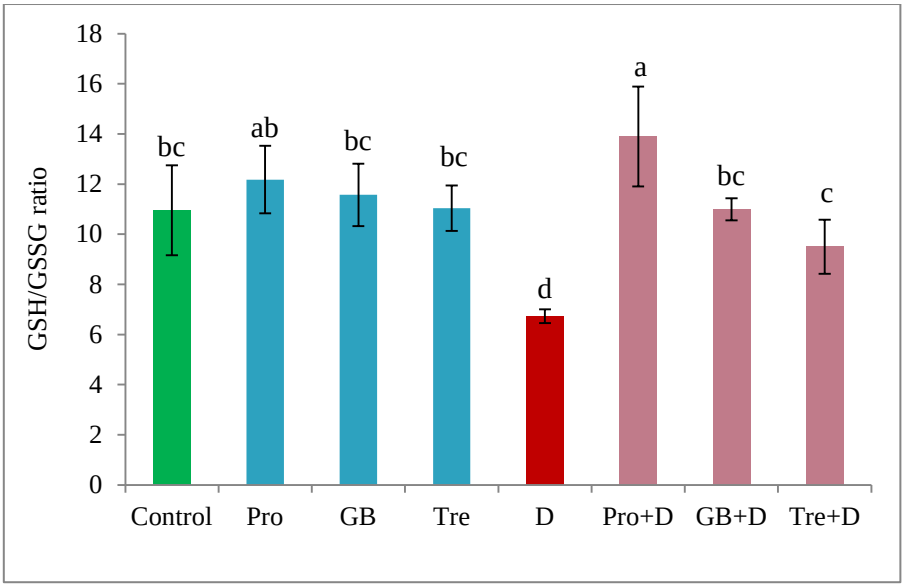

Figure 15. Effect of exogenous Pro, GB and Tre on GSH/GSSG raio of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

Ascorbate (AsA) is an important water-soluble antioxidant that scavenges a range of ROS including $^1O_2$, $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$ and at the same time able to donate electron to many enzymatic and non enzymatic reactions what makes it an important ROS scavenging molecule (Gill and Tuteja, 2010). GSH is another non enzymatic antioxidant especially important for photosynthetic organelles such as chloroplast. Ascorbate, GSH with the enzymes APX, MDHAR, DHAR and GR predominantly constitute the AsA-GSH cycle (Hasanuzzaman *et al*., 2012a). AsA and GSH have vital roles in development of plant stress tolerance to adverse environmental conditions (Ball *et al*., 2004). Increased AsA or GSH content can effectively reduce ROS produced under stress conditions including drought stress and thus prevents oxidative stress (Nahar *et al.*, 2016). In the present study, it was observed that under drought stress condition AsA and GSH content increased significantly (Figure 12, 13). Our results are consistent with the results of other studies where increases in AsA and GSH levels were found (Liu *et al.*, 2010; Chugh *et al*., 2013). The AsA content in the Pro, GB and Tre pre-treated seedlings were significantly increased when compared with controls (Figure 12). The similar results regarding modulation of AsA pool and its related enzymes by exogenous Pro, GB and Tre under salt and drought

stresses were previously reported (Hasanuzzaman *et al*., 2014; Alam *et al.*, 2014). They also mentioned Pro as more efficient protectant than GB and Tre.

In the present experiment, GSH content increased significantly under drought stress. Similar results were reported by Hossain *et al*. (2014) and Alam *et al*. (2014). The increased GSH content might be due to the increase in GR activities as well as higher GSH biosynthesis (Mittova *et al*., 2004). In our experiment supplementation of Pro, GB, Tre under drought stress showed further increasement of GSH content. It might be due to GR activity is desirable to recycle GSH and increase its content, which our findings corroborate. In the present study, the GR activity of the rapeseed seedlings significantly increased under drought stress which was restored after the Pro, GB and Tre supplementation. This higher GR activity is correlated with higher GSH content in the seedlings treated either with drought stress alone or with the combination of Pro, GB and Tre and drought stress.

In our study, drought stress resulted in an abrupt increase in GSSG level (Figure 14), which is very detrimental to the plant cell. The increase in GSSG content indicates the higher oxidative load in the drought stressed seedlings. This increase might be due to the decreased regeneration as well as increased degradation rate of GSH (Noctor and Foyer, 1998). However, exogenous Pro, GB and Tre significantly reduced the osmotic stress-induced increase in GSSG level (Figure 14).

The GSH/GSSG ratio has immense functions in cell redox status and stress signaling processes. Thus a higher GSH/GSSG is considered as supportive for improved abiotic stress tolerances including drought stress. Exogenous supplementation of Pro, GB and Tre in our experiment maintained higher GSH/GSSG ratio (Figure 15). Importantly, the higher level of GSH/GSSG ratio in Pro, GB and Tre pre-treated drought stressed seedlings indicates that osmolyte pre-treatments suppress the increase of GSSG accumulation probably due to higher GR and Gly II activites. Therefore, other research findings also support the results of this study (Hossain *et al.*, 2014; Alam *et al.*, 2014).

4.13 Activities of antioxidant enzymes

4.13.1 APX activity

Drought stress resulted in a significant increase (23%) in APX activity as compared to control. Whereas, drought-stressed seedlings pre-treated with Pro, GB and Tre also showed a non-significant increase by 5, 3 and 3%, respectively, relative to drought stress alone (Figure 16).

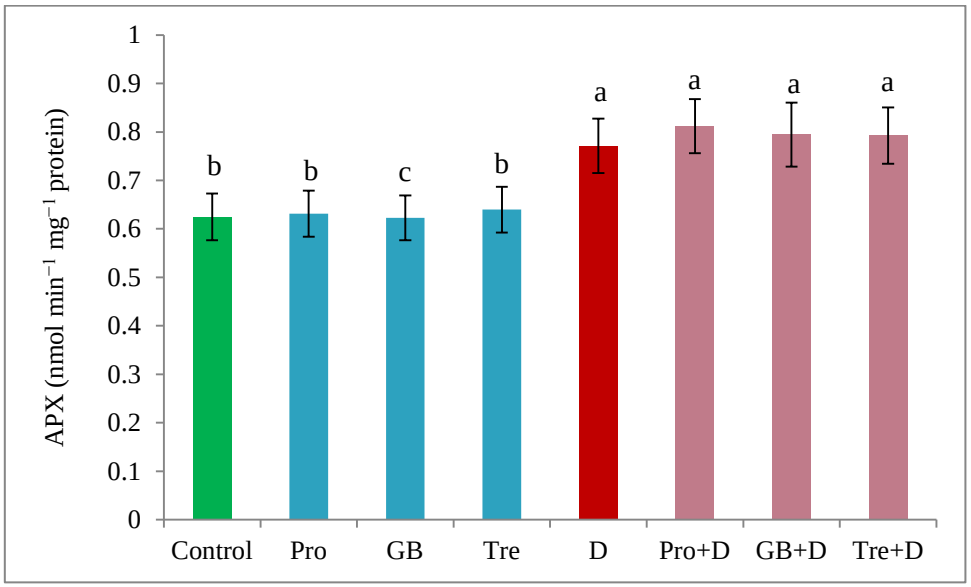

Figure 16. Effect of exogenous Pro, GB and Tre on (A) APX activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.13.2 MDHAR activity

As compared to the control group, drought stress led to a significant increase in MDHAR activity by 17%. Drought-stressed seedlings pre-treated with Pro significanty increase the MDHAR activity by 14% but seedlings pre-treated with GB and Tre showed a non-significant increase by 8 and 2%, respectively, over drought stress alone (Figure 17).

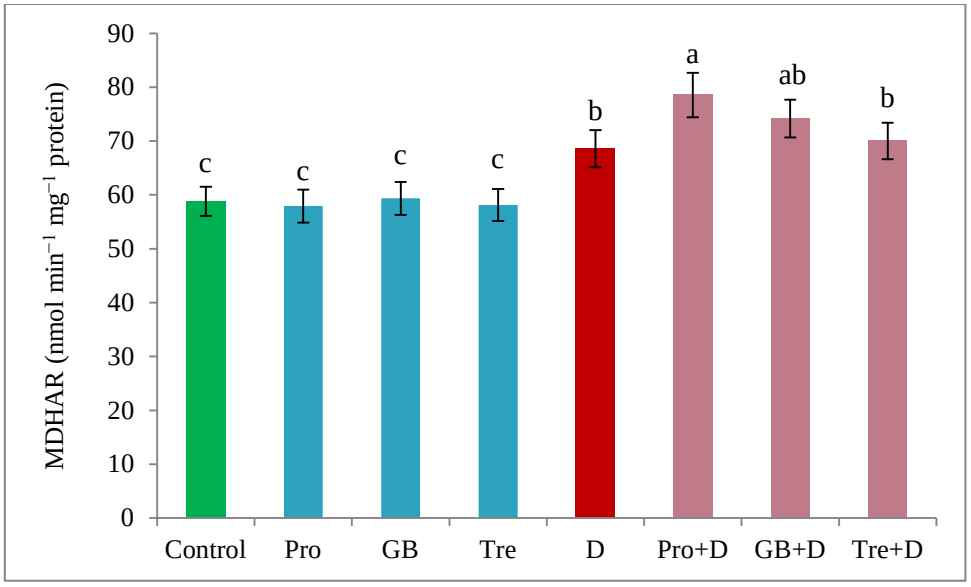

Figure 17. Effect of exogenous Pro, GB and Tre on MDHAR activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.13.3 DHAR activity

DHAR activity decreased significantly by 22% as compared to control. The seedling supplemented with Pro, GB and Tre, on the other hand, increased the activity by 15, 7 and 4% compared to drought stress alone (Figure 18).

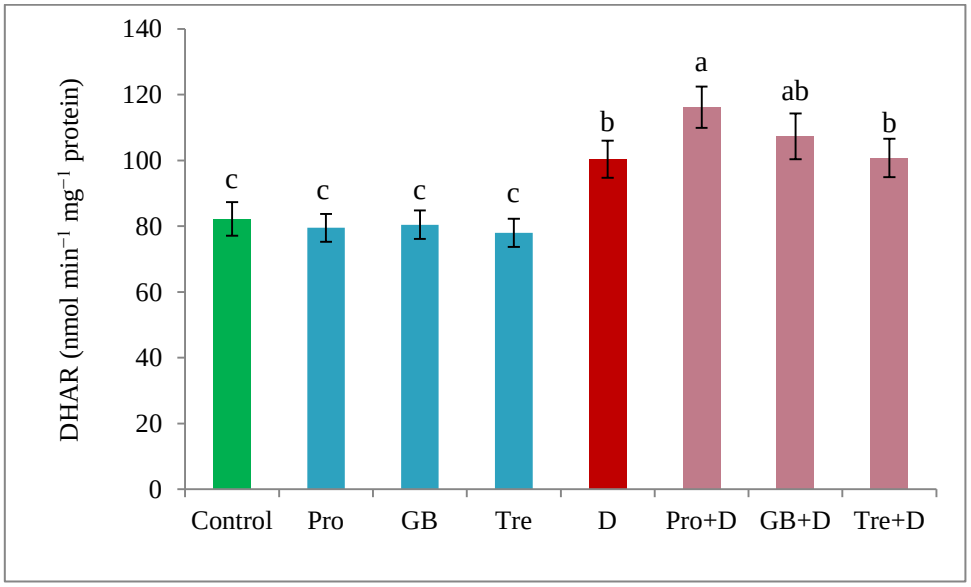

Figure 18. Effect of exogenous Pro, GB and Tre on DHAR activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.13.4 GR activity

The GR activity was significantly increased by 81% in drought-stressed *B. campestris* over control. The Pro, GB and Tre treated drought-stressed seedlings had reduced GR activities by 12, 7 and 12%, respectively, compared to the seedlings exposed to drought stress without pre-treatment (Figure 19).

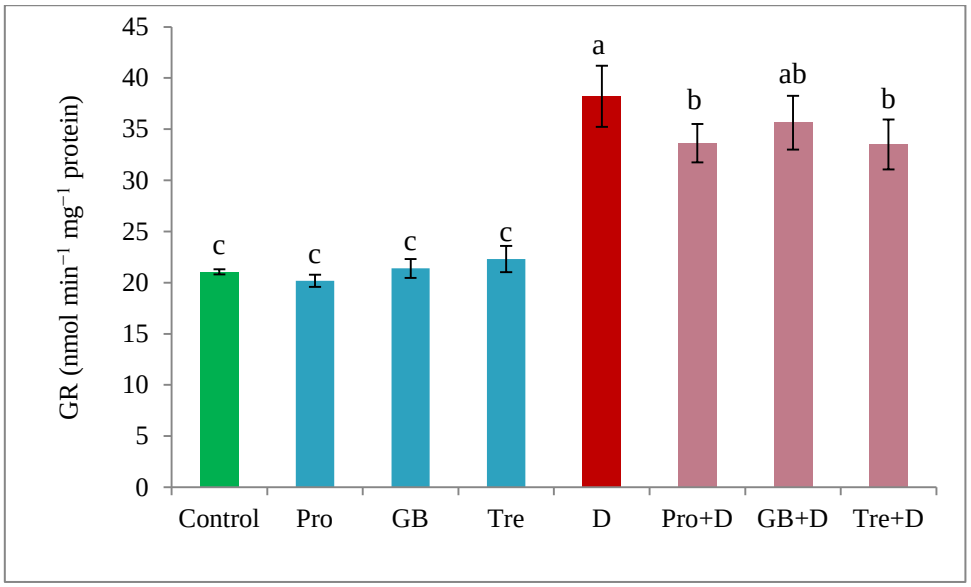

Figure 19. Effect of exogenous Pro, GB and Tre on GR activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.13.5 CAT activity

Drought stress resulted in a significant increase by 29% in CAT activity in relation to control, whereas, Pro, GB and Tre pre-treated drought-stressed seedlings showed increase CAT activity by 8, 4 and 3% as compared to the seedlings subjected to drought stress without pre-treatment (Figure 20).

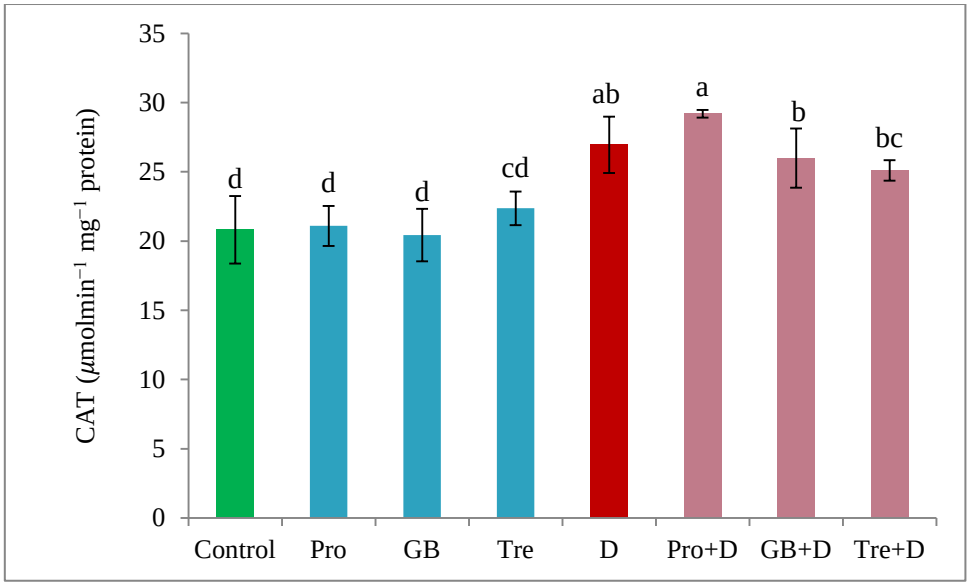

Figure 20. Effect of exogenous Pro, GB and Tre on CAT activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

4.13.6 GPX activity

Compared with control, drought stress led to a significant increase in GPX activity by 26%. In contrast, Pro, GB and Tre treatment further augment the activity by 19, 12 and 7%, respectively as compared to drought treatment alone (Figure 21).

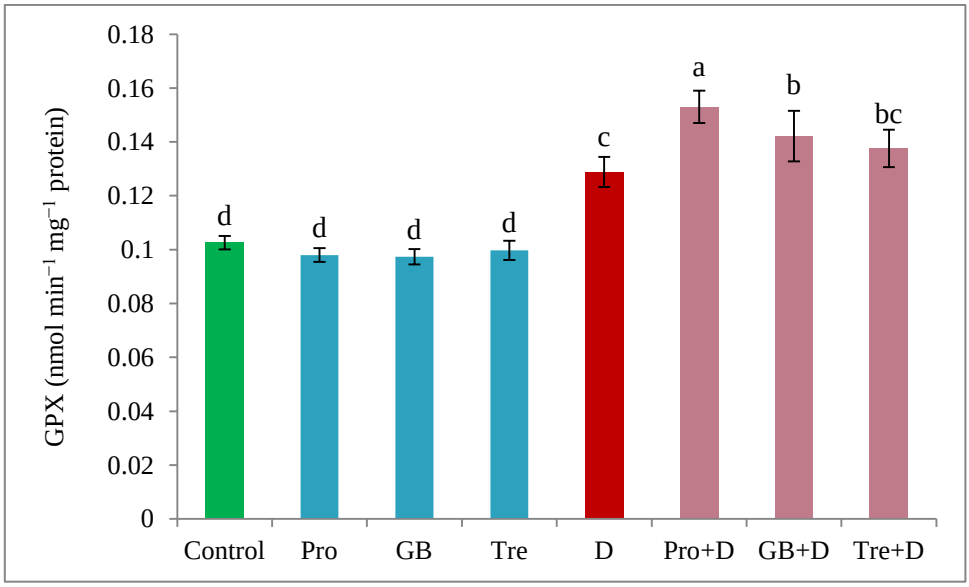

Figure 21. Effect of exogenous Pro, GB and Tre on GPX activity of rapeseed seedlings under drought stress. Means (±SD) were calculated from three replicates for each treatment. Values in the bars with different letters are significantly different at $p \leq 0.05$ applying Fisher's LSD test

To counteract the damaging effects of different stesses, plants activate their well established antioxidant defense system comprising of several enzymes. The four enzymes of AsA-GSH, namely, APX, MDHAR, DHAR, and GR are vital for antioxidant defense because they are involved in maintaining the AsA and GSH pool. 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. Drought stress-induced excess generation of ROS and subsequent enhanced activities of many antioxidant enzymes during stress have been reported in many plant species (Hasanuzzaman *et al.,* 2014; Nahar *et al.,* 2016). In the present study APX activity increased significantly in rapeseed seedlings subjected to drought stress and exogenous Pro, GB and Tre application slightly enhanced the activity further which indicated the H_2O_2 scavenging role of Pro, GB and Tre (Figure 16). This result is in agreement with other findings (Hasanuzzaman *et al.,* 2012a; Patade *et al.,* 2014).

Another two most important enzymes MDHAR and DHAR are related to the regeneration of AsA which is a strong antioxidant. Both enzymes are equally important in regulating AsA level and its redox state under oxidative stress condition (Eltayeb *et al*., 2006, 2007; Wang *et al.,* 2010). In our findings, the activity of MDHAR and DHAR clearly increased under drought stress condition (Figures 17, 18). Enhanced MDHAR and DHAR activities under drought stress were also reported in earlier studies (Alam *et al*., 2014). Exogenous Pro and GB supplemented seedlings, on the other hand, enhanced the activity of both MDHAR and DHAR enzymes which helped the plants in efficient regeneration of AsA (Figure 17, 18). But no significant variation in both enzymes activities were observed in Tre pre-treated and non-treated drought stressed seedlings which might be the reason behind reduced regeneration of AsA in case of Tre supplementation (Figures 17, 18).

Glutathione reductase is another important enzyme of AsA-GSH cycle, which is important for maintaining high ratio of GSH/GSSG in plant cells, also necessary for accelerating the H_2O_2 scavenging (Rao and Reddy, 2008; Pang and Wang, 2010). Significantly higher activity of GR in drought stress condition was observed in our study (Figure 19) which is in agreement with some previous findings (Hasanuzzaman *et al*., 2014, Nahar *et al*., 2013). This increase in GR activity might be due to

enhanced antioxidative capacity under drought stress. However, supplementation of Pro, GB and Tre in drought stressed seedlings showed reduced activities of GR (Figure 19), which might be due to the contributions in regulating the H_2O_2 level under drought stress. Present result is corroborated with another study in rice under Cu stress (Mostofa *et al.*, 2015).

Catalase is a potential enzyme which has higher turnover rate and is capable to dismutase two molecules of H_2O_2 to water and oxygen and thus is considered as an efficient ROS detoxifier (Hasanuzzaman *et al*, 2012a). In the present study CAT activity significantly increased under drought stress condition (Figure 20). Different stress induced elevation of CAT activity was also found in some previous studies (Rahman *et al.*, 2015; Dixit *et al*., 2015). Similarly, increased CAT activity was observed in Pro pre-treated seedlings. Zarei *et al*. (2012) showed that transgenic plants over-expressing Pro biosynthetic gene under drought stress conditions showed higher APX and CAT activity under drought stress conditions. However, its activity under drought stress was restored by GB and Tre supplementation. Several findings are corroborated to our study where water stress was imposed in *Zea mays* (Ali and Ashraf, 2011) and Cd stress in *Lemna gibba* (Duman *et al.*, 2011).

The GPX is another vital enzyme of antioxidant defense system and due to substrate specifications and stronger affinity for H_2O_2, it can efficiently scavenge especially, H_2O_2 and thus provide protection against stress (Hasanuzzaman *et al*., 2011). Drought stress significantly increased GPX activities in rapeseed seedlings, compared to untreated control seedlings (Figure 21). Similar observations of increased GPX under drought stress were reported by several researchers (Liu *et al*., 2010; Nahar *et al*., 2013). But compared to drought stress alone, Pro, GB and Tre supplemented drought treatment improved GPX activity further (Figure 21). Similarly, it was found that application of organic compatible solutes increased GPX activity in *Brassica* sp. under drought and *Nicotiana tabacum* under salt stress (Hoque *et al.*, 2008; Alam *et al*., 2014).

From the above discussion, it can be concluded that exogenous application of osmolytes (Pro, GB and Tre) ameliorated the drought stress-induced adverse effects on the growth of rapeseed plants by increasing the plant photosynthetic activity,

adjusting plant water status, improving plant defense mechanism against oxidative stress by enhancing the activities of some key antioxidant enzymes. Furthermore, of these osmolytes, Pro was more effective in inducing drought stress tolerance in rapeseed plant.

Chapter V

SUMMARY AND CONCLUSION

This research work was conducted in the Laboratory of Plant Stress Responses, Kagawa University, Japan, to test the relative effectiveness of the three osmolytes in alleviating the adverse effects of drought stress on rapeseed plants. The experiments were arranged in a CRD design with three replications. Seedlings were grown in petridishes in a semi hydroponic medium and drought stress was imposed artificially by using 20% PEG. The experiment consisted of 8 treatments: control (well-watered), 0.5 mM Pro, 0.5 mM GB, 0.5mM Tre, Drought (20% PEG, D), Pro+D, GB+D and Tre+D. There were about 60 seedlings maintained in each petriplate. The osmolytes were applied as pre-treatment in rapeseed plants under non-stress or drought-stress conditions. In the present experiment, 0.5 mM concentration of each of the three osmolytes (Pro, GB and Tre) were applied as seedling pre-treatment at 6 days and as plant material rapeseed (*B. campestris* L. cv. BARI Sharisha-15) was used. Drought was artificially imposed on 8-day-old seedlings by using 20% PEG (polyethylene glycol). Seedlings were then allowed to grow under controlled condition (light, 350 μmol photon m^{-2} s^{-1}; temperature, 25±2°C; RH, 65–70%) and 5000-fold diluted Hyponex solution (Hyponex, Japan) was used as nutrient. Data were taken by sampling the leaves of 10-day-old seedlings.

Different data of growth, physiology and biochemical parameters were measured. Plant height, fresh weight $plant^{-1}$ and dry weight $plant^{-1}$ were measured for growth. As physiological parameters leaf chl contents, RWC were measured. Biochemical parameters included MDA content, H_2O_2, Pro, GB, Tre, AsA, GSH, GSSG, MG and antioxidant enzyme like CAT, APX, MDHAR, DHAR, GR, Gly I and Gly II activities and histochemical detection of H_2O_2 and $O_2^{\bullet-}$ generation.

In the present study drought stress significantly reduced plant height, fresh and dry weight $Plant^{-1}$. However, exogenous Application of Pro, GB and Tre improved the above mentioned growth parameters. Among three protectants Pro was the most effective in alleviating the drought induced growth reduction and in promoting seedling growth under drought stress conditions.

Drought stress results in significant reduction of Leaf RWC and chl content. But exogenous supplementation of Pro, GB and Tre restored the tissue water status as well as the photosynthetic pigmens. Here, Pro and GB showed almost the similar effects in restoring the water status and chl contents. Tre showed lesser effect on RWC and chl content than Pro and GB.

Drought induced oxidative damage resulted in a drastic increase in MDA and H_2O_2 contents. Increased level of oxidative damages was also evident from histochemical detection which showed highest generation of H_2O_2 and $O_2^{\bullet-}$ under drought stress. Exogenous osmolyte application reduced the oxidative damage by reducing the MDA and H_2O_2 contents and $O_2^{\bullet-}$ generation. Increased Pro, GB and Tre contents were studied in *Brassica* species which proved the ability to possess some adaptive mechanisms against drought.

Drought stress also found to enhance MG content, decreased GSH/GSSG ratio and alters the antioxidant defense system in rapeseed plants. It can be conclude that burden of drought stress in rapeseed seedlings led to a severe oxidative damage due to inappropriate induction of ROS and MG detoxification systems. Antioxidant defense system comprises non-enzymatic antioxidants such as AsA and GSH and enzymatic antioxidants such as CAT, APX, MDHAR, DHAR, GR, GPX etc. Most importantly, pretreatment of seedlings with Pro, GB and Tre modulated the activities of CAT, APX, MDHAR, DHAR, GR, GPX, Gly I 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 any pretreatment. Exogenously applied different compatible solutes (Pro, GB, Tre) as pretreatment improved growth and survival of rapeseed pants. Osmolyte-induced enhancement in growth under water stress conditions was associated with increased net photosynthetic rate, plant water status, antioxidant capacity and reduced oxidative damages.

The present experimental results confirmed the beneficial effects of exogenous Pro, GB and Tre in alleviating drought induced oxidative damage. These results conclude that co-ordinate stimulation of glyoxalase system and antioxidant defense system is an important determinant for the acquisition of drought stress tolerance. The results also suggest that the performance of Pro was best as a protectant against drought stress

because Pro showed better effects on all growth, physiological and oxidative stress parameters, compared to GB and Tre. However, identification of additional key factors involved in Pro, GB and Tre induced-drought stress tolerance and the underlying signaling roles of Pro, GB and Tre warrant further research.

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APPENDICES

Appendix I: Mean square values and degree of freedom (DF) of plant height, FW, DW, RWC (%), chl *a*, chl *b* and total chl *(a+b)* of rapeseed as influenced by exogenous Pro, GB and Tre under drought stress condition

Source	DF	Mean square values						
		Plant height	FW	DW	RWC (%)	chl *a*	chl *b*	chl (*a*+*b*)
Model	7	0.091	0.000052	0.00000035	209.662	18.837	2.539	27.927
Error	16	0.008	0.0000060	0.000000069	6.654	0.456	0.242	0.345

Appendix II: Mean square values and degree of freedom (DF) of Pro, GB, Tre, MDA, H_2O_2 and MG of rapeseed as influenced by exogenous Pro, GB and Tre under drought stress condition

Source	DF	Mean square values					
		Pro	GB	Tre	MDA	H_2O_2	MG
Model	7	16.635	7.206	0.210	171.644	54.645	19.987
Error	16	0.064	0.155	0.002	5.096	0.482	2.029

Appendix III: Mean square values and degree of freedom (DF) of AsA, GSH, GSSG and GSH/GSSG of rapeseed as influenced by exogenous Pro, GB and Tre under drought stress condition

Source	DF	Mean square values			
		AsA	GSH	GSSG	GSH/GSSSG
Model	7	132412.651	27690.911	709.372	13.040
Error	16	23430.102	828.229	12.931	1.597

Appendix IV. Mean square values and degree of freedom (DF) of Gly I, Gly II, APX, MDHAR, DHAR, GR, CAT and GPX of rapeseed as influenced by exogenous Pro, GB and Tre under drought stress condition

Source	DF	Mean square values							
		Gly-I	Gly-II	APX	MDHAR	DHAR	GR	CAT	GPX
Model	7	0.002	0.001	0.023	200.992	659.740	175.680	31.983	0.002
Error	16	0.00025	0.000036	0.003	10.973	29.279	3.532	2.800	0.000029