INVESTIGATION OF GLYOXALASES IN B*rassica* spp UNDER ABIOTIC STRESS AND PURIFICATION OF GLYOXALASE-I

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CERTIFICATE

This is to certify that thesis entitled, "INVESTIGATION OF GLYOXALASES Brassica spp UNDER ABIOTIC STRESS AND PURIFICATION OF GLYOXALASE-I" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE (MS) IN BIOTECHNOLOGY, embodies the result of a piece of bona-fide research work carried out by MD. SHAHADAT HOSSAIN, Registration no. 09-03324 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 of during the course of this investigation has duly been acknowledged.

Date:

Place: Dhaka, Bangladesh

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DEDICATED
TO
MY BELOVED PARENTS

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SAU, Dhaka

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ABSTRACT

This study was carried out in the Molecular Breeding Laboratory of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur during the period from March, 2014 to March, 2015 to investigate the role of Glyoxalase system in contrast plants of *Brassica* spp. Ten prescreened genotypes were subjected to test the variation among them with 5 known SSR markers, and among them, E5 (Tori 7) and E6 (BARI sarisha 16) were selected based on their variation. The comparative phenotyping study of E5 and E6 under salinity and drought stress revealed that E6 is more tolerant than E5 in both stresses. To investigate the responses of Glyoxalase-I (Gly-I) and Glyoxalase-II (Gly-II) in detoxification of methylglyoxal (MG) under salinity and drought stress, 5 day old Brassica seedlings were subjected to impose NaCl-induced salinity level (16dS/m) and drought stress and data were observed at 2 day, 4 day and 6 day of stress. The contents of (MG) increased 2.0, 2.5 and 3.0 fold over control under salinity at 2, 4 and 6 day, respectively in E6 and 0.5, 0.3 and 0.6 fold in E5. On the other hand, in drought stress, methylglyoxal (MG) level increased 2, 3 and 3.5 fold over control at 2, 4 and 6 day, respectively in E6 and 2, 2.5 and 3 fold in E5. Under salinity stress, Gly-I activity increased by 41%, 69% and 105% in E6, at 2, 4 and 6 day, respectively, whereas 12%, 43% and 41% in E5. On the contrary, in drought stress, Gly-I activity increased by 70%, 76% and 61% over control at 2, 4 and 6 day, respectively in E6, and 37%, 32% and 53% in E5. In E6, Gly-II activity increased 144%, 128% and 101% at 2, 4 and 6 day of salinity stress, respectively in E6, and 52%, 59% and 50% in E5. Notably, concomitant increased activities of Gly-I and Gly-II with increased MG suggested their MG detoxification role in *Brassica*

Spp. At the same time, it was remarkable that both Gly-I and Gly-II activities were higher in E6, and hence, Gly-I was purified from E6 seedlings. In purification, Gly-I were found to be highly purified and migrated as a single band on SDS-PAGE with an apparent molecular mass of 27 kDa in silver staining and coumassie brilliant blue staining with purification fold of 113 and recovery of 0.38%.

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LIST OF ABBREVIATIONS

APX Ascorbate peroxidase

BSA Bovine seram albumin

CAT Catalase

cDNA Complementary DNA

EDTA Ethylene Diamine Tetra-acetic Acid

GPX Glutathione peroxidase

GR Glutathione reductase

GSH Reduced glutathione

Gly-I Glyoxalase-I

Gly-II Glyoxalase-II

GST Glutathione S-transferase

HNE 4-hydroxynonenal

kDa Kilo Dalton

Min Minute

PAGE Polyacrylamide gel electrophoresis

ROS Reactive Oxygen Species

Rpm Revolutions per minute

SDS Sodium Dodecyl Sulfate

SOD Superoxide dismutase

TAE Tris-Acetate-EDTA

v/v Volume Per Volume

w/v Weight Per Volume

CHAPTER 1

INTRODUCTION

Due to climate change, crop plants being sessile exposes to a number of adverse condition known as abiotic stress including salinity, water deficit, extremely high or low temperatures, toxic metals, waterlogging, elevated ozone, and ultraviolet radiation which adversely affect proper growth, metabolism and productivity of crop plants. Fifty percent yield reduction is the ultimate consequence of abiotic stress and in case staple food yield may be reduced up to 70% due to negative effect on crop survival, biomass production and yield (Acquaah, 2007; Kaur *et al.,* 2008). Reactive oxygen species (ROS) and methylglyoxal (MG) production in plant cell is the ultimate result under any kind of abiotic stresses (Singla-pareek *et al.,* 2006; Hossain *et al.,* 2009). MG is highly toxic to plant cells, and in absence of adequate protective mechanism, they can react with proteins, lipids, and nucleic acid and inactivate the vital defense system leading to irreparable metabolic disorder and cell death (Hossain *et al*., 2009).

During conversion of glyceraldehyde-3-phosphate (G3P) from dihydroxyacetone phosphate (DHAP) in glycolysis, MG is formed spontaneously in plants by non-enzymatic mechanisms under physiological conditions (Espartero *et al.,* 1995; Yadav *et al.,* 2005a). The rate of glycolysis increases under stress conditions, leading to an imbalance (in the initial and latter five reactions) in the pathway. Triose phosphates are very unstable metabolites, and removal of the phosphoryl group by β-elimination from 1, 2-enediolate of these trioses leads to the formation of MG (Yadav *et al.,*

2005b). Therefore, MG production cannot be stopped as it is an unavoidable consequence of the glycolysis pathway during stress. From G3P and DHAP, MG can also be formed enzymatically by Triosephosphate isomerase that removes phosphate to yield MG (Pompliano *et al.,* 1990).

Glyoxalases in animals and microbial systems have been known for more than 100 years. Glyoxalase enzyme first reported in 1913 (Neuberg, 1913; Dakin and Dudley, 1913) which can detoxify MG. The Glyoxalase system is ubiquitous in nature and consists of two enzymes: Glyoxalase-I (Gly-I, EC 4.4.1.5) and Glyoxalase-II (Gly-II, EC 3.1.2.6). These two enzymes catalyze 2-oxoaldehydes coordinately to convert into 2-hydroxyacids when reduced glutathione used as a cofactor (reviewed in Thornalley,

1993). The reaction catalyzed by Gly-I (Aronsson *et al.*, 1978) and Gly-II (Uotila *et al.*, 1989) is shown in figure 1. Methylglyoxal is a primary physiological substrate for Gly-I (Thornalley, 1993). It is a potent cytotoxic compound known to arrest growth and react with DNA and protein, and increases sister chromatid exchanges (Thornalley, 1990). However, the physiological significance of this system is still unclear. The Glyoxalase system has been proposed to be involved in protection against α-oxoaldehyde cytotoxicity, regulation of cell division and proliferation, microtubular assembly, vesicle mobilization, growth of tumors, and clinical complications associated with various diseases, for example diabetes mellitus (reviewed in Thornalley, 1990, 1993). These properties of Glyoxalase system make it important tool for crop improvement against abiotic stress. Thus Gly-I and Gly-II has already been purified and characterized. It is proved that transgenic with genes encoding Gly-I and Gly-II perform well than non-transgenic under abiotic stress.

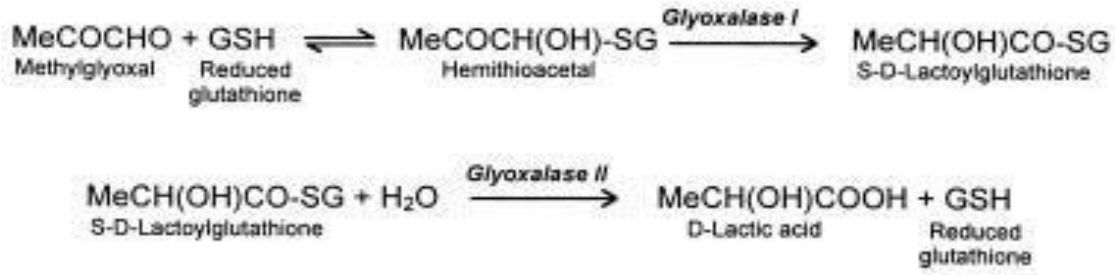

Figure 1. Mechanism of Glyoxalase reaction to detoxify MG in living system

Understanding the importance of Glyoxalases in protection of plants under stress condition, this study was designed to investigate the role of Glyoxalase system in contrast plants of *Brassica* spp under salinity and drought stresses. Previously, a good number of genotypes were studied for salinity tolerance in Oil seed Research Centre, Bangladesh Agricultural Research Institute (BARI) on the dry matter basis, it was reported E5 (Tori 7) is very susceptible to salinity (Anonymous, 2014). In this study, at first the genetic variability was examined in 10 *Brassica* genotypes including E5 and E6 (BARI Sarisha 16) by SSR markers, and the Glyoxalase system was studied in two contrast *Brassica* spp. Finally, Glyoxalase-I enzyme was purified from E6, a comparative saline tolerant genotype. With this view, this study was undertaken with the following objectives:

- To study the polymorphism of DNA among the Brassica genotypes by SSR markers.
- To investigate the role of Glyoxalase system under salinity and drought stress in two genotypes
- To purify the Glyoxalase-I from tolerant genotype of Brassica.

CHAPTER 2

REVIEW OF LITERATURE

Understanding the importance of Glyoxalase system, as a background work, I studied some articles related the role and purification of Glyoxalase to develop a foundation of my study. Here the results of those reports are summarized.

Racker (1951) reported that two enzymes participate in the Glyoxalase reaction. He proposed that Glyoxalase-I catalyzes the condensation of methylglyoxal and glutathione to an intermediate which is split by Glyoxalase-II to lactic acid and glutathione. A scheme for the stepwise process, resulting in an internal oxidation reduction, is proposed. He suggested that the use of the purified enzymes provides a sensitive assay method for reduced and oxidized glutathione.

Deswal *et al.,* (1991) purified the Glyoxalase-I enzyme from *Brassica juncea* by affinity chromatography on S-hexyl GSH sepharose 4B. They confirmed homogeneity of the protein electrophoretically by a silver stained gel. Activity staining on a native starch gel also showed a single band. They also studied the effect of glutathione, methylglyoxal and pH on enzyme kinetics. They found Magnesium which stimulated the enzyme activity.

Hossain *et al.,* (2009) purified Glyoxalase-I from onion bulbs using DEAE- cellulose, hydroxyapatite, *S*-hexylglutathion agarose chromatography. They reported highest specific activity 356 µmol min^{-1} mg^{-1} protein and the purified enzyme showed a single major band with a relative molecular mass of approximately 25000 on SDS-PAGE. They cloned and sequenced a cDNA encoding Glyoxalase-I and sequence comparison suggested that it is to be classified as a short type Glyoxalase. They also reported increased activity of Gly-I and Gly-II under salinity stress and an increase in Gly-I protein was also seen by immunoblotting.

Sneh *et al.,* (2006) suggested that transgenic plant with Glyoxalase-I and Glyoxalase-II may provide better tolerance to heavy metal stress based on their previous studies where over expressed Glyoxalase-I provide better tolerance under salinity stress.

Thornalley (1990) reviewed recent investigations that have brought new developments in the involvement of the Glyoxalase-I in cell growth and vesicle mobilization, with increasing evidence of changes in the Glyoxalase system during

tumor growth and diabetes mellitus, particularly relating to the development of associated clinical complications.

Thornalley (2003) stated that methylglyoxal forms during glycolysis through Embden- Meyerhof pathways. This methylglyoxal is highly reactive in glycation reaction and finally form AGEs (advanced glycation end-products) of protein, nucleotides and probably also basic phospholipids. He also said that Glyoxalase-I suppresses the formation of AGEs came from studies of endothelial cells in normoglycaemic and hyperglycaemic culture. Hyperglycaemia induced increases in the concentrations of methylglyoxal, D-lactate and cellular protein AGEs. He reported that Overexpression of Glyoxalase-I prevented totally the increase in methylglyoxal and cellular protein AGEs, and increased the concentration of D-lactate. This indicated that Glyoxalase-I has a critical role in suppressing the formation of protein AGEs.

Veena *et al.,* (1999) reported that Glyoxalase-I plays important role under stress condition tolerance. They characterized a cDNA from *Brassica juncea* encoding Glyoxalase-I (Gly-I) and made transgenic tobacco plants harbouring Gly-I in both sense and antisense orientation. They found that the expression of Glyoxalase-I in *B. juncea* was upregulated in response to salt, water and heavy metal stresses. In response to a high concentration of salt, they found the transcript level averaged three fold higher in 72 h, and the protein also increased seen by immunoblotting. A comparison of plants expressing high and low levels of Glyoxalase-I showed that the tolerance.

Saxena *et al.,* (2005) cloned cDNA Bj Gly-II from *Brassica juncea* which showed 92% and 56% identity with *Pennisetum* and rice Glyoxalase-II, respectively, and 30% identity was observed with the human Glyoxalase-II due to presence of highly conserved THHHXDH domain which is involved in zinc binding. p-NN and pSORT analysis of this sequence revealed a N-terminal mitochondrial target peptide. Under different abiotic stress, they found Bj gly-II was upregulated.

According to Skipsey *et al.,* (2000) Glyoxalase-I and glutathione S- transferase act coordinately to detoxify methylglyoxal under abiotic stress. In soybean, a further connection between these two enzymes has been suggested by a clone (Accession No. X68819) resembling a GST being described as a Glyoxalase-I. They tried to clarify the identity of GSTs and Glyoxalase-I in soybean, they confirmed on the characterization of the recombinant protein encoded by Accession No. X68819 as

well as the cloning and detailed analysis of a Glyoxalase-I from soybean resembling the dimeric Glyoxalase-I from animals. They also confirmed the clone X68819 was expressed in *E.coli*, the respective recombinant enzyme was active as a GST rather than a Glyoxalase and was termed GmGST 3.

Sneh *et al.,* (2008) transferred Gly-I gene in tobacco to get stress tolerant plant. After successful transformation, they cloned the rice Gly-II cloned in pCAMBIA1304 and transformed into rice (Oryza sativa cv PB1) via Agrobacterium. The transgenic plants showed higher constitutive activity of Glyoxalase-II that increased further upon salt stress, reflecting the upregulation of endogenous Glyoxalase-II. Their transgenic rice showed higher tolerance to toxic concentrations of methylglyoxal (MG) and NaCl. Compared with non-transgenics, transgenic plants at the T1 generation exhibited sustained growth and more favorable ion balance under salt stress conditions.

The Glyoxalase pathway involving Glyoxalase-I (Gly-I) and Glyoxalase-II (Gly-II) enzymes is required for glutathione-based detoxification of methylglyoxal. Singla-Pareek *et al.,* (2003) reported that overexpression of Gly-I and Gly-II together confers improved salinity tolerance, thus offering another effective strategy for manipulating stress tolerance in crop plants and overexpression of the Gly-II gene either alone in untransformed plants or with Gly-I transgenic plants stably expressed the foreign protein, and the enzyme activity was also higher. Compared with nontransformants, they found several independent Gly-II transgenic lines showed improved capability for tolerating exposure to high methylglyoxal and NaCl concentration and were able to grow, flower, and set normal viable seeds under continuous salinity stress conditions. They suggested that the double transgenic lines always showed a better response than either of the single gene-transformed lines and WT plants under salinity stress. They concluded that the potential of manipulation of the Glyoxalase pathway for increased salinity tolerance without affecting yield in crop plants.

Hossain *et al.,* (2007) investigated three ezymes (Glutathione S-Transferases, Glyoxalase-I and Alliinase) in soluble extracts of vegetable crops, including pumpkin, cabbage, broccoli, radish, carrot, potato, sweet potato, mungbean, and onion. The highest specific activity for glutathion *S*-tranferase and Glyoxalase-I were 648 nmol/min/mgP and 4540 nmol/min/mgP, respectively exhibited in the extract of onion bulb. They found moderate specific activities for Glyoxalase-I in radish and carrot, and the extracts of other vegetables had rather low levels of activities.

Alam *et al.,* (2013) studied the protective role of salicylic acid (SA) in relation to the water status, chlorophyll content, antioxidant defense and Glyoxalase system was investigated in drought stressed Brassica (*Brassica juncea* L. cv. BARI Sharisha 11) seedlings. They found the activity of glutathione *S*-transferase (GST) increased at any level of stress and the activities of glutathione peroxidase (GPX) and Glyoxalase-II (Gly- II) decreased only at severe stress while Glyoxalase-I (Gly-I) activities decreased at any level of stress. They found that spraying with SA alone had little influence on the non-enzymatic antioxidants and the activities of antioxidant enzymes. Salicylic acid supplemented drought stressed seedlings also enhanced the activities of Gly-I, and Gly-II as compared to the drought-stressed plants without SA supplementation. They suggested that the exogenous application of SA assisted the plants to become more tolerant to drought stress-induced oxidative damage by enhancing their antioxidant defense and Glyoxalase systems.

Espartero *et al.,* (1995) isolated a cDNA, GLX1, encoding Glyoxalase-I by differential screening of salt-induced genes in tomato. They reported that the protein encoded by GLX1 shared 49.4% and 58.5% identity with Glyoxalase-I isolated from bacteria and human, respectively. Yeast cells expressing GLX1 showed 20-fold higher Glyoxalase-I specific activity than non-transformed cells. Both GLX1 mRNA and Glyoxalase-I polypeptide levels increased 2-to 3-fold in roots, stems and leaves of plants treated with NaCl, mannitol, or abscisic acid. They suggested that the increased expression of Glyoxalase-I may be linked to a higher demand for ATP generation and to enhanced glycolysis in salt-stressed plants.

Lin *et al.,* (2010) isolated an EST encoding a Glyoxalase-I from a SSH (suppression subtractive hybridization) cDNA library of wheat spike inoculated by *Fusarium graminearum*. After cloning, sequencing and characterization of TaGly I, they reported its genomic sequence consists of 2,719 bp, including seven exons and six introns, and its coding sequence is 929 bp with an open reading frame encoding 291 amino acids. Sequence alignments showed that there were two Glyoxalase-I domains in the deduced protein sequence. By using specific primers, TaGly I was mapped to chromosome 7D of wheat via a set of durum wheat „Langdon" D-genomedisomic-substitution lines. The result of Real-time quantitative polymerase chain reaction demonstrated that TaGly I was induced by the inoculation of *Fusarium graminearum* in wheat spikes. Additionally, it was also induced by high concentration of NaCl and $ZnCl_2$. TaGly I overexpressed in tobacco leaves via *Agrobacterium tumefaciens*

infection, their transgenic tobacco showed stronger tolerance to $ZnCl_2$ stress relative to transgenic control with GFP. They concluded that TaGly I might play a role in response to diverse stresses in plants.

Saxena *et al.,* (2011) studied engineering of salinity tolerance of agronomically important crop plants. Since Glyoxalase system has been shown to impart salinity tolerance in the model plant tobacco, they used the Glyoxalase-II gene for engineering salinity tolerance in an important oil yielding crop, *Brassica juncea*. In their study, the transgenic plants of *B. juncea* overexpressing the Glyoxalase-II gene showed higher salinity tolerance as compared to the untransformed control plants as observed by delayed senescence in leaf discs at 400 mM and 800 mM NaCl in T1 generation. The percentage of germination of the T2 transgenic seeds was higher at 150 mM and 200 mM NaCl as compared to the seeds of untransformed plants. For the first time, they demonstrated the applicability of utilizing the Glyoxalase-II gene for enhanced salinity tolerance in an oilseed crop plant *B. juncea.*

Using a strictly auxin-dependent soybean *(Glycine max* (L.) cell suspension, Paulus *et al.,* (1993) studied the correlation of auxin-dependent cell proliferation and the activity of Glyoxalase-I, an enzyme generally associated with cell proliferation in animal, microbial and, as reported recently, also plant systems. They found the activity of Glyoxalase-I to be modulated during the proliferation cycle, with a maximal activity between day 2 and day 4 of culture, growth. After starving the culture of auxins for three subsequent periods, both the enzyme activity and cell growth could be re-initiated with auxin. Enzyme activity reached its maximum one day before cell number was at a maximum. They purified the enzyme to homogeneity and characterized the enzyme.

Hossain *et al.,* (2009) studied the effects of various abiotic stresses on the upregulation of methylglyoxal levels and Glyoxalase-I activities in pumpkin seedlings (*Cucurbita maxima* Duch.). They reported that most of the stresses caused significant increases in methylglyoxal level and Glyoxalase-I activity. They showed that accumulation of methylglyoxal in plants under various stressful conditions is a common phenomenon, and methylglyoxal could therefore act as a signal for plants to respond to stress. They isolated, subcloned and determined nucleotide sequence a cDNA encoding Glyoxalase-I. They reported that the pumpkin Glyoxalase-I cDNA consists of 975-bp nucleotides encoding a polypeptide of 185 amino acids having a predicted molecular weight of 20,772.14 Da. Based on the number of amino acids, it

was categorized as short-type Glyoxalase-I and the nucleotide sequence of pumpkin Glyoxalase-I showed significant homology with other known Glyoxalase-I sequences of plants.

In this study, Sneh *et al.,* (2006) discussed the extended suitability of this engineering strategy for improved heavy metal tolerance in transgenic tobacco (*Nicotiana tabacum*). They found Glyoxalase transgenics were able to grow, flower, and set normal viable seeds in the presence of 5 mM $ZnCl_2$ without any yield penalty. They reported that the endogenous ion content measurements revealed roots to be the major sink for excess zinc accumulation, with negligible amounts in seeds in transgenic plants. Their investigations indicated restricted methylglyoxal accumulation and less lipid peroxidation under high zinc conditions in transgenic plants. They finally reported that an increase in the level of phytochelatins and maintenance of glutathione homeostasis in transgenic plants during exposure to excess zinc as the possible mechanism behind this tolerance.

Kaur *et al.,* (2014) discussed the suitability of Glyoxalases and methylglyoxal as potential markers for stress tolerance. Methylglyoxal initiates stress-induced signaling cascade via reactive oxygen species, resulting in the modifications of proteins involved in various signal transduction pathways, that eventually culminates in cell death or growth arrest. The associated mechanism of tolerance conferred by over-expression of methylglyoxal-detoxifying Glyoxalase pathway mainly involves lowering of methylglyoxal levels, thereby reducing subsequently induced cellular toxicity. They stated in this review about the multiple stress-inducible nature of these enzymes play a vital role for Glyoxalases, associating them with plant defense mechanisms. In this context, they summarized available transcriptome, proteome and genetic engineering-based reports in order to highlight the involvement of Glyoxalases as important components of plant stress response.

Uotila *et al.,* (1975) purified Glyoxalase-I from sheep liver by a procedure which includes ammonium sulfate and polyethylene glycol fractionations and column chromatographies on hydroxyapatite, Cibacron Blue - Sephadex G-100 and DEAE-cellulose. They reported that the specific activity of the homogeneous preparations was about 4000 units/mg of protein. Three separate peaks of activity were obtained in the last column on DEAE-cellulose. They did not find any signs of heterogeneity in the previous steps. Purified but not crude preparations gave two activity peaks on disc gel electrophoresis.

Tommasini *et al.,* (2003) assessed the potential of multiplex SSR markers for testing distinctness, uniformity and stability of rape (*Brassica napus* L.) varieties and developed three multiplex SSR sets composed of five markers each. These were used to measure the extent of diversity within and between the varieties using fluorescence based semi-automated detection technology. They also evaluated the significance of any correlation between SSRs, pedigree and five of the morphological characters currently used for statutory distinctness, uniformity and stability testing of rape varieties. They found no significant correlation between SSR and morphological data. However, genetic distances measured by SSRs were correlated to pedigree. They suggested that SSRs could be used for pre-screening or grouping of existing and candidate varieties, allowing the number of varieties that need to be grown for comparison to be reduced. Multiplex SSR sets are a promising way forward for complementing the current variety testing system in *B. napus*.

Chakravarthi *et al.*, (2006) investigated the genetic diversity and DNA fingerprinting of 15 elite rice genotypes using 30 SSR primers on chromosome numbers 7-12. The results revealed that all the primers showed distinct polymorphism among the cultivars studied indicating the robust nature of microsatellites in revealing polymorphism. They suggested that the larger range of similarity values for related cultivars using microsatellites provides greater confidence for the assessment of genetic diversity and relationships. The information obtained from the DNA fingerprinting studies helps to distinctly identify and Characterize 9 varieties using 18 different RM primers.

Hasan *et al.*,(2006) examined Genetic diversity throughout the rapeseed (*Brassica napus*) primary gene pool by obtaining detailed molecular genetic information at simple sequence repeat (SSR) loci for a broad range of winter and spring oilseed, fodder and leaf rape gene bank accessions. A set of 96 genotypes was characterized using publicly available mapped SSR markers spread over the *B. napus* genome. Allelic information from 30 SSR primer combinations amplifying 220 alleles at 51 polymorphic loci provided unique genetic fingerprints for all genotypes. UPGMA clustering enabled identification of four general groups with increasing genetic diversity as follows (1) spring oilseed and fodder; (2) winter oilseed; (3) winter fodder; (4) vegetable genotypes. The most extreme allelic variation was observed in a spring kale from the United Kingdom and a Japanese spring vegetable genotype, and two winter rape accessions from Korea and Japan, respectively. Unexpectedly the

next most distinct genotypes were two old winter oilseed varieties from Germany and Ukraine, respectively. The molecular genetic information gained enables the identification of untapped genetic variability for rapeseed breeding and is potentially interesting with respect to increasing heterosis in oilseed rape hybrids.

Guang *et al.,* (2006) studied the genetic diversity of 43 sources of upland cotton germplasm with different parental origins, breeding periods, and ecological growing areas in China on the basis of simple sequence repeat (SSR) markers. They detected a total of 130 gene alleles with 80% polymorphism from 36 SSR primers. The number of alleles per primer ranged from two to eight with an average of 3.6 and the polymorphism information content (*PIC*) range was 0.278-0.865, with an average of 0.62. The average genotype diversity index (*H*) was 1.102, the highest was 2.039 and the lowest was 0.451. The average coefficient of the genetic similarity of SSR markers among source germplasm was 0.610, ranging from 0.409 to 0.865.

Lian *et al.,* (2006) described an approach for developing codominant polymorphic markers (compound microsatellite (SSR) markers), with substantial time and cost savings. In this technique, fragments flanked by a compound SSR sequence at one end were amplified from the constructed DNA library using compound SSR primer$(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adaptor primer for the suppression-PCR. A locus-specific primer was designed from the sequence flanking the compound SSR. The primer pairs of the locus-specific and compound SSR primers were used as a compound SSR marker. Because only one locus-specific primer was needed for design of each marker and only a common compound SSR primer was needed as the fluorescence-labeled primer for analyzing all the compound SSR markers, substantially reducing the cost of developing co-dominant markers and analyzing their polymorphism. They demonstrated this technique for *Dendropanax trifidus* and easily developed 11 codominant markers with high polymorphism for *D. trifidus*. They suggested that use of the technique for successful isolation of codominant compound SSR markers for several other plant species will be possible.

Hoque *et al.,* (2012) reported that under environmental stresses plants accumulates methylglyoxal. Being a reactive oxoaldehyde, MG may act as a signaling molecule in plants during stresses. They investigated whether MG induces stomatal closure, reactive oxygen species (ROS) production, and cytosolic free calcium concentration ([Ca2+]cyt) to clarify roles of MG in Arabidopsis guard cells. They also reported that

the MG induced production of ROS and [Ca2+]cyt oscillations, leading to stomatal closure.

Yadav *et al.*, (2005) studied the level of methylglyoxal (MG) in various tobacco plant species by using the substrate Glyoxalase-I and glutathione which has not been reported earlier. Methylglyoxal (MG), a cytotoxic by-product produced mainly from triose phosphates, is used as a substrate by Glyoxalase-I. They showed that MG concentration varies in the range of 30–75 μM in various plant species and it increases 2 to 6 fold in response to salinity, drought, and cold stress conditions. Transgenic tobacco under expressing Glyoxalase enhanced accumulation of MG which resulted in the inhibition of seed germination. In the Glyoxalase-I overexpressing transgenic tobacco, MG levels did not increase in response to stress compared to the untransformed plants, however, with the addition of exogenous GSH there was a decrease in MG levels in WT to 50% both untransformed and transgenic plants whereas in the transgenic plants a 5-fold decrease was observed. Finally they demonstrated an important role of Glyoxalase-I along with GSH concentration in maintaining MG levels in plants under normal and abiotic stress.

Chang *et al.*, (2004) was investigated that methylglyoxal (MG) causes the generation of nitric oxide (NO) and superoxide anion (O_2 S^-), leading to peroxynitrite ($ONOO^-$) formation in VSMCs methylglyoxal (MG) is a metabolite of glucose. Cultured rat thoracic aortic SMCs (A-10) were treated with MG or other different agents. Oxidized DCF, reflecting H_2O_2 and $ONOO^-$ production, was significantly increased in a concentration- and time-dependent manner after the treatment of SMCs with MG (3–300 AM) for 45 min–18 h (n=12). MG increased oxidized DCF was effectively blocked by reduced glutathione or N-acetyl-L-cysteine, as well as L-NAME (p b 0.05, n = 12). Both O_2 S^- scavenger SOD and NADPH oxidase inhibitor DPI significantly decreased MG-induced oxidized DCF formation. MG significantly and concentration-dependently increased NO and O_2 S^- generation in A-10 cells, which was significantly inhibited by L-NAME and SOD or DPI, respectively. In conclusion, MG induces significant generation of NO and O_2 S^- in rat VSMCs, which in turn causes $ONOO^-$formation. An elevated MG level and the consequential ROS/RNS generation would alter cellular signaling pathways, contributing to the development of different insulin resistance states such as diabetes or hypertension.

In a review paper Distler (2012) discussed about the Glyoxalase-I and methylglyoxal and their effect on animal bodies. He found that Glyoxalase-I is a ubiquitous in nature and participate in the process of detoxification of MG which is cytotoxic in nature and produced under oxidative stress. He reported that MG level increases with an increase in glucose level in blood.

Takatsume *et al.,* (2006) found that MG activates transcription factors such as Yap1 and Msn2, and triggers aHog1 mitogen-activated protein kinase cascade in

Saccharomyces cerevisiae. Regarding the activation of Hog1 by MG, they found that Sln1, an osmosensor possessing histidine kinase activity, functions as a sensor of MG. To gain further insight into the role of MG as a signal initiator, they analyzed the response of *Schizosaccharomyces pombe* to extracellular MG. Finally they suggested that *S.pombe* has an alternative module(s) that directs the MG signal to the SAPK pathway via Mcs4. Additionally, they found that the transcription factor Pap1 is concentrated in the nucleus in response to MG, independent of the Spc1-SAPK pathway.

Kalapos (1999) reviewed some important characteristics of methylglyoxal metabolism and toxicity in a wide variety of species, and emphasizing the action of methylglyoxal on energy production, free radical generation and cell killing. He emphasized on the discussion of α-oxoaldehyde production in the environment as a potential risk factor and to the possible role of this α –dicarbonyl in diseases. At the conclusion, he stated that since the early stage of evolution the function of methylglyoxalase pathway has been related to carbohydrate metabolism, but its significance has been changed over the thousands of years. Namely, at the beginning of evolution methylglyoxalase path was essential for the reductive citric acid cycle as an anaplerotic route, while in the extant metabolism it concerns with the detoxification of methylglyoxal and plays some regulatory role in triose-phosphate household.

Being a reactive α-oxoaldehyde, methylglyoxal (MG) may act as a signaling molecule in plants during stresses Hoque *et al*., (2012) investigated whether MG induces stomatal closure, reactive oxygen species (ROS) production, and cytosolic free calcium concentration ([Ca2+]cyt) to clarify roles of MG in Arabidopsis guard cells. MG induced production of ROS and [Ca2+]cyt oscillations, leading to stomatal closure. The MG-induced stomatal closure and ROS production were completely inhibited by a peroxidase inhibitor, salicyl hydroxamic acid (SHAM). Based on the results they suggested that intrinsic metabolite MG can induce stomatal closure in

Arabidopsis accompanied by extracellular ROS production mediated by SHAM-sensitive peroxidases, intracellular ROS accumulation, and [Ca2+] cyt oscillations. Abbas *et al.,* (2009) studied the genetic diversity of Brassica to improve yield and quality of oil content of *Brassica*. In that experiment, on an average, 45.8 and 25.8 alleles were amplified using RAPD and *Brassica* specific SSR primer sets, respectively. They estimated mean genetic distance ranged from 26-89% and 5-61%, respectively. Size of scorable fragments ranged from approximately 250 to >2000 bp. A high level of genetic dissimilarity (GD=up to 100%) was estimated among the 14 genotypes. Entries were grouped in clusters using cluster analysis. On the basis of dendrogram, they identified most diverse genotypes that can be used in future brassica breeding program.

Turi *et al.*, (2012) characterized the Genetic diversity among 120 different accessions of *Brassica* species with the help of SSR markers. These species include *Brassica rapa, B. juncea* and *B. napus*. Using 39 SSR primers and they produced 162 scorable bands in which 105 were polymorphic. They found the average rate of polymorphic loci was 46%, which indicates high genetic diversity among the accessions. The UPGMA cluster analysis revealed two main clusters and nine sub-clusters. Based on their study, they suggested that SSR analysis proved to be a useful tool in assessing the genetic diversity of leafy Brassica germplasm.

As methylglyoxal (MG) is one of the aldehydes that accumulate in plants under environmental stress and Glutathione S-transferases (GSTs) play important roles, including detoxification, in the stress tolerance systems of plants. To determine the effects of MG, Hoque *et al.,* (2006) characterized recombinant GST. They reported that MG decreased GST activity and thiol contents with increasing *Km*. They suggest that GST can serve as a target of MG modification, which is suppressed by application of reduced glutathione.

To identify biochemical markers for salt tolerance, El-Shabrawi *et al.,* (2010) analyzed two contrasting cultivars of rice (*Oryza sativa L.*) differing in salt tolerance for various parameters. They found Pokkali, a salt-tolerant cultivar, showed considerably lower level of H_2O_2 as compared to IR64, a sensitive cultivar. Enzyme activities and the isoenzyme pattern of antioxidant enzymes also showed higher activity of different types and forms in Pokkali as compared to IR64, suggesting that Pokkali possesses a more efficient antioxidant defense system to cope up with salt-induced oxidative stress. Further, Pokkali exhibited a higher GSH/GSSG ratio along

with a higher ratio of reduced ascorbate/oxidized ascorbate as compared to IR64 under NaCl stress. Finally they suggested that both ascorbate and glutathione homeostasis, modulated also via Glyoxalase enzymes, can be considered as biomarkers for salt tolerance in Pokkali rice. In addition, status of reactive oxygen species and oxidative DNA damage can serve as a quick and sensitive biomarker for screening against salt and other abiotic stresses in crop plants.

To understand the protective mechanisms of proline and glycinebetaine (betaine) against NaCl stress, Banu *et al.,* (2010) investigated intracellular levels of hydrogen peroxide (H_2O_2), superoxide (O_2^-), NO, and MG in tobacco Bright Yellow-2 cells. They found higher Levels of H_2O_2, O_2^-, NO and MG in the short-term and long-term NaCl-stressed cells than in the non-stressed cells, whereas the O_2^- level was higher in the long-term stressed cells and also found that exogenous proline and betaine decreased the H_2O_2 level in both the short-term and the long-term NaCl-stressed cells and the MG level in the long-term NaCl-stressed cells.

Hoque *et al.,* (2008) investigated tobacco Bright Yellow-2 cells grown in suspension culture to assess the protection offered by proline and glycinebetaine against salt stress. Salt stress increased the activity of glutathione peroxidase and Glyoxalase-I enzymes involved in the ROS and MG detoxification systems. Exogenous application of proline or glycinebetaine resulted in a reduction of protein carbonylation, and in an increase in glutathione redox state and activity of glutathione peroxidase, glutathione *S*-transferase and Glyoxalase-I under salt stress. They suggested that both proline and glycinebetaine provide a protective action against NaCl-induced oxidative damage by reducing protein carbonylation, and enhancing antioxidant defense and MG detoxification systems.

Inoue *et al*., (2011) reviewed that the budding yeast has the sole gene (GLO1) encoding the structural gene for Glyoxalase-I. This yeast has two isoforms of Glyoxalase-I I encoded by GLO2 and GLO4. They found that the expression of GLO1in *Saccharomyces cerevisiae* is regulated by Hog1 mitogen-activated protein kinase and Msn2/Msn4 transcription factors under highly osmotic stress conditions. Thus the physiological significance of GLO1 expression in response to osmotic stress is to combat the increase in the levels of methylglyoxal in cells during the production of glycerol as a compatible osmolyte. Deficiency in GLO1 in *S. cerevisiae* causes pleiotropic phenotypes in terms of stress response, because the steady state level of

methylglyoxal increases in glo1 cells thereby constitutively activating Yap1 transcription factor and Yap1 is crucial for oxidative stress response, although methylglyoxal per se does not enhance the intracellular oxidation level in yeast, but it directly modifies cysteine residues of Yap1 that are critical for the nucleocytoplasmic localization of this b-ZIP transcription factor. Finally, they concluded that Glyoxalase-I can be defined as a negative regulator of Yap1 through modulating the intracellular methylglyoxal level.

Inoue *et al*., (1996) identified the structural gene for Glyoxalase-I (*GLO1*) of *Saccharomyces cerevisiae*. The *GLO1* gene contained an open reading frame with 326 amino acids, and the molecular weight of the gene product (Glo1p) deduced from the DNA sequence was 37,207.06. They reported that *GLO1* gene containing species showed increased Glyoxalase-I activity approximately 95-fold and increased resistance against methylglyoxal. They suggested that purified Glyoxalase-I from yeast could use g-glutamyl cysteine as a substrate (*k*cat/*Km* 5 1.89 3 107 M21 s21, glutathione; 3.47 3 104 M21 s21, g-glutamylcysteine).

Clugston *et al.*, (1997) isolated the Glyoxalase-I gene (*gloA*) from *Salmonella typhimurium* in *Escherichia coli* on a multi-copy pBR322-derived plasmid, selecting for resistance to 3 mM methylglyoxal on Luria-Bertani agar. They sequenced the region of the plasmid which confers the methylglyoxal resistance in *E. coli*. Comparing to the known sequences of the *Homo sapiens* and *Pseudomonas putida* Glyoxalase-I (GlxI) enzymes sequence, they found strong homology. They presented the clustal alignments of the sequences, indicating possible Zn^{2+} ligands and active site regions. Suggesting that the structures of the yeast enzymes are those of fused dimers, they concluded that the *S. typhimurium* sequence aligns with both the N-terminal half and the C-terminal half of the proposed GlxI sequences from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

Rhee *et al.,* (1988) determined the nucleotide sequence of the Glyoxalase-I gene of *Pseudomonas putida* IFO 3738.They calculated molecular weight of 18,442 for a polypeptide of 164-amino acids encoded by the Glyoxalase-I gene, which was closely similar to that obtained with purified Glyoxalase-I (M.W. 19,500) from *P. putida.* The gene for Glyoxalase-I was expressed in Escherichia coli cells. Comparison of the nucleotide sequence of the gene with the N-terminal amino acid sequence of the enzyme synthesized in *E. coli* cells, they revealed that the N-terminal methionine residue was removed after translation, possibly by methionine

aminopeptidase in *E. coli*. They reported that Glyoxalase-I contained zinc atom per enzyme and its pi was 4.0.

Cooper *et al*., (1970) reviewed that methylglyoxal was once believed to be involved in glucose catabolism as a component of the so-called non-phosphorylating glycolysis

.When phosphorrylated compounds were identified as intermediates in glucose breakdown the formation of methylglyoxal was considered to be due to non-enzymic side-reactions and thus of little or no importance. They showed that *E. coil* contains enzymes which convert dihydroxyacetone phosphate (DHAP) to pyruvate via methylglyoxal and D-lactate. They suggested this sequence provides a by-pass of the normal glycolytic reactions for the conversion of DHAP to pyruvate and as such represents a combination of the phosphorylated and non-phosphorylated pathways of glucose breakdown.

Wild *et al*., (2012) considering methylglyoxal (MG) concentrations as an indicator of various health impairments including complications of diabetes in vivo is gaining increasing importance as high it is necessary to precisely determine the concentration of MG stock solutions used as analytical standards. The "gold standard" method where purified Glyoxalase-I is required thus this method is expensive. Another method uses a derivation reaction with 2,4-dinitrophenylhydrazine, but this substance is explosive and needs special handling and storage. They reported a new method of MG determination based on the previously published fast reaction between MG and N-acetyl-L-cysteine at room temperature which yields an easily detectable condensation product, N-α-acetyl-S-cysteine. They suggested that the N acetyl- L-cysteine assay is the most favorable, providing an economical and robust assay without the need for the use of hazardous or expensive reagents.

Hasanuzzaman *et al*., (2011a) investigated the possible regulatory role of exogenous nitric oxide NO) in antioxidant defense and methylglyoxal (MG) detoxification systems of wheat seedlings exposed to salt stress (150 and300 mM NaCl, 4 days). They found that Glyoxalase-I (Gly-I), and Glyoxalase-II (Gly-II) activities decreased upon the imposition of salt stress, especially at 300 mM NaCl. Their study reveal that NO pre-treatment had a synergistic effect; that is, the pre-treatment increased the AsA and GSH content and the GSH/GSSG ratio, as well as the activities of MDHAR, DHAR, GR, GST, GPX, Gly-I, and Gly-II in most of the seedlings subjected to salt stress. They suggested that the exogenous application of NO rendered the plants more

tolerant to salinity-induced oxidative damage by enhancing their antioxidant defense and MG detoxification systems.

Hasanuzzaman *et al*., (2011b) investigated the possible regulatory role of selenium (Se) in relation to the changes in ascorbate (AsA) glutathione (GSH) levels and to the activities of antioxidant and Glyoxalase pathway enzymes, rapeseed (*Brassica napus*) seedlings. They observed that Glyoxalase-I (Gly-I) activity significantly increased under any level of drought stress, while catalase (CAT) and Glyoxalase-II (Gly-II) activity decreased. They reported that Se-pretreated seedlings exposed to drought stress showed a rise in AsA and GSH content, maintained a high GSH/GSSG ratio, and evidenced increased activities of, CAT, Gly-I, and Gly-II as compared with the drought-stressed plants without Se. They suggested that the exogenous application of Se increased the tolerance of the plants to drought-induced oxidative damage by enhancing their antioxidant defense and methylglyoxal detoxification systems.

Kumar *et al.,* (2013) reported the efficacy of an aldose reductase (ALDRXV4) enzyme from *Xerophyta viscosa* Baker in enhancing the prospects of plant‟s survival under abiotic stress. Transgenic tobacco plants overexpressing ALDRXV4 cDNA showed alleviation of NaCl and mannitol induced abiotic stress. They observed that the transgenic plants survived longer periods of drought and salinity stress as compared to the wild type plants. The increased synthesis of aldose reductase in transgenic plants correlated with reduced methylglyoxal and malondialdehyde accumulation and an elevated level of sorbitol under stress conditions. In addition, the transgenic lines showed better photosynthetic efficiency, less electrolyte damage, greater water retention, higher proline accumulation, and favorable ionic balance under stress conditions. They suggested that the potential of engineering aldose reductase levels for better performance of crop plants growing under drought and salt stress conditions.

Upadhyaya *et al*., (2011) studied salt-tolerance in transgenic potato. It was conferred by overexpression of ascorbate pathway enzyme. They reported the comprehensive aptness of this engineering approach for enhanced salt tolerance in transgenic potato (Solanum tuberosum L.cv. Taedong Valley). Potatoes overexpressing GalUR grew and tuberized in continuous presence of 200 mM of NaCl. They found the transgenic plants maintained a higher reduced to oxidized glutathione (GSH: GSSG) ratio together with enhanced activity of glutathione dependent antioxidative and Glyoxalase enzymes under salinity stress. They observed transgenes resisted an

increase in methylglyoxal that increased radically in untransformed control plants under salinity stress. They reported firstly of genetic engineering of ascorbate pathway gene in maintaining higher level of GSH homeostasis along with higher Glyoxalase activity inhibiting the accumulation in methylglyoxal (a potent cytotoxic compound) under salt stress. They suggested the engineering of ascorbate pathway enzymes as a major step towards developing salinity tolerant crop plants.

Sommer *et al*., (2001) cloned and characterized the cDNA for Glyoxalase-I from the filarial nematode *Onchocerca volvulus* (designated *O*v-GloI). They isolated cDNA which contained an open reading frame of 579 bp encoding a protein with a calculated molecular mass of 21930 Da. Owing to the high degree of sequence identity (60%) with human Glyoxalase-I, they built a three-dimensional model of *O*v-GloI. They obtained a 22 kDa protein by heterologous expression in *Escherichia coli*. They also obtained a homogeneous enzyme preparation by affinity purification and functional characterization of the recombinant enzyme included the determination of kinetic constants for methylglyoxal and phenylglyoxal as well as inhibition studies. Gel filtration demonstrated a dimeric structure. By using a semi-quantitative PCR ELISA, they observed that *O*v-GloI was expressed at elevated levels under conditions of oxidative stress.

Kumar *et al*., (2009) studied the influence of proline and betaine exposure on antioxidant and methylglyoxal (MG) detoxification system during cold stress in

Camellia sinensis (L.). Cold stress enhanced MG and lipid peroxidation levels in tea bud (youngest topmost leaf). They found that MG increase was resisted up on the exposure of tea bud to proline and betaine. They further studied on Glyoxalase pathway enzymes that are involved in MG detoxification and comprise of two enzymes Glyoxalase-I and Glyoxalase-II. Both proline and betaine showed protective effect on Glyoxalase-I and activating effect on Glyoxalase-II during cold stress in tea bud. Based on this study they suggested that proline and betaine might provide protection to cold stress in tea by regulating MG and lipid peroxidation formation as well as by activating or protecting some of antioxidant and Glyoxalase pathway enzymes.

Hasanuzzaman *et al*., (2014) investigated the roles of exogenous proline (Pro, 5mM) and glycinebetaine (GB, 5mM) in improving salt stress tolerance in salt sensitive (BRRI dhan49) and salt tolerant (BRRI dhan54) rice (*Oryza sativa* L.) varieties. They reported that salt stresses increased glutathione peroxidase (GPX), catalase (CAT) and

Glyoxalase-I (Gly-I) activities were reduced in sensitive variety and these were increased in tolerant variety due to salt stress. They found that the Glyoxalase-II (Gly II), glutathione S-transferase (GST) and superoxide dismutase (SOD) activities were increased in both cultivars by salt stress. Exogenous Pro and GB application with salt stress improved physiological parameters and reduced oxidative damage in both cultivars where BRRI dhan54 showed better tolerance. They suggested that exogenous application of Pro and GB increased rice seedlings‟ tolerance to salt-induced oxidative damage by upregulating their antioxidant defense system where these protectants rendered better performance to BRRI dhan54 and Pro can be considered as better protectant than GB.

Hossain *et al.*, (2013) examined the potential biochemical mechanisms of hydrogen peroxide pre-treatment-induced drought tolerance in Brassica (*Brassica juncea* L.) seedlings by analyzing numerous vital components of methylglyoxal and reactive oxygen species detoxification systems. They pre-treated eight-day-old seedlings with low concentration (50 μM) of hydrogen peroxide for 24 h prior to the imposition of drought stress (20% PEG-6000) for 48 h. They observed a declination in the activities of, catalase and Glyoxalase-II were observed in response to drought stress whereas, glutathione peroxidase and Glyoxalase-I activities significantly increased. Surprisingly, hydrogen peroxide pre-treated drought-stressed seedlings maintained a significantly higher, glutathione reductase, catalase, glutathione *S*-trasnferase, and Glyoxalase-II activities when compared with the seedlings subjected to drought stress without hydrogen peroxide pre-treatment. They suggested that hydrogen peroxide primed a defense response in the seedlings that could trigger the activation of both ROS and MG detoxification pathways.

Bartling *et al.*, (1993) isolated a full-length cDNA clone for a novel glutathione *S*-transferase from *A. thaliana* and characterized it. The cDNA encoded a polypeptide of 218 amino acids with a calculated molecular mass of 24,363 Da. They reported that the enzyme function as glutathione peroxidase and showed that it efficiently converts the natural substrates 13-hydroperoxy-9,11-octadecadienoic acid (13-hydroperoxylinoleic acid) as well as 13-hydroperoxy-9,11,15-octadecatrienoic acid (13-hydroperoxylinolenic acid) into the corresponding hydroxy-acids with concomitant production of GSSG. On the basis of results, they proposed that glutathione *S*-transferases may have evolved as part of the system protecting the cell from oxygen toxicity.

Dean *et al.*, (1995) purified a glutathione *S*-transferase from corn with activity toward *ρ*-coumaric acid and other unsaturated phenyl propanoids. They reported that the native enzyme is a monomer with a molecular mass of approximately 30 kDa and an apparent isoelectric point at pH 5.2. The enzyme had a pH optimum between 7.5 and 8.0 and apparent *K*m values of 4.4 and 1.9 mM for GSH and *ρ*-coumaric acid, respectively.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental site

This study was carried out in the Molecular Breeding Laboratory of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur during the period from March, 2014 to March, 2015.

3.2 Plant materials

Ten plant materials of *Brassica*, BD7114 (*B. rapa*) (E1), BD 7115 (*B. rapa*) (E2), BD7014 (*B. juncea*) (E3), BD6757 (*B. juncea*) (E4), Tori 7 (*B. rapa*) (E5), BARI sarisha 16 (*B. juncea*) (E6), BD10108 (*B. napus*) (E7), BARI sarisha 13 (*B. napus*) (E8), JBC05117 (*B. rapa*) (E9), and BD6950 (*B. juncea*) (E10) were used for this study. Seedlings of E5 and E6 were used to investigate Gly-I and Gly-II response under abiotic stress. Finally, Gly-I purified from leaf of E6 seedlings.

3.3 Sources of plant materials

All plant materials were collected from the field laboratory of Oil seed Research Centre, BARI.

3.4 Chemicals

All of the chemicals used in this study are listed in Appendix- I.

3.5 Stress treatments

Seeds of E5 and E6 were grown in pot under greenhouse conditions. Five day old seedlings were subjected to impose salinity and drought stresses.

Five day old seedlings were treated with NaCl solution to induce saline stress. The salinity level 16 dS/m was arisen by adding saline water prepared by NaCl, and the concentration of salinity in soil was measured by electrical conductivity meter (H993300). For all the treatments, one set of control was maintained. After arising the salinity level seedlings were observed for 2 day, 4 day and 6 day. For drought stress, the plants were kept without watering to induce drought stress. Data were taken after 2, 4 and 4 days of stress implementation. In case of visual symptom study, stress was implemented in 15 days old seedlings.

3.6 Preparation of soluble protein

To assay the activity level of Glyoxalase in seedlings, five gram of sample was homogenized in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10 % (w/v) glycerol with mortar and pestle. The homogenates were centrifuged at 11,500 x *g* for 10 min and the supernatant was used as a soluble protein solutions.

3.7 Gly-I assay

Gly-I assay was carried out according to the method of Hossain *et al*., (2009) with slight modification. Briefly, the assay mixture contained 100mM sodium phosphate buffer (pH 7.5), 15 mM magnesium sulphate, 1.7 mM glutathione and 3.5 mM methylglyoxal. The reaction was started by the addition of enzyme solution. The formation of thioester was measured by observing absorption at 240 nm for 1min. The activity was calculated using the extinction coefficient of 2.37 $mM^{-1}cm^{-1}$.

3.8 Gly-II assay

Glyoxalase-II (EC: 3.1.2.6) activity was determined according to the method of Hasanuzzaman *et al*., (2011b) by monitoring the formation of reduced glutathione (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 $mM^{-1}cm^{-1}$.

3.9 Sample preparation for MG estimation

About 0.3 g leaf tissue was extracted in 3 ml of 5% perchloric acid. After incubating for 15 min on ice, the mixture was centrifuged at 4 °C at 11,000×*g* for 10 min. The colored supernatant was decolorized by adding charcoal (10 mg/ml), kept for 15 min at room temperature, and centrifuged at 11,000×*g* for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000×*g* for 10 min. Neutralized supernatant was used for MG estimation.

3.10 MG assay

An aqueous 500 mM N-acetyl-L-cysteine solution was freshly prepared in 100 mM sodium dihydrogen phosphate buffer (adjusted to pH 7.0 with 10 M NaOH) at 25 °C.

The reaction was started by adding 20 µl of the N-acetyl-L-cysteine solution (final concentration up to 10 mM) in 980 µl neutralized solution, and the formation of the product N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine was recorded at a wave length of 288 nm. Data was calculated with standard curve of MG solutions in sodium dihydrogen phosphate.

3.11 Preparation of crude protein for Gly-I purification

3.11.1 Preparation of crude protein

Fifty gram fresh leaves were homogenized in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10 % (w/v) glycerol with a waring blender. The homogenates squeezed in a nylon cloth and was centrifuged at 11,500×*g* for 10 min and the supernatant was used as a crude enzyme solution.

3.12 Purification of Gly-I

3.12.1 Anion exchange chromatography-1

Protein in the soluble protein solution was precipitated with ammonium sulfate at 65% saturation. The proteins were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) that contained 0.01% (v/v) β-mercaptoethanol and 1 mM EDTA (buffer A) overnight. The dialyzate was applied to a column (1.77cm i.d. × 20 cm) of DEAE-cellulose (DE-52; Whatman, Kent, UK) that had been equilibrated with buffer A. The column was washed with buffer A, and eluted with a linear gradient of 0 to 0.2 M KCl in 800 ml of buffer A. High active fractions of 5.0 ml were collected. The fractions corresponding to the high Gly-I active peaks were combined as the Gly-I pool for further purification.

3.12.2 Anion exchange chromatography-2

The Gly-I pool was subjected to pass through another DEAE-cellulose anion exchange chromatography (1.77 cm i.d. × 10 cm) and eluted with potassium-phosphate buffer (K-P buffer) 0-2 mM KCl gradient solution. Fractions of 5 ml high

active fractions were collected and activity and absorbance (A_{280}) were measured spectrophotometrically.

3.12.3 Affinity Chromatography

The high active Gly-I peak of anion exchange chromatography-2 were applied to a column (0.76 cm i.d. ×4.0 cm) of *S*-hexylglutathione-agarose (Sigma, St. Louis, MO) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.01% (v/v) β-mercaptoethanol (buffer B). The column was washed with buffer B containing 0.2 M KCl and eluted with buffer B containing 1.2 mM *S*-hexylglutathione. Fractions of 2.5 ml were collected. Protein fractions eluted with Gly-I were combined and dialyzed against buffer B. The dialysate was used as the purified Gly-I solution.

3.13 Assay of enzymatic activities and protein quantification

Gly-I activity assay was carried out according to method mentioned in Hasanuzzaman *et al*., (2011). 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 µl. 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 2.37 $mM^{-1}cm^{-1}$.The protein concentration of each sample was determined by the method of Bradford (1976) using BSA as protein standard.

3.14 SDS-PAGE and Silver Staining

To check the homogeneity of the purified enzyme and to estimate its molecular mass SDS-PAGE was done in 12.5%(w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970) followed by silver staining.

Silver staining was done as follows:

1. After electrophoresis, the gel was dipped into 50% ethanol in a plastic box and was shaken gently overnight at room temperature
2. Following morning, ethanol solution was changed twice with an interval of 2 hours and 1 hour
3. Gel was transferred to freshly made 67.3 ml 46.36 mM silver nitrate solution containing 0.83% NH_3 and 0.019 N NaOH and treated with gentle shaking for 15 minutes at room temperature

4. After removing silver nitrate solution, distilled water was added to the tray containing gel and was shaken gently for 5 minutes. Within this time, distilled water was changed once

5. Gel was transferred to freshly made 500 ml color developing solution [0.26 mM citric acid anhydrous and 0.019% (v/v) formaldehyde] in the second tray and was shaken gently. Shaking was continued until protein bands appeared clearly

6. Gel was transferred to a box containing 50% ethanol and was shaken gently for several hours at room temperature

7. Gel was incubated in distilled water for 30 minutes with gentle shaking

8. To maintain a permanent gel record, the gel was taken in between two transparent OHP sheet and scanned.

3.15 CBB Staining

The gel was shacked in concentrated CBB solution overnight. In next day, the gel was destained and scanned.

3.16 Measurement of Molecular Weight

The Molecular weight was measure by gel documentation system (Alpha-Inotech)

3.17 Data Analysis

Data generated from this study were analyzed by STATISTIX 10 software where need. The graphs were prepared in MS Excel, 2010. Mean values ± standard errors (SE) were presented in graphs from at least three independent experiments. $P<0.05$ was considered as significance level.

3.18 DNA extraction

Seeds of the entries were obtained from BARI. Genomic DNA was isolated following protocol described by Saghai-Maroof *et al*. (1984) with some modifications. Juvenile leaves (unfolded) of 5 days old plants were used in genomic DNA isolation. Leaf tissues were cut into small pieces, homogenized and digested with extraction buffer 262 (pH= 8.0): 50 mM Tris-HCl, 25 mM EDTA (Ethylene diamine tetra acetic acid), 300 mM NaCl and TEN buffer + 5% SDS (Sodium Dodecyl Sulfate) +10% PVP (Poly Vinyl Pyrolideone) +20% CTAB (Cetyl Trimethyl Ammonium Bromide). After incubation for 20 minutes at 65°C

with intermittent swirling, the mixture was emulsified with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v/v). DNA was precipitated using two volume of absolute alcohol in presence of 0.3 M sodium acetate and pelleted by centrifugation. The pellets were then washed with 70% ethanol, air dried and resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0) and finally treated with 2 µl of RNAse. Quality and quantity of DNA were controlled via gel electrophoresis and spectrophotometer, repectively (SpectronicGenesis™, Spectronic Instruments Inc., USA).

3.19 Microsatellite Markers and PCR amplification

A set of ten microsatellite loci (Ra2-A11, Ra2-B02 , Ra2-D04, Ra2-E03, Ra2-E07, Ra2-E11, Ra2-E12, Ra2-F11, Ra2-G09) (see table.1) have been selected from the literature cited by Rungis *et al.* (2005) and Liu *et al.* (2006) to determine the potential of these markers for variety identification. Finally five primers, Ra2-A11, Ra2-D04, Ra2-E03, Ra2-E12 and Ra2-F11were selected based on their performance for SSR data analysis. Polymerase Chain Reactions were done in a volume of 10 µl containing 10 x PCR Buffer, 0.25 mM each of the dNTPs, 1 µM of each of primer, 1 unit ampli *Taq* DNA polymerase, 50 ng template DNA. Amplification were carried out in oil free thermal cycler (Thermal cycler gradient, Eppendorf) with the following thermal profile: initial denaturation step at 94^{O}C for 3 min followed by 35 cycles at 95^{O}C for 1 min, 57^{O}C for 45 sec, and 72^{O}C for 1.5 min and a final cycle at 70^{O}C for 7 min. PCR was the confirmed by electrophoresis on 2% agarose gel.

Table 1. List of SSR Primer of *Brassica* spp.

Locus code	Forward	Reverse
Ra2-A11	GACCTATTTTAATATGCTGTTTTACG	ACCTCACCGGAGAGAAATCC
Ra2-B02	GATGGTTTTTCGTTTTCACG	TCAGCTGTCACGTCTTGTCG
Ra2-D04	TGGATTCTCTTTACACACGCC	CAAACCAAAATGTGTGAAGCC
Ra2-E03	AGGTAGGCCCATCTCTCTCC	CCAAAACTTGCTCAAAACCC
Ra2-E07	ATTGCTGAGATTGGCTCAGG	CCTACACTTGCGATCTTCACC
Ra2-E11	GGAGCCAGGAGAGAAGAAGG	CCCAAAACTTCCAAGAAAAGC
Ra2-E12	TGTCAGTGTGTCCACTTCGC	AAGAGAAACCCAATAAAGTAGAACC
Ra2-F11	TGAAACTAGGGTTTCCAGCC	CTTCACCATGGTTTTGTCCC
Ra2-G09	ACAGCAAGGATGTGTTGACG	GATGAGCCTCTGGTTCAAGC
Ra2-H06	GAATTCAGAGGTATCTACACGGC	TAACAAAGACCCTGCGTTCC

3.20 Agarose gel electrophoresis

Agarose gel electrophoresis was performed in a Tris acetate buffer containing 40 mM Tris, 20 mM acetic acid, and 2 mM Na_2EDTA (pH 8.1). Gels contained 0.6% agarose, and electrophoresis was performed at 100 V (3.6 V/cm) for 5 h. Gels were stained with 0.5 µg of ethidium bromide per ml and gel picture was taken through gel documentation system.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Genetic variation among Brassica genotypes

In this study five primers were used. In most of cases, the allele positions were different between E6 and E5 (Fig. 2). Considering these, E6 and E5 were selected for test their tolerance under salinity and drought.

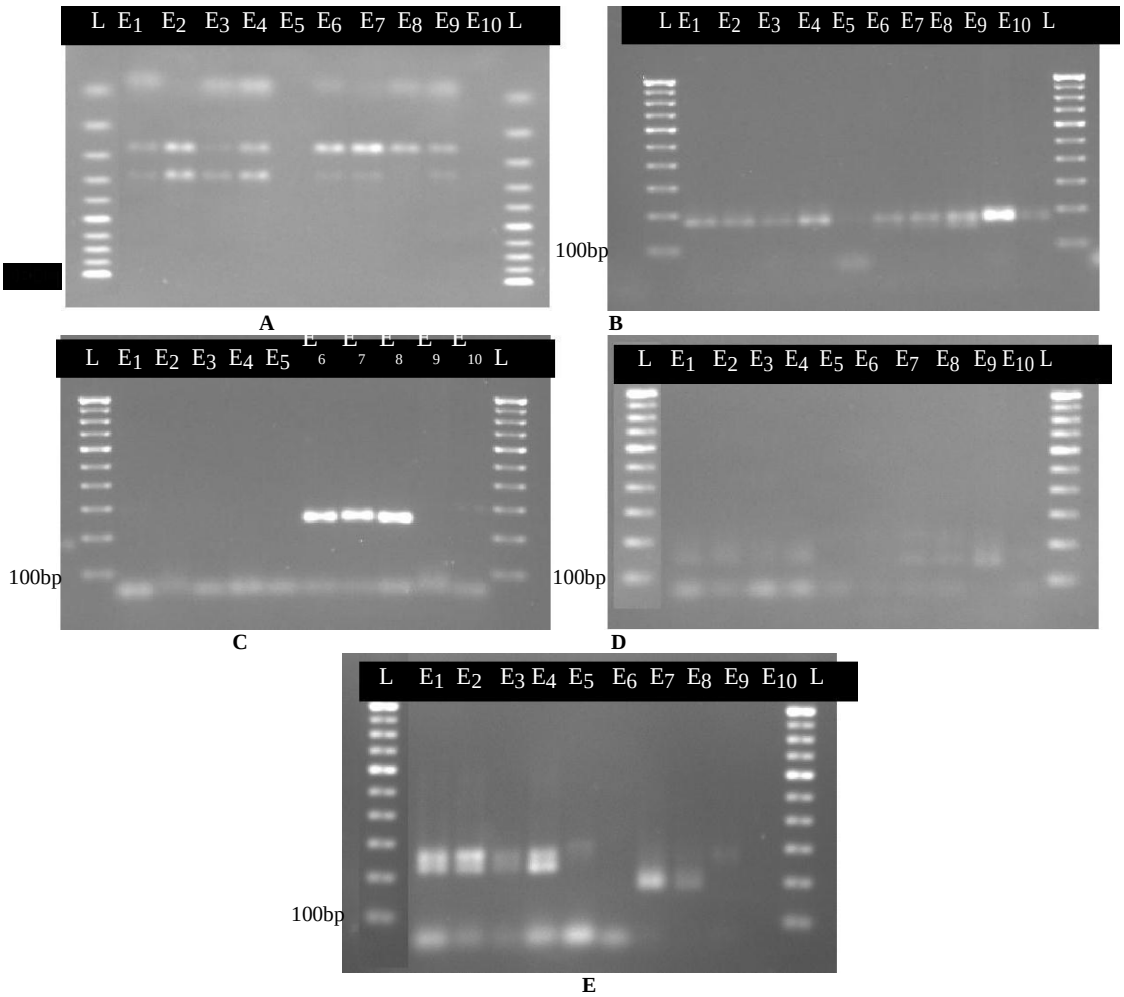

Figure 2. Allele position PCR product of Brassica sp by agarose gel electrophoresis using five primers (A: Ra2-A11, B: Ra2-D04, C: Ra2-E03, D: Ra2-E12 and E: Ra2-F11).

Now-a-days SSR marker has become a useful tool for plant breeder, plant physiologist and molecular biologist to study variation among plant species in molecular level. In present study, SSR marker was used to just find out the variation between two brassica species. This type of work has been reported previously by different research group and similar results were found. Characterization of plant

germplasm using molecular techniques has an important role in the management and utilization of plant genetic resources. It can also enhance plant breeding in selection of diverse parents to widen the breeding gene pool . A total of 60 SSR markers were used for the analysis of genetic relationships of fifty Brassicaceae accessions. Genetic diversity among 120 different accessions of *Brassica* species were characterized with the help of SSR markers. These species include *Brassica rapa*, *B. juncea* and *B. napus*. 39 SSR primers were used and they produced 162 scorable bands in which 105 were polymorphic (Turi *et al*., 2012). Islam *et al*., (2012) worked on molecular characterization of eight cotton varieties in Bangladesh using simple sequence repeat (SSR) or microsatellite DNA markers. All the three microsatellite DNA markers were found to be polymorphic, extracting a total of eight alleles with an average of 2.67 alleles per locus. In this study 5 known SSR markers was us used just to have a look in the allele position to avoid repetition the Brassica genotypes. In most of the cases E6 and E5 were different in terms of allele position (Figure 2) and hence these two genotypes were chosen for study.

4.2 Morphological changes of E5 and E6 under salinity and drought stress

Seedlings of E5 and E6 were subjected to saline and drought stress on 15 days old seedlings. In case of salinity the level 16dS/m was arisen by adding saline water and in case of drought water was stopped. The visual symptoms of after 30 days are shown in Figure 3. Here also E6 showed better performance than E5.

D S C D S C

Figure 3. Visual symptom of E6 (Left) and E5 (Right) after 30 day of stress. D, drought; S, salinity and C, control.

4.3 MG level in under salinity stress

In control seedlings, the contents of MG were 12.30 and 16.81µmolg^{-1}FW at 2 day in E5 and E6, respectively. Changes in MG level under salinity stress at 2, 4 and 6 days in E6 and E5 presented in Figure 4. To check whether the upregulation of MG happened in plants in response to various stress duration, its levels were measured in five-day-old seedlings under control as well as various stress conditions, including drought and salinity stresses.

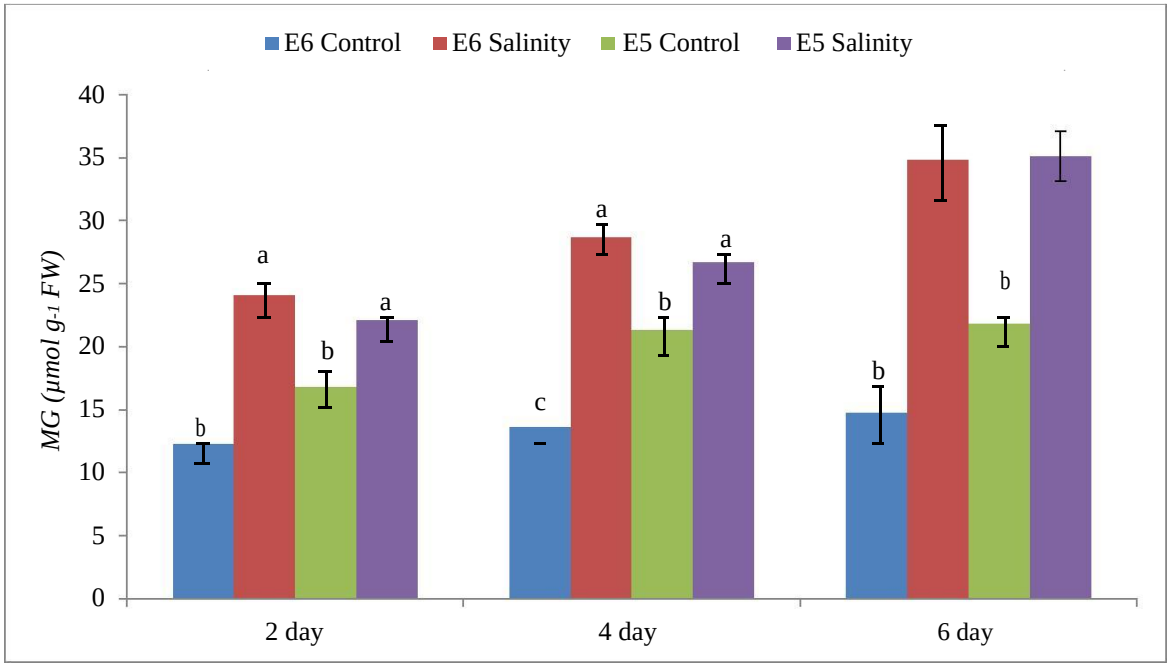

Figure 4. Changes in MG level under salinity stress at 2,4 and 6 days. Values present in the bar are mean±SE. Similar letter between two bars in a particular day are not significant at 5% level of significance.

The graph (Figure 4) showed that the contents of MG level were higher in E5 as compared to those in E6. Salinity stress increased MG level sharply increased with the duration of salinity stress in both genotypes. However, there was no significant difference in MG contents between E6 and E5 under salinity stress. At 2 day, MG level increased almost 103% in E6 over control. At 4 and day stress, MG level increased by 110% and 25% over control in E6 and E5, respectively, while at 6 day, the increments were 136% and 61% over control in E6 and E5, respectively.

4.4 MG level under drought stress

At control condition, the contents of MG were 15.5 and 17.0 µmolg^{-1}FW at 2 day in the seedlings of E5 and E6, respectively. Changes in MG levels under salinity stress at 2, 4 and 6 days in E6 and E5 presented in Figure 5. Methylglyoxal level gradually and significantly increased with the duration of drought stress. Under control condition, MG level was higher in E5. Interestingly, unlike saline stress, MG contents were higher in E5 as compared to E6 under drought stress and the contents were significantly higher in E5 than E6 at 4 and 6 day.

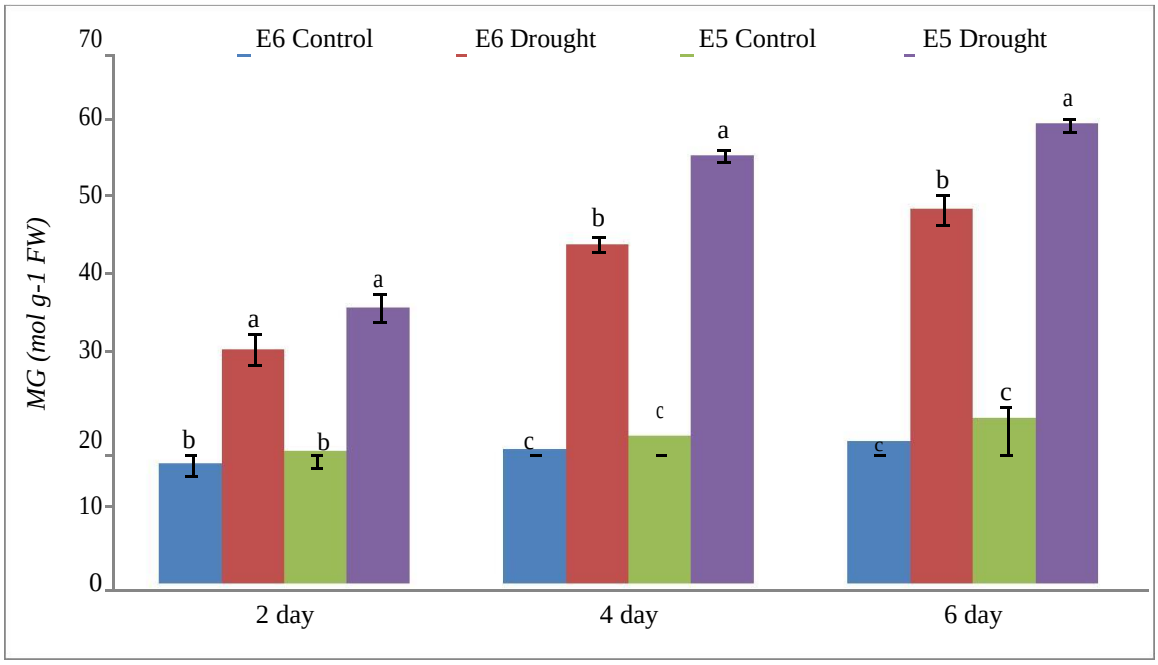

Figure 5. Changes in MG level under drought stress for 2, 4 and 6 days. Values present in the bar are mean±SE. Similar letter between two bars in a particular day are not significant at 5% level of significance.

Two day drought stress increased MG contents by 95 and 105% in E6 and E5, respectively. After two days the MG contents increased more sharply than 2 day stress. Four day drought stress increased MG level by 153% and 189% over control in E6 and E5 respectively. At 6 day stress, MG contents increased by 164.7% and 178% in the leaves of E6 and E5, respectively. At 2, 4, and 6 day of drought stress, the contents of MG were 18, 26 and 23% higher in E5 than those in E6.

4.5 Changes in MG level under salinity and drought stress

In this study, it is observed that level of MG was higher under stress than control and the level increased with the increase in the duration of stress (Figure 4 & 5). MG level increased 96%, 109% and 135% in E6 whereas 31%, 25% and 61% in E5 at 2, 4 and 6 day stress as shown in Figure 4. In drought stress, MG level increased 2, 3 and 3.5 fold over control at 2, 4 and 6 day, respectively in E6 and 2, 2.5 and 3 fold in E5 as shown in Figure 5. MG is synthesized naturally by three kinds of enzymes: methylglyoxal synthase; cytochrome P450 IIE1 isozyme and amine oxidase participating in glycolytic bypass, acetone metabolism and amino acid breakdown, respectively. MG could be generated by removal of the phosphoryl group of triose phosphates produced during glycolysis or following the degradation of lipid peroxides (as in animals), which generates products like 4-hydroxynon-2-enal and MG (Vander *et al*., 2001). Under stress conditions, cells become metabolically active, which is mirrored by upregulation of enzymes involved in glycolysis and TCA cycles (Umeda *et al*., 1994; Espartero *et al*., 1995; Sommer *et al*., 2001) and as a result flux of triose phosphates increases which, instead of giving only pyruvate could be converted to MG. Hossain *et al.* (2009) measured MG under various stressful conditions. Significant increases of MG levels were observed due to different stress treatments. They reported higher level of MG under salinity stress that drought stress and the MG level was 70 μmolg^{-1} FW for salinity and 65 μmolg^{-1} FW for drought. Elevated levels of MG due to stress treatments reported previously in plant systems (Yadav *et al*., 2005b; Singla-Pareek *et al*., 2006). Kaur *et al* (2014) reported a basal level of MG (30–75 mM) in plants under control conditions which increases under stress; resulting in the possibility of MG acting as a signal molecule in plants as well. Yadav *et al* (2005a) measured MG in leaves and roots of two monocot (*Oryza sativa* and *Pennisetum glaucum*) and two dicot (*Nicotiana tabacum* and *Brassica juncea*) plants under control and salt stress conditions and found that the basal level of MG was the same in leaves and roots, except in rice where its level was lower in roots. Under normal conditions, the MG level was lower in *Pennisetum* and tobacco than in rice and Brassica reflecting species-specific variations. They reported the MG levels range from 40 to 75 μmolg^{-1} FW under normal conditions and from 75 to 200 μmol g^{-1} FW under salinity stress. In my study, the range was 10 to 30 μmol g^{-1} FW under normal conditions and from 20 to 60 μmol g^{-1} FW under salinity stress. The range of MG

reported is very wide in nature with as high as 300 µmolg^{-1} FW MG in cultured Chinese hamster ovary cells and as low as 0.3l mM in yeast and in other animal systems (Chaplen *et al*., 1998; Martins *et al*., 2001). A minimal level of MG is retained in the system under non stressed conditions seems to be important for normal development. Now question, under salinity and drought, how the high concentration of MG in this study is detoxified in *Brassica* seedlings to survive. Therefore, the glyoxalase system should discuss.

4.6 Responses of Gly-I under salinity stress

At control condition, the activities of Gly-I were 1.47 and 1.34 µmol min^{-1}mg^{-1} protein in the seedlings of E5 and E6, respectively. The activities of Gly-I in E6 and E5 responses significantly under salinity stress at various durations (Figure 6).

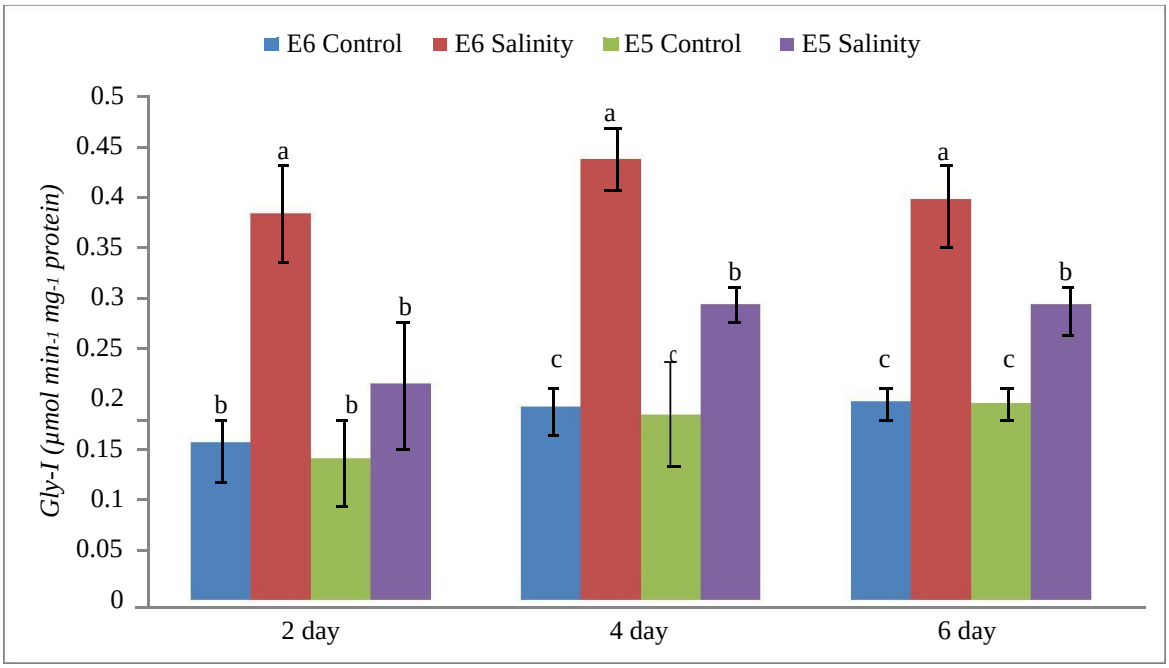

Figure 6. Changes in activity of Gly-I under salinity stress. Values present in the bar are mean±SE. Similar letter between two bars in a particular day are not significant at 5% level of significance.

At early stage of stress (2 day), though the Gly-I activity increased significantly in E6, the activity did not differ significantly in E5. At this stress, Gly-I activities increased 41% and 12% over control in E6 and E5, respectively. At 4 and 6 day of salinity, the activities increased significantly over control. At 4 day, 61% and, 43% higher activities were observed due to salinity stress in E6 and E5, respectively over control, whereas 105% and 41% increased activities were observed at 6 day. It was

remarkable that the activities differed between the seedlings of E6 and E5 under all the stress treatments (Figure 6.). This difference was remarkable at 2, 4 and 6 day where the activity in E6 was 27%, 16% and 33% higher than that in E5.

Various studies on animal and microbial systems have suggested a possible role of Glyoxalase system during cell division and proliferation (Thornalley, 1993). The activity of Gly-I has also been shown to be affected by various exogenous factors (Deswal *et al*., 1993). Previously Gly-I from tomato was shown to be upregulated under salt and water stress (Espartero *et al*., 1995). In this study Gly-I activities were higher in E6 than control and E5. Gly-I activity increased 41%, 69% and 105% in E6 whereas 12%, 43% and 41% in E5 under salinity as shown in Figure 6. These results suggested that the higher Gly-I activity in E6 is involved in tolerance through detoxification of high level of MG. This suggested that the upregulation of Gly-I may be a general effect in response to abiotic stresses to survive. The existence of Gly-I activity in vegetables suggested their tolerance mechanisms against MG, which occurs endogenously and is toxic to cells as it arrests growth (Szent-Gyorgi *et al*., 1967), reacts with proteins and nucleic acids (Thornally, 1998) and inactivates the antioxidant defense system (Martins, 2001). Gly-I activity was measured by various groups under different stimuli. Initial studies revealed that Gly-I activity is high during the cell division and proliferation state (Deswal *et al*., 1993). Further, studies in onion bulb determined a significant increase in Gly-I activity under various stress treatments (Hossain and Fujita, 2009). Beside detoxification, the Glyoxalase system has been reported to influence cell division and proliferation (Paulus *et al*., 1993; Thornalley, 1993). The studies determining the potential role of antioxidant and various enzymes in increasing tolerance to different stresses have also shown enhanced Gly-I activity has been described as an indicator of improved stress tolerance (Hoque *et al*., 2008; Hossain *et al*., 2010; 2011, Hasanuzzaman and Fujita, 2011). MG, substrate for Gly-I, is produced from triosephosphates, increased activity of Gly-I might be required whenever the rates of glycolysis or photosynthesis are enhanced. Therefore, beside MG detoxification, the higher Gly-I activities in E6 and E5 might involve in different physiological process.

4.7 Responses of Gly-I under drought stress

Drought stress significantly increased the Gly-I activities E6 and E5 (Figure 7).

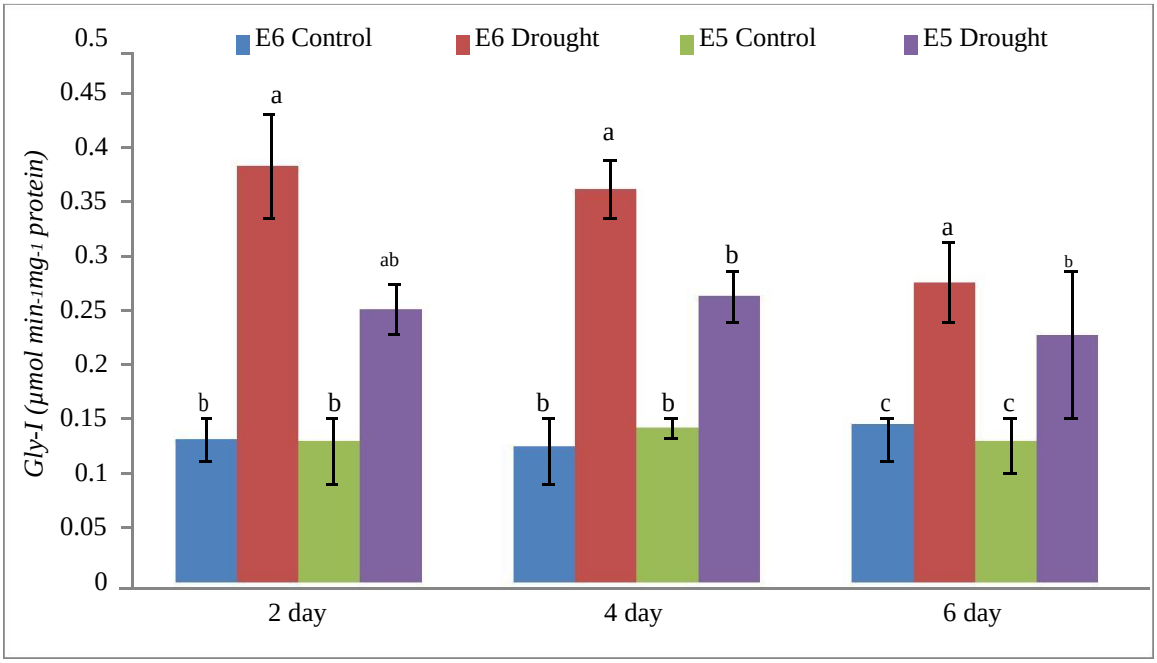

Figure 7. Changes in activity level of Gly-I under drought stress. Values present in the bar are mean±SE. Similar letter between two bars in a particular day are not significant at 5% level of significance.

After two days of drought stress, Gly-I activities increased 71% and 37% over control in E6 and E5, respectively. However, though the Gly-I activity was higher in E6 than E5, the difference was not statistically significant. At 4 day and 6 day stress, there were significant differences in Gly-I activities between E6 and E5. At four days of drought stress, Gly-I activity increased 76% and 32% over control in E6 and E5, respectively. At 6 day the activity decreased as compared to 4 day tress. At 6 day Gly-I activities were 62% and 53 % higher over control in E6 and E5, respectively.

Several research groups have reported that the activity of Gly-I was affected by various exogenous factors and abiotic stress treatments including salt, water and heavy metal stresses (Espartero *et al*., 1995; Veena *et al*., 1999). In present study, under drought stress, Gly-I activity increased 70%, 76% and 61% fold over control at 2, 4 and 6 day, respectively in E6 and 37%, 32% and 53% fold in E5 as shown in Figure 7. In the previous study, it was observed a significant increase of Gly-I activity as well as Gly-I transcript level due to different stress treatments, and the results were in accordance with the MG levels. Significant positive correlation between MG level

and Gly-I activity indicating that the Glyoxalase system might be required for the detoxification of MG formed, both spontaneously and enzymatically, from triose phosphates. In this regard, Gly-I could be expected to be a house-keeping protein present in all cells. In this study the high MG and Gly-I in Brassica under drought implicated the detoxification role of Gly-I. The efficient manipulation of Glyoxalase pathway enzymes in different plants inhibits an increase in MG level under oxidative stress and confers tolerance (Yadav *et al*. 2005; Singla-Pareek *et al*. 2006, 2008). The results of this study also addressed the situation.

4.8 Responses of Gly-II under salinity stress

Gly-II activities were found to increase abundantly under salinity stress in E6, and the increase in E5 was comparatively lower (Figure 8).

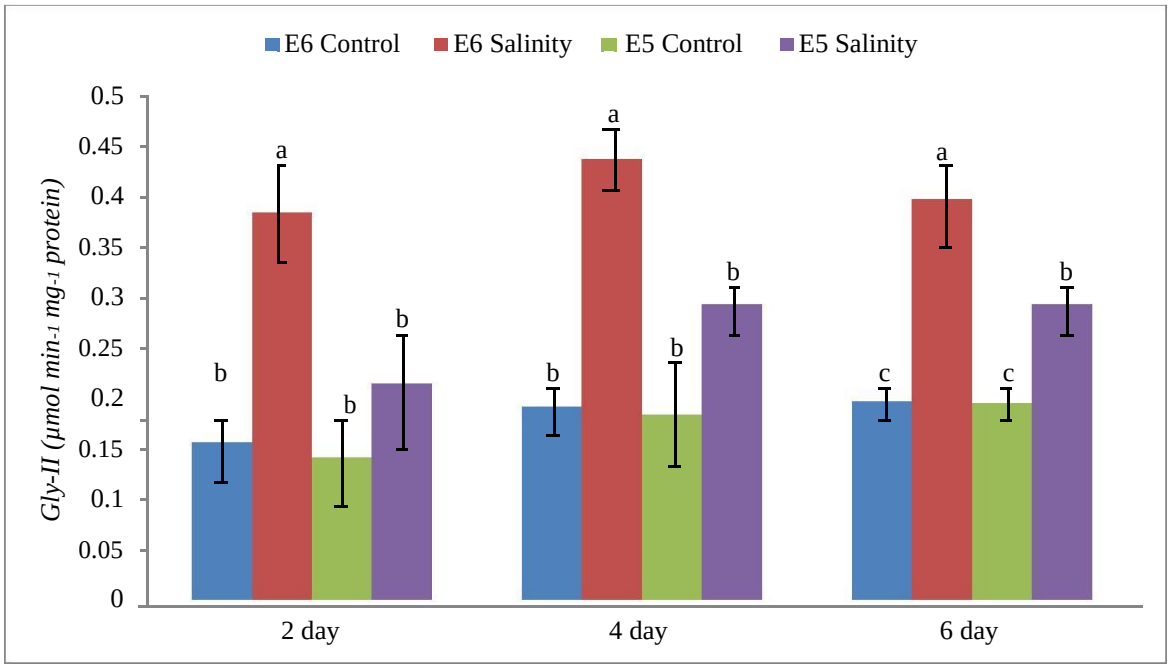

Figure 8. Changes in activity level of Gly-II under salinity stress. Values present in the bar are mean±SE. Similar letter between two bars in a particular day are not significant at 5% level of significance.

As a part of Glyoxalase system, Gly-II takes part in MG detoxification. To investigate its role, Gly-II activity measured under salinity and drought stress. Higher Gly-II activity found in all stress induced plant than control. At 2 day salinity stress, Gly-II activity increased 145% and 52% over control in E6 and E5 respectively, and the Gly-II activity of E6 and E5 were 0.384 and 0.215 µmol min^{-1} mg^{-1} protein, respectively. At 4 day salinity stress, Gly-II activities increased 189% in E6 and 86% in E5 over

control. At 6 day of salinity, the activities were comparatively lower than those in 4 day. At 6 day, the activities were 102% and 50% higher over control in E6 and E5, respectively. The activity in E6 was 44%, 33% and 26% higher than that in E5 at 2, 4 and 6 day of stress, respectively.

Gly-II converts the intermediate product produced by Gly-I to D-lactate and release GSH to glutathione pool (Singla-Pareek *et al*. 2006, 2008). Therefore, in stress environment it plays important role in MG detoxification and GSH maintenance as well. In response to salinity stress, Gly-II activity increased 144%, 128% and 101% in E6 at 2, 4 and 6 day stress. On the other hand 52%, 59% and 50% activity increased in E5 over control (Figure 8). Previously salt sensitive BRRI dhan49 was reported to show slight increase in the activity of Gly-II and conversely, in salt tolerant BRRI dhan54 treatment with 150 and 300 mM NaCl showed significant increase in Gly-II activity as compared to control. (Hasanuzzaman *et al*., 2014). The increased Gly-II activity was also in tobacco (Hoque *et al*., 2008). In this study, Gly-II activity was found higher in E6 than that in E5 suggesting that the MG detoxification ability in higher in E6. On the other hand, GSH recycling ability might also be in E6.

4.9 Responses of Gly-II under drought stress

Changes in Gly-II activity under drought stress at 2, 4 and 6 days in E6 and E5 presented in Figure 9. There was a significant increase in Gly-II activity in response to drought stress (Figure 9). A sharp increase of Gly-II activity (191% and 93% over control) was observed in E6 and E5 due to salinity stress within 2 days. In case of E6 Gly-II activity decreased slowly upon exposure to 4 and 6 day drought, and the activities were 0.361 and 0.276 µmol min^{-1} mg^{-1} protein, respectively. On the other hand, in E5, the activity decreased after 4 day. At 4 and 6 day drought stress, Gly-II activities increased 189% and 90%, respectively in E6 and 86% and 76% in E5, respectively over control.

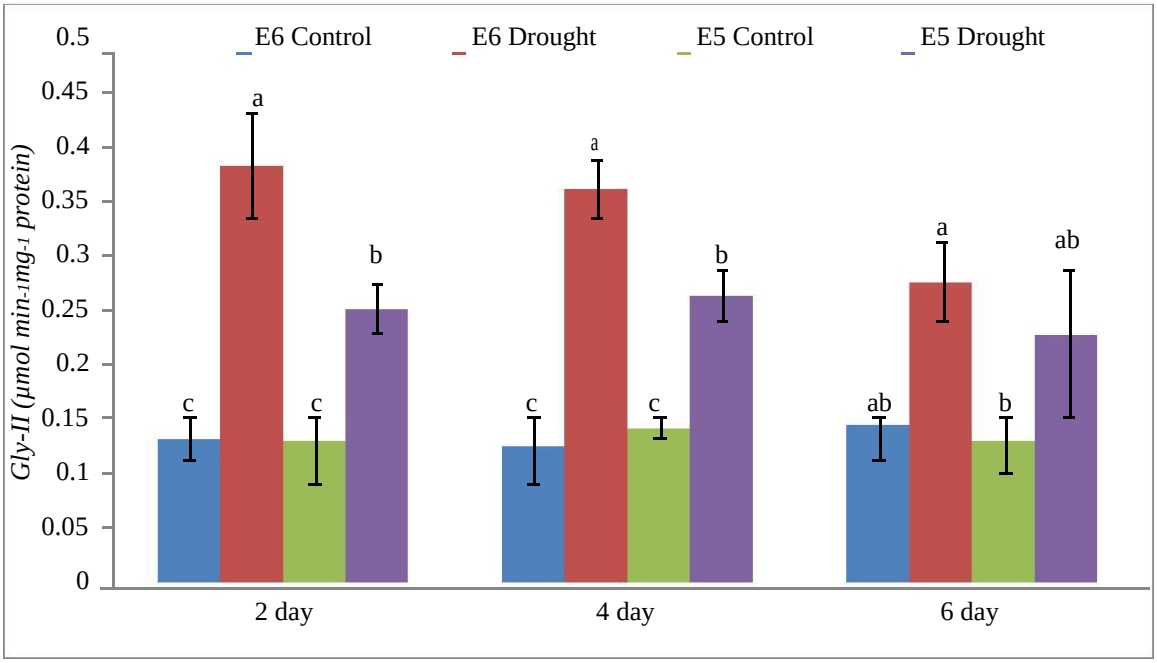

Figure 9. Changes in activity level of Gly-II under drought stress. Values present in the bar are mean±SE. Similar letter between two bars in a particular day are not significant at 5% level of significance.

Increased Gly-I and Gly-II activity during stress may also indicate active metabolic status of the cell, in which cell division and growth are compromised in order to conserve energy for mobilization of resources towards stress tolerance and defense strategies. The gene expression profile of Gly-I and II also showed true reflection of possible changes in activity levels due to different abiotic stresses. Besides detoxification of methylglyoxal, the Glyoxalase system might also play a role in providing tolerance to stress by recycling glutathione that would be „trapped' spontaneously by methylglyoxal to form hemithioacetal (Thornalley,1990), thereby maintaining glutathione homeostasis. In response to drought stress, Gly-II activity increased 94%, 152% and 164% in E6 at 2, 4 and 6 day stress on the other hand 108%, 189% and 177% activity increased in E5 over control (Figure 9). In previous study, the activity of Gly-II varied with genotype (Alam *et al*., 2013). In this study, Gly-II activity was higher in E6 than that in E5. These results suggested that E6 has better MG detoxification ability under drought stress.

4.10 Purification of Gly-I

Since Gly-I was found to increase considerably under stress, an attempt was taken to purify the Gly-I from E6. The soluble protein fraction prepared from 50 g fresh

seedlings was precipitated by 65% $(NH_4)_2SO_4$ and the dializate was applied on DEAE-cellulose column chromatography (i.d. 1.7 × 20 cm) and eluted with a liner gradient of KCl (0-0.2 M) (Figure 10). Total 140 fractions, each containing 5 ml, were collected. The Gly-I activities of each fraction and absorbance at 280 nm were measured. Gly-I peak eluted at 76 mM of KCl (Figure 10).

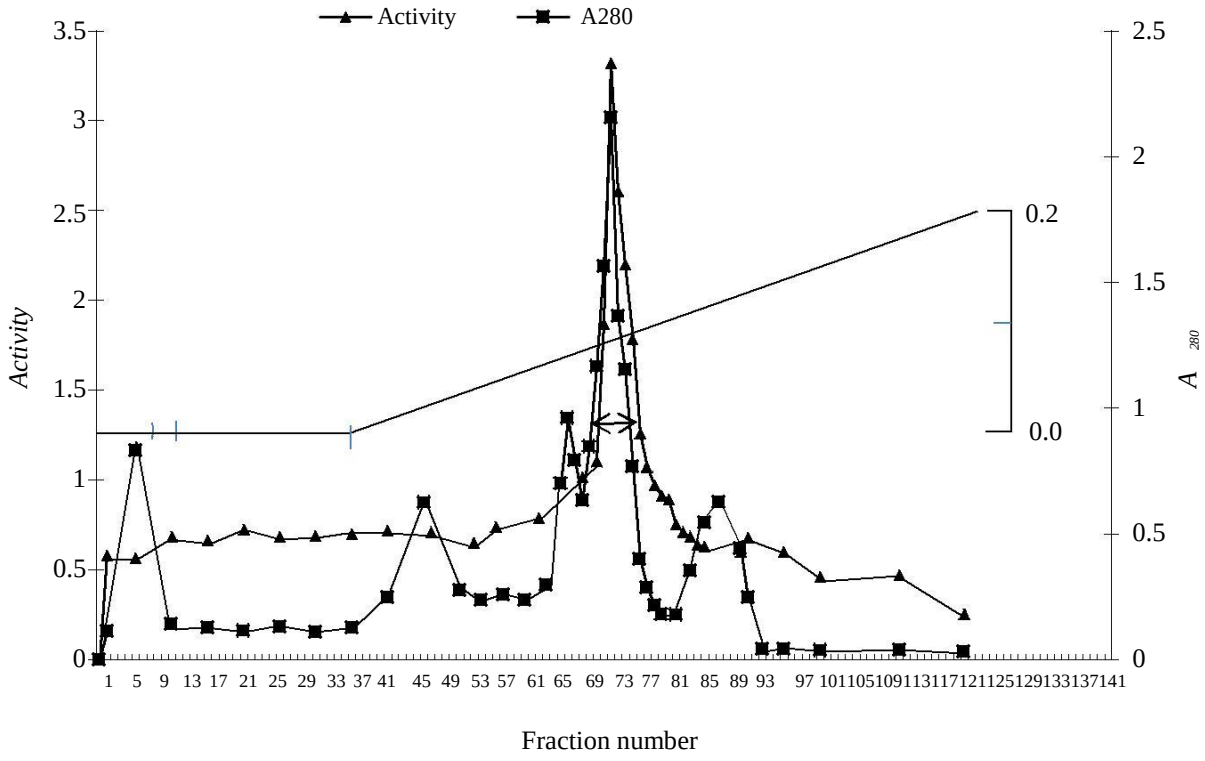

Figure 10. A typical column chromatography of DEAE-cellulose of soluble proteins prepared from 50 g fresh leaf of seedlings of E6. For each fraction, absorbance at 280 nm (■) and Gly-I activity (▲) at A_{240} were determined. Activity is expressed as μmol min^{-1} ml^{-1}. Bars indicate the high active peak fractions of Gly I. The fractions under the bar of Gly I, peak was pooled for subsequent purification. The curve shows the concentration of KCl (0-0.2 M).

The high fractions containing high Gly-I activity was collected from the peak and pooled for measuring its activity. Among them, Gly-I showed total activity of 4.31mmol min^{-1} with purification fold 9.23 and recovery 3.73% (Table 2).

The Gly-I pools was applied on a DEAE column again for further purification. The Gly-I activities of each fraction towards model substrate MG and absorbance at 280 nm were measured. Again, one Gly-I peak eluted at around 76 mM of KCl (Figure

11). The high active fractions were pooled again for further purification. The pooled sample (15 ml) showed total activity of 2.3 mmol min^{-1} with purification fold 19.8 and recovery 2.06% (Table 2).

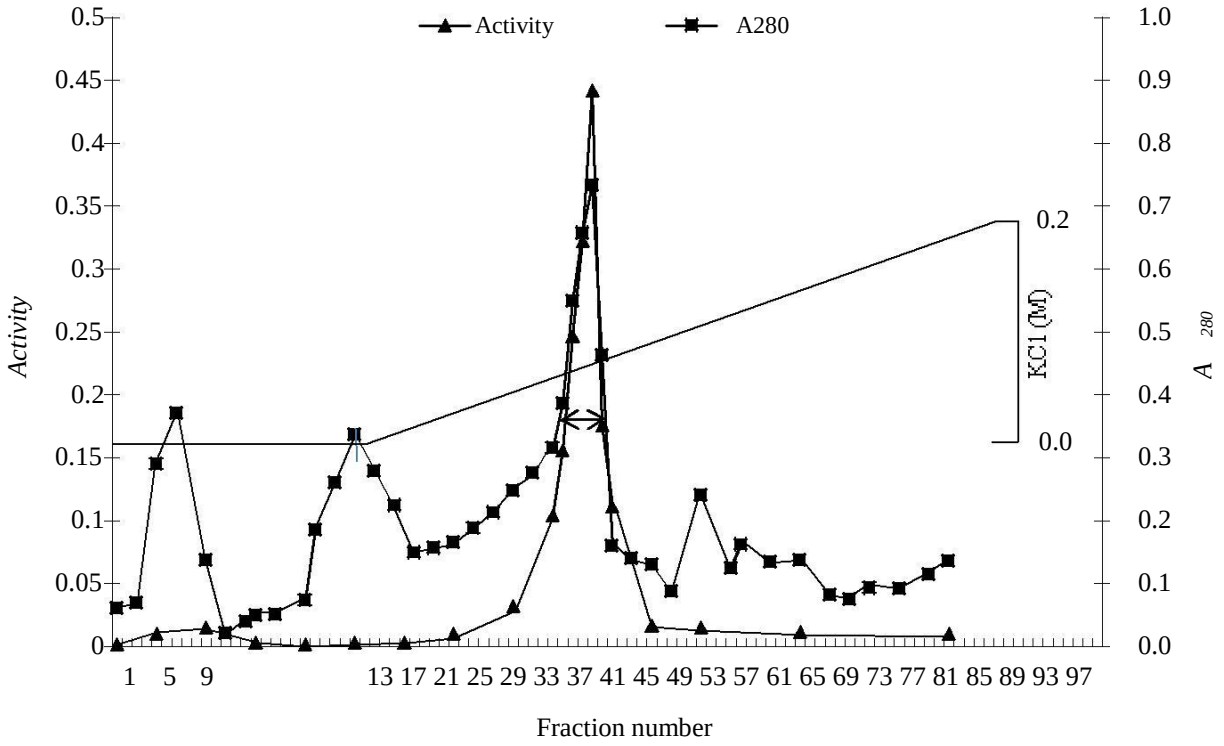

Figure 11. DEAE column-2 of Gly-I pool from DEAE column-1. For each fraction, absorbance at 280 nm (●) and Gly-I activity toward MG (■) at A_{240} were determined. Activity is expressed as μmol min^{-1} ml^{-1}. Bars indicate the high active peak fractions of Gly-I. The fractions under the bar of Gly-I, peak was pooled for subsequent purification. The curve shows the concentration of KCl (0-0.2 M).

The active Gly-I pools from DEAE chromatography-2 were applied on an affinity column chromatography of *S*-hexylglutathione-agarose to complete the purification. The Gly-I sample were passed through the column following 10 ml 0.2 ml KCl for washing. The Gly-I was eluted with 15 ml *S*-hexylglutathione. Activities of affinity fractions and absorbance A_{280} were taken (Figure 12). The active fractions were pooled and dialyzed in B buffer overnight. The active fractions had specific activity 173.7 $\mu mol^{-1}mg^{-1}$ protein, total activity 0.44, purification fold 113 and recovery 0.38% (Table 2).

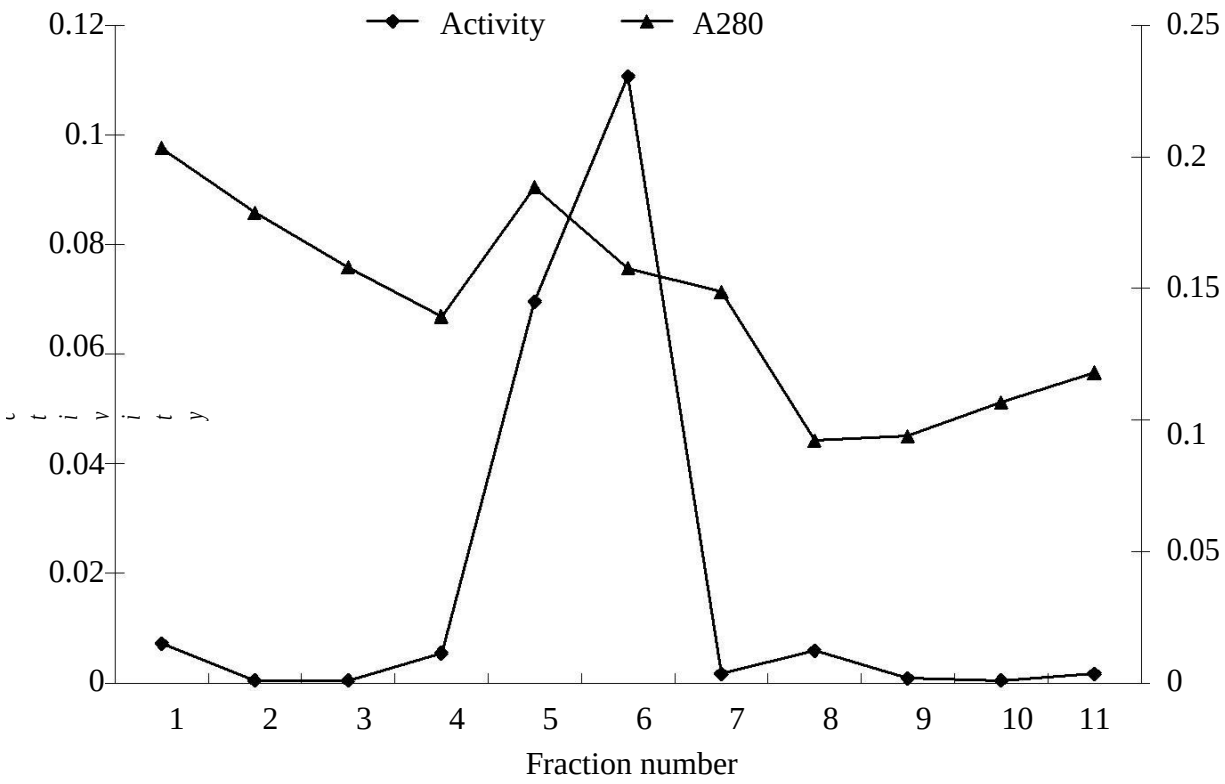

Figure 12. Elution profile of GSTs from affinity column chromatography. The line indicates the linear gradient of potassium - phosphate (K-P) buffer. For each fraction, absorbance at 280 nm (▲) and Gly-I activity toward MG (♦) at A_{240} were determined. Activity is expressed as μmol min^{-1} ml^{-1}.

The purities and molecular masses of the purified Gly-I were examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The silver staining and CBB staining of the purified Gly-I indicated that final product of Gly-I were highly purified and migrated as a single band on SDS-PAGE with an apparent molecular mass of 27 kDa (Figure 13).

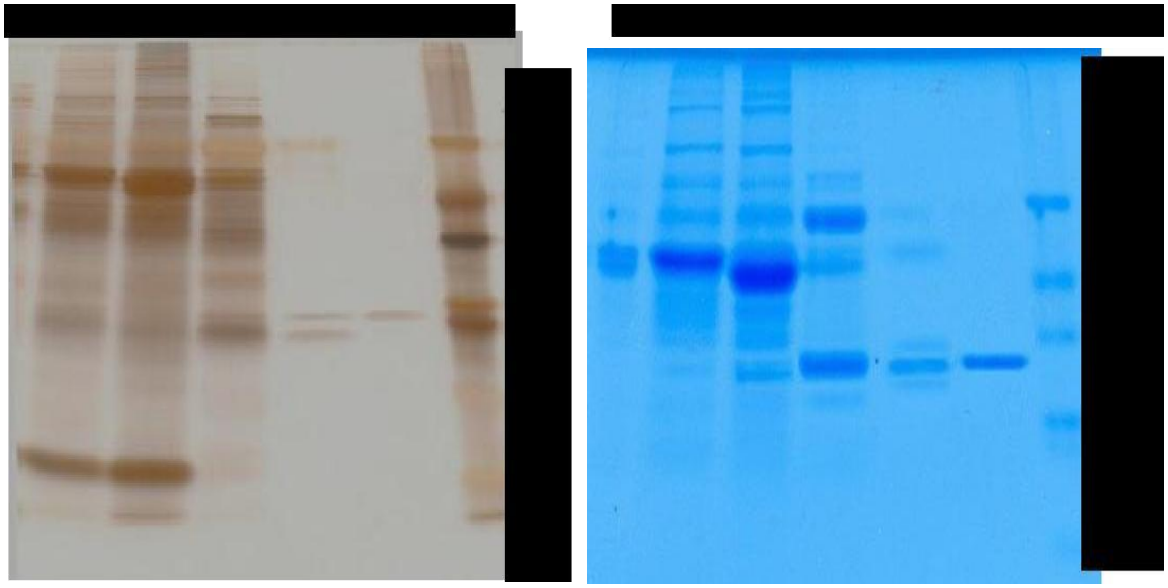

Figure 13. Silver staining and CBB staining of different fraction of Gly-I purification from *Brassica* seedlings. Lane 1, Homogenous; Lane 2, $(NH_4)_2SO_4$ ppt; Lane 3, DEAE fraction-1; Lane 4, DEAE fraction-2, Lane 5, Purified Gly-I ; and Lane 6, Molecular weight marker.

Table 2. Purification fraction of Gly-I from *Brassica*.

Fraction	Total Volume (ml)	Total Protein (mg)	Total Activity (mmol min^{-1})	Specific Activity (µmol $^{-1}mg^{-1}p$)	Purification Fold	Yield (%)
Crude protein	192	375.36	115.68	1.54	1	100
$(NH_4)_2SO_4$ ppt	35	200.07	97.27	2.43	1.57	84.08
DEAE (1)	23	6.06	4.31	14.22	9.23	3.73
DEAE (2)	15	1.56	2.38	30.51	19.80	2.06
Affinity	5	0.051	0.44	173.74	112.7	0.38

During purification, homogeneous showed the lowest specific activity 1.54 µmol^{-1} mg-1 protein whereas the product from affinity column showed highest the specific activity 174 µmol^{-1}mg^{-1} protein with 113purification fold and 0.38% recovery. According to Table 2. $(NH_4)_2SO_4$ ppt had lower total activity, total protein and recovery than homogeneous. The final product of DEAE fraction-1 showed 14.22 µmol^{-1}mg^{-1} protein specific activity with 9.23 purification fold and 3.73% recovery on

the other hand the final product of 2nd DEAE fraction showed 30.52 µmol^{-1}mg^{-1}p specific activity with 20 purification fold and 2% recovery. Total activity, total protein was the highest in the homogeneous and gradually decreased as the product purified further.

Glyoxalases are known to play a very important role in abiotic stress tolerance. This two-step pathway detoxifies ubiquitously present cytotoxic metabolite MG, which otherwise increases to lethal concentrations under various stress conditions. Methylglyoxal initiates stress-induced signaling cascade via reactive oxygen species, resulting in the modifications of proteins involved in various signal transduction pathways, that eventually culminates in cell death or growth arrest. In this study, Using affinity chromatography, purified and homogeneous enzyme judged by SDS-PAGE showing single band upon staining. The molecular weight was calculated to be 27 kDa (Figure 13 and 14). The specific activity of homogenous was 1.54 µmol^{-1}mg^{-1}p and purified sample was 174 µmol^{-1}mg^{-1}p. Total activity of homogenous was 115.68 mmol min^{-1} whereas 0.44 mmol min^{-1} in purified sample as in Table 2. As the sample passed through the columns specific activity increased, total activity decreased as the different proteins were removed through successive use of column chromatography. In purification, Gly-I were found to be highly purified with purification fold 113 and recovery 0.38%. The associated mechanism of tolerance conferred by over-expression of methylglyoxal-detoxifying glyoxalase pathway mainly involves lowering of methylglyoxal levels, thereby reducing subsequently induced cellular toxicity (Kaur *et al*., 2014). Brassica Gly-I had no isoforms (Veena *et al*., 1999). In this study similar results were found. Gly-I has three types, short medium and long (Thornalley, 1990). The size of the protein was 27 kDa and 29 kDa confirmed after SDS-PAGE. These molecular masses were in the same range as those reported for Glyoxalases from animal systems where the subunits range from 21 to 29.5 kDa. The size of the protein was in onion is 25 kDa confirmed after SDS-PAGE (Hossain *et al*., 2009). Gly-I purified from soybean cell suspension showed 1000-fold with a yield of 3.5%. The Gly-I purified is lied with in the sizes reported above.

CHAPTER 5

SUMMARY AND CONCLUSION

In this study, ten different genotypes of *Brassica* species were studied to identify dissimilar genotype. In response of five primers and visual symptoms under salinity and drought, E6 (BARI sarisha 16) and E5 (Tori 7) were chosen for study.

MG levels increased with the increased duration of salinity and drought stress. In E6, MG levels increased 96%, 109.8% and 135.7% over control under salinity stress at 2, 4 and 6 day, respectively. In E5, MG levels increased 31%, 25% and 61% over control under salinity stress at 2, 4 and 6 day. Under drought stress, MG levels increased 94.5%, 152% and 164% in E6 whereas 108%, 189% and 177% in E5 compared to control.

Gly-I activity levels increased with the increased duration of salinity and drought stress. In E6, Gly-I activity levels increased 41%, 69% and 105% compared to control under salinity stress at 2, 4 and 6 day. In E5, Gly-I activity levels increased 12%, 43% and 41.7% compared to control under salinity stress at 2, 4 and 6 day. Under drought stress, Gly-I activities increased 70%, 76% and 61% in E6 whereas 37%, 32% and 53% in E5 over control. In most cases higher Gly-I activity observed in E6 than E5.

In E6, Gly-II activity levels increased 144%, 128% and 101% compared to control under salinity stress at 2, 4 and 6 day. In E5, Gly-II activity levels increased 52%, 59% and 50% compared to control under salinity stress at 2, 4 and 6 day. In case of E6, Gly-II activity upon exposure to 2, 4 and 6 day drought were 0.382, 0.361 and 0.275 µmol min^{-1} mg^{-1} protein, respectively. In E5, Gly-II the activities were 0.251, 0.263 and 0.227 µmol min^{-1} mg^{-1} protein for 2, 4 and 6 day drought treatment, respectively. In most cases E6 showed higher Gly-II activity than E5.

Since Brassica Gly-I was found to increase considerably; an attempt was taken to purify the active Gly-I from E6 (BARI sarisha 16) seedling. The soluble protein fraction prepared from 50 g fresh Brassica leaf was applied on DEAE-cellulose column chromatography (i.d. 1.7 × 20 cm) and eluted with a liner gradient of KCl (0-0.2 M). One Gly-I peak eluted at 76 mM of KCl .

Following the method of Rohman *et al.* (2009), soluble protein from plant materials was extracted. By using a series of protein separating columns proteins were purified. Using DEAE twice provides better purification fold. To check the homogeneity of purified Gly-I enzyme and to estimate its molecular mass, SDS-PAGE was done in

12.5% (w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970) followed by silver staining and CBB sating. In purification, the final product was purified 112.7 fold with a yield of 0.38%. The specific activity of purified Gly-I was 173.74 μmolmin^{-1} mg^{-1} proteins. The table also showed that due to use of successive chromatography, the undesired proteins removed successfully. Finally a single band of 27 kDa was observed upon silver staining and CBB staining of SDS-PAGE gel.

The following conclusions have been made on the basis of the findings of the investigations:

MG level increased under stress condition and it increases with the increase in duration of stress. But there was no significant difference between E6 and E5 in terms of MG level. Plant produces more MG at drought than salinity which indicates the severity of drought stress. MG level in control plant ranges from 12 to 21 μmol g^{-1} FW and under stress it varies from 22 to 59 μmol g^{-1} FW. Gly-I activity increased under salinity and drought stress to detoxify MG at different duration of stresses. However, higher Gly-I activity observed in E6 than E5 under salinity and drought stress. Gly-II activity also increased under stress condition. In E6, Gly-II activity levels increased 144%, 128% and 101% compared to control under salinity stress at 2, 4 and 6 day, respectively. In E5, Gly-II activity levels increased 52%, 59% and 50% compared to control under salinity stress at 2, 4 and 6 day, respectively. On the basis of results, it can be said that E6 (BARI sarisha 16) showed better tolerance than E5 (tori 7) under salinity and drought stress. During purification, single Gly-I peak was eluted by using DEAE column chromatography. The purity and molecular mass of the fraction was examined by SDS-PAGE. The silver staining of the purified Gly-I indicated that final product of Gly-I was highly purified and migrated as a single band on SDS-PAGE with an apparent molecular mass of 27 kDa. However, use of DEAE twice better purification fold instead of using hydroxylapatite column because there is a chance of losing activity enzyme when using hydroxylapatite column.

Based on conclusions, the following recommendations can be made:

- Since, Gly-I were found to be induced under saline and drought it needs to examine their accumulation. Therefore, production of polyclonal antibody of Gly-I should be developed and western blot analysis should be perform under different abiotic stresses.
- More study on the scenario of GST (another detoxification enzyme in plant) activity in relation to Gly-I under different abiotic stresses as GST are also GSH depended elute sometimes together with Gly-I.
- Abiotic stress tolerance is a complex system and includes other antioxidant system. Therefore, whole antioxidant system might provide more information.
- In this study, the role of glyoxalases was checked under short-term salinity. It may be examined under long-term salinity.

CHAPTER 6

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APPENDICES

Appendix - I

List of chemicals

- Acrylamide
- Agarose
- Albumin Bovine Serum (BSA)
- Ammonium per-oxisulfate
- Ammonium Sulfate
- Beta mercaptoethanol (β-ME)
- Citric acid (Monohydrate)
- CTAB (Cetyl Trimethyl Ammonium Bromide)
- Coumassie Brilliant Blue
- DEAE (DE-52, UK)
- Dye (Bromophenol Blue)
- dNTPs
- EDTA (Ethylene diamine tetraacitic acid)
- Ethanol
- Formaldehyde
- Glutathione (Reduced)
- Glycerol
- Glysine
- HCl
- Hydroxyapatite

- KCl
- KOH
- L-Ascorbic acid
- Methanol
- Methylglyoxal
- $MgSO_4.7H_2O$
- N, N' Methylen Biss
- $Na_2H_2PO_4.2H_2O$
- $Na_2HPO_4.12H_2O$
- NaCl
- NaOH
- NH_3 Solution
- NH_4SO_4
- Phosphoric acid
- Potassium (Dibasic)
- Potassium (Monobasic)
- PVP (Poly Vinyl Pyrolideone)
- S-hexylglutathine
- S-hexylglutathine-agarose
- Silver nitrate
- Sodium Dodecile Sulfate (SDS)
- Sugar
- *Taq* DNA polymerase

- TEMED
- Tris-buffer