Project ID 389

Competitive Research Grant

Sub-Project Completion Report

on

Oxidative stress tolerance of maize under drought and salinity: Mechanism and identification of stress inducible proteins

Project Duration

May 2017 to September 2018

Plant Breeding Division
Bangladesh Agricultural Research Institute

Submitted to
Project Implementation Unit-BARC, NATP-2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215

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Project Implementation Unit
National Agricultural Technology Program-Phase II Project (NATP-2)
Bangladesh Agricultural Research Council (BARC)
New Airport Road, Farmgate, Dhaka – 1215
Bangladesh

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Bangladesh

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Acronyms

^{0}C- Degree Celsius
$^{1}O_2$ -Singlet oxygen
ADH- Alcohol dehydrogenase
AO- Ascorbate oxidase
ASA- Ascorbic acid
APX- Ascorbate peroxidase
BSA- Bovine albumin serum
CAT- Catalase
df- Dilution factor
DHA- Dehydroascorbate
DHAR- Dehydroascorbate reductase
DNA-Deoxyribonucleic acid
EC- Enzyme Commission
EDTA- Ethylenediaminetetraacetic acid
ETC- Electron transport chain
FC- Field capacity
FW- Fresh weight
Gly-I- Glyoxalase-I
Gly-II- Glyoxalase-II
GPX-Glutathione peroxidase
GSH- Reduced glutathione
GSSG- Oxidized glutathione
GST- Glutathione *S*-transferase
GR- Glutathione reductase
H_2O_2- Hydrogen peroxide
$HO_2^{\bullet}$-Perhydroxy radical
K-P- Potassium-Phosphate
L- Liter
LDH- Lactate dehydrogenase
MDA- Melondialdehyde
MDHAR- Monodehydroascorbate reductase
MG- Methylglyoxal
min- Minute
mM- Milli molar
NAD- Nicotinamide adenine dinucleotide
NBT-Nitroblue tetrazolium
NAPDH- Nicotinamide adenine dinucleotidephosphate
NOX- NADPH oxidase
$O_2^{-\bullet}$-Superoxide radicals
$OH^{\bullet}$-Hydroxylradical
PCD- Programmed cell death
PDC- Pyruvate decarboxylase
POD- Peroxidase
PSI- Photosystem I
PSII- Photosystem II
PUFA- Polyunsaturated fatty acid
$RO^{\bullet}$-Alkoxy radicals
ROS- Reactive oxygen species
SOD- Superoxide dismutase
TBA- Thiobarbituric acid
TMED- Tetramethylethylenediamine

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Executive Summary

Drought and salinity lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic causing damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress. The ROS comprises both free radical ($O_2^{-\bullet}$, superoxide radicals; $OH^{\bullet}$, hydroxylradical; $HO_2^{\bullet}$, perhydroxy radical and $RO^{\bullet}$, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen). Among them, the most studied common and cytotoxic ROS are $O_2^{\bullet-}$ and H_2O_2.The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to cell death. To study the nature of oxidative damage and its possible protective mechanism needs phenotypically differently sensitive maize genotypes. One tolerant genotype (P1×P7), two moderately tolerant genotypes (BHM-7 and BHM-9) and one susceptible genotype (BHM-5) to salinity were selected. On the other hand, BHM-12, BHM-13, BHM-5, BHM-9 and P1×P7 were used for drought related study. Their tolerant level was confirmed by biochemical analysis of proline, melondialdehyde (MDA), H_2O_2, methylglyoxal (MG), accumulation of Na^+ (for saline experiment) as well as histochemical detection of $O_2^{-\bullet}$, $H_2O_2$and NADPH-oxidase (NOX) synthesis in leaves. For salinity screening, five days old seedlings grown on rock media were transferred for screening in a hydroponic system containing Hoagland's solution in plastic pots, each pot connected to oxygen channel. After two days hardening, the seedlings were subjected to NaCl induced 16 dSm^{-1} equivalent salinity to observe their tolerance till to death. Anaerobic condition was created by stopping O_2 supply. On the other hand, for drought screening, one month old seedlings were subjected to water withdrawal, and observed till attaining a soil moisture of 12.5% of field capacity (FC).Fully expanded leaves were used to record data on ROS ($O_2^{-\bullet}$ and H_2O_2) and their metabolizing stress inducible enzymatic proteins like superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbic peroxidase (APX), glutathione peroxidase (GPX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) as well as MG detoxification system to understand the oxidative stress tolerance mechanism. In addition, anaerobic enzymes like alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH) and pyruvate decarboxylase (PDC) as well as ascorbate- (ASA) and glutathione- (GSH) homeostasis were also analyzed. For identification of stress inducible proteins to drought and saline, in-gel separation protocols for SOD, POD, CAT, APX and GPX were developed to find highly expressed isozymes under drought and salinity. The spectrophotometrically assay protocols of anaerobic enzymes were also developed. Importantly APX1 and APX2 are identified as important ROS scavenger under salinity. At the same time, CAT3 played important role in tolerant genotypes. POD2 and POD3 also played important role for H_2O_2 decomposition in both tolerant and susceptible genotypes under salinity and drought. In contrast,GPX2 indicated as important H_2O_2decomposer in drought tolerant genotypes. The ASA- and GSH Redox homeostasis indicated that susceptible genotype speeded up the oxidation of ASA and GSH to reduce the activity of enzymatic antioxidants. Both Gly-I and Gly-II showed higher activity in tolerant genotypes than susceptible genotypes in both stresses. However, the anaerobic enzymes might have involve metabolic role in all the genotypes under anaerobic saline as well as drought stress. Based on oxidative stress mitigation, APX1, APX2, CAT3, POD2 and POD3 were identified as saline inducible proteins while GPX2 as drought stress inducible protein. Among them, APX1, APX2, CAT3 and GPX2 were recommended for further research through biotechnological approaches.

CRG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Sub-Project title: Oxidative stress tolerance of maize under drought and salinity: Mechanism and identification of stress inducible proteins
2. Implementing organization: Bangladesh agricultural Research Institute
3. Name and full address with phone, cell and E-mail of PI/Co-PI (s): Principal Investigator (Full address with phone and e-mail): Dr. Md. Motiar Rohman, Senior Scientific Officer, Plant Breeding Division, BARI, Gazipur 1701. Cell: +88 01716374405; Co-PI: Dr. Kamrun Nahar, Senior Scientific Officer, Biotechnology Division, BARI, Gazipur 1701.
4. Sub-project budget (Tk):
   4.1 Total: 28,00,000.00 (Twenty eight lac taka only).
   4.2 Revised (if any): N/A
5. Duration of the sub-project:
   5.1 Start date (based on LoA signed): 11 May, 2017
   5.2 End date: 30 September 2018
6. Justification of undertaking the sub-project:
   Drought and salinity are major constraints in crop production in Northern and Southern part of Bangladesh. The forthcoming climate change will affect the production of crop unless there would have any drought and saline tolerant crop variety. Maize is a C_4 crop supposed to have wider adaptability to such stressful areas. However, till today, no such variety has been developed due to complexity of drought and salinity tolerance trait. Therefore, to develop a tolerance and sustainable variety, it is essential to understand the tolerance mechanism. Both drought and salinity tolerance are polygenic traits associated with numerous physio-molecular, biochemical and metabolic responses.

   Drought and salinity lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic causing damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress. The ROS comprises both free radical ($O_2^{-\bullet}$, superoxide radicals; $OH^{\bullet}$, hydroxyl radical; $HO_2^{\bullet}$, perhydroxy radical and $RO^{\bullet}$, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $^1O_2$, singlet oxygen) (reviewed in Gill and Tujeta, 2010). Among them, the most common and cytotoxic ROS are singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$). In chloroplasts, photosystem I and II (PSI and PSII) are the major sites for the production of $^1O_2$ and $O_2^{-\bullet}$. In mitochondria, complex I, ubiquinone and complex III of electron transport chain (ETC) are the major sites for the generation of $O_2^{-\bullet}$. H_2O_2 and $OH^{\bullet}$ are produced through Haber-Weiss and Fenton reactions. The enhanced production of ROS during environmental stresses can pose a threat to cells by causing peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death (PCD) pathway and ultimately leading to cell death (Mittler, 2002; Sharma et al., 2012). In addition, methylglyoxal (MG) is potential cytotoxic compound

produced under abiotic stress which can react with and modify other molecules including DNA and proteins (Yadav et al., 2005a, b).

Plants possess internal antioxidative defense machinery to protect cells against oxidative stress damages. Plants have very efficient enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; peroxidase, POD and glutathione *S*-transferase, GST) and non-enzymatic (ascorbic acid, ASH; glutathione, GSH; phenolic compounds, alkaloids, non-protein amino acids and a-tocopherols) antioxidant defense systems working in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS (reviewed in Das and Roychoudhury, 2014). However, the regulation of enzymatic and non-enzymatic antioxidant in reducing oxidative damage is interrelated and complex (Apel and Hirt, 2004; Noctor et al., 2012; Rai et al., 2013) (Fig. 1).

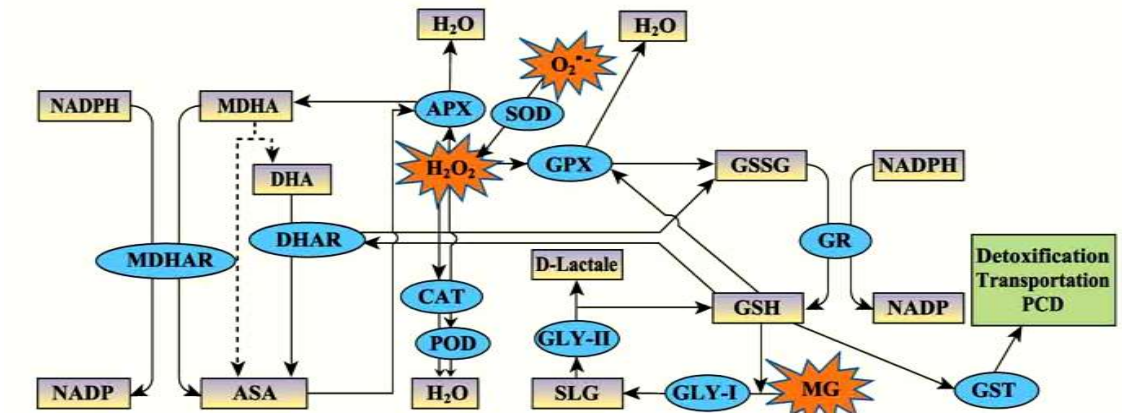

Fig. 1. Schematic presentation of antioxidant protective system in plant. SOD-Superoxide dismutase; POD- Peroxidase; CAT- Catalase; GPX-Glutathione peroxidase; GST-Glutathione *S*-transferase; APX- Ascorbate peroxidase; MDHAR-Monodehydroascorbate reductase; DHAR- Dehydroascorbate reductase; GR-Glutathione reductase; ASA- Ascorbic acid; DHA- Dehydroascorbate; GSH-Reduced glutathione; GSSG- Oxidized glutathione; MG- Methylglyoxal; Gly-I-Glyoxalase-I; Gly-II- Glyoxalase-II. (Hossain et al., 2011)

It is established that, oxidative stress under drought and salinity causes expressions of new genes and antioxidative apparatus in combating/reducing the damage caused by stress (reviewed in Gill and Tujeta, 2010; AbdElgawad et al., 2016). Although a significant knowledge has been achieved, the tolerance mechanism is not fully understood. Recently, we have been investigating the oxidative stress tolerance mechanism under salinity and drought in maize in Molecular Breeding Lab of Plant Breeding Division at Bangladesh Agricultural Research Institute. We have also achieved some results at activity level of stress related antioxidants, and they have been published in international reputed journals (Rohman et al., 2016a, b). It is interesting that different isozymes under stress are inhibited or changes into another form with higher activity. In-gel activity analysis provides such type data to identify active enzyme(s) related to stress tolerance. At the same time, expression of genes related to

antioxidative defense depends on many transcriptional and post transcriptional factors. Consequently, the relation between gene expression and protein accumulation might important information in combating oxidative stress under drought and salinity. Although a good number of studied demonstrated the regulation of specific activities enzymatic antioxidative protein, very few studies demonstrated specific isozymes responsible to regulate the specific activities. With this view, to identify specific stress inducible enzymatic proteins in stress signaling pathway, further work on separation of isozymes is essential. This work could provide important causes of susceptibility and tolerance of a genotype along with the frontiers to improve the tolerance of maize to drought and salinity.

7. Sub-project goal: Insight into oxidative stress tolerance mechanism in maize under drought and salinity
8. Sub-project objective (s):
    1. Understanding oxidative stress tolerance in maize at physiological, biochemical and molecular level in contrast maize genotypes in relation to tolerance to drought and salinity.
    2. Protocol development for measuring expression of inducible protein.
    3. Identification of important inducible protein under drought and saline condition for further biotechnological work.

9. Implementing location (s): Plant Breeding Division, Bangladesh Agricultural Research Institute, Gazipur

10. Methodology in brief:

Screening of plant materials: Screening for salinity and drought tolerance was done to obtain relatively tolerant genotypes for each stress. For salinity stress, seedlings of fifty hybrids and inbreds were screened. Seedlings of each genotype were grown on rock media in tray under greenhouse condition. Five days old seedlings were transferred to screening pots containing Hoagland's solution, each pot is set with oxygen channel. After two days hardening, the seedlings were subjected to NaCl induced 16 dSm^{-1} salinity to observe their tolerance. The seedlings were observed for 30 days. After every 3 days, the solution was changed. Tolerant and susceptible genotypes were selected based on phenotypic difference. On the other hand, drought screening was imposed against 40 maize genotypes. For drought tolerance screening, seedlings were grown in 35 L bucket where soil was used as growing media. One month old seedlings were subjected to water withdrawal for observing drought tolerance, and were observed till attaining soil moisture of 12.5% field capacity (FC).

Stress for isozyme analysis: For salinity stress, five days old seedlings of selected genotypes were transferred to a hydroponic system containing Hoagland's solution. After two days hardening, the seedlings were subjected to NaCl induced 12 dSm^{-1} salinity, and observed for 9 days. After every 3 days, the treatment solution was changed. On 3, 6 and 9 day the data were recorded from fully expanded leaves and the seedlings were allowed to grow in saline free media for recovery. For drought stress, similar method was followed as stated above. The

selected genotypes were subjected to water withdrawal to impose drought stress, and samplings were done for data analysis when the soil moisture reached to 12.5% of FC.

Enzyme extraction and protein measurement:
Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 mL of 50 mM ice-cold K-P buffer (pH 7.0) containing 100 mM KCl, 1mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500×g for 10 min and the supernatants were used for determination of enzyme activity. All procedures were performed at 0–4^{0}C. Protein was measured following Bradford (1976) using BSA as standard.

MDHAR (EC: 1.6.5.4): MDHAR activity was determined following Hossain et al. (2010). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM ASA, and 0.5 unit of AO and enzyme solution in a final volume of 0.7 ml. The reaction was started by the addition of AO. The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

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

GR (EC: 1.6.4.2): GR activity was measured by the method of Hossain et al. (2010). The reaction mixture contained 0.1 M K-P buffer (pH 7.8), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 ml. The reaction was initiated with GSSG, and the decrease in absorbance at 340 nm due to NADPH oxidation was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 mM^{-1} cm^{-1}**.**

GST (EC: 2.5.1.18): GST activity was determined following Rohman et al. (2010). The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and enzyme solution in a final volume of 0.7 ml. The enzyme reaction was initiated by the addition of CDNB, and the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Gly-I (EC: 4.4.1.5): Gly-I assay was carried out according to Yadav et al. (2005a). Briefly, the assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM reduced glutathione, and 3.5 mM MG in a final volume of 0.7 ml. The reaction was started by the addition of MG, and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 mM^{-1} cm^{-1}.

Measurement of the $O_2^{\bullet-}$ generation rate and H_2O_2
$O_2^{\bullet-}$was determined according to Elstner and Heupel (1976). The $O_2^{\bullet-}$ concentration was calculated from a standard curve of $NaNO_2$. H_2O_2 was assayed according to the method

described by Yu et al. (2003). The H_2O_2 content was determined by using extinction co-efficient of 0.28 μM^{-1} cm^{-1} and expressed as micromoles per gram FW.

Measurement of proline

Ninhydrin was used to produce proline's reaction which was used to estimate proline following the method of Bates et al. (1973).

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating melondialdehyde (MDA), a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material for MDHAR (EC: 1.6.5.4): MDHAR activity was determined by the method of Hossain et al. (2010). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM ASA, and 0.5 unit of AO and enzyme solution in a final volume of 0.7 ml. The reaction was started by the addition of AO. The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

Measurement of MG

About 0.3 g leaf tissue was extracted in 3 ml of 5% perchloric acid and centrifuged at 4°C at 11,000×*g* for 10 min. The supernatant was decolorized by adding charcoal (10 mg/ml) and centrifuged at 11,000×*g* for 10 min. The supernatant was neutralized by 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 following Wild et al. (2012). 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.

Measurement of K^+/Na^+

The sap was extracted from leaves and was put on compact Na+ ion meter (Horiba-731, Japan) and compact K^+ ion meter (Horiba722, Japan) to estimate the Na^+ and K^+ ions in leaves. The K^+/Na^+ ratio was measured from the estimated values.

11. Results and discussion:

Selection of suitable genotypes for salinity tolerance

For salinity stress, seedlings of fifty hybrids and inbreds, collected from Plant Breeding Division, Bangladesh Agricultural Research Institute, were screened. Seedlings of each genotype were grown on rock media under greenhouse condition. Five days old seedlings were transferred to hydroponic pots containing Hoagland's solution, each pot is set with oxygen channel. After two days hardening, the seedlings were subjected to NaCl induced 16 dSm^{-1} equivalent salinity for primary screening against salinity. Based on phenotype, one relatively tolerant hybrid (P1xP7), one susceptible hybrid (BHM-5) and two moderately tolerant hybrids (BHM-7 and BHM-9) were selected for salinity inducible protein analysis (Fig. 1). Importantly, in leaves of susceptible genotype, ROS, Na^+/K^+ and MDA were much higher that other genotypes (1C,D,F). At the same time, proline, a crucial low molecular substance with antioxidant and gene activation role, was higher in tolerant genotypes (Fig.

1F). Finally, higher production of ROS was conformed NOX analysis (Fig. 1E), a cell membrane bound enzymatic protein responsible for oxidative stress.

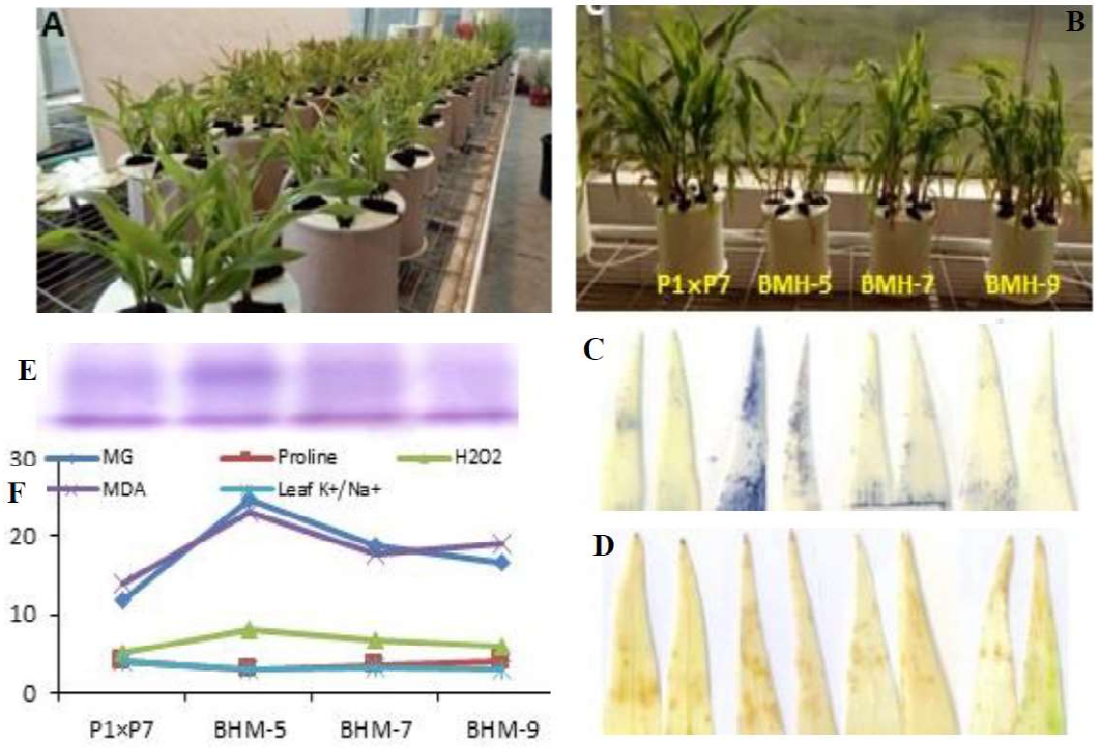

Fig. 1. Comparative phenotypes and stress markers for selecting salinity tolerance genotypes of maize: A) salinity screening block, B) Selected genotypes, C) histochemical detection of $O_2^{\bullet-}$, D) histochemical detection of H_2O_2. E) NOX activity and F) biochemical stress markers

Selection of drought tolerance genotypes

For drought tolerance, 40 maize genotypes were screened. One month old seedlings were subjected to water withdrawal for observing drought tolerance. Among them, three genotypes BHM-12 and BHM-13 were considered as relatively tolerant genotypes. Whereas, BHM-5, CZ-42 and P4×P7 were selected as susceptible andBHM-9 and P1xP7 were found as moderately tolerant. Accordingly, BHM-12, BHM-13, BHM-5, BHM-9 and P1xP7 were used for isozyme detection. These five genotypes were analysed for tolerance markers where, susceptible genotypes showed higher ROS, MDA and MG accumulation (Fig. 2). Here also susceptible genotype showed comparatively lower amount of proline synthesis.

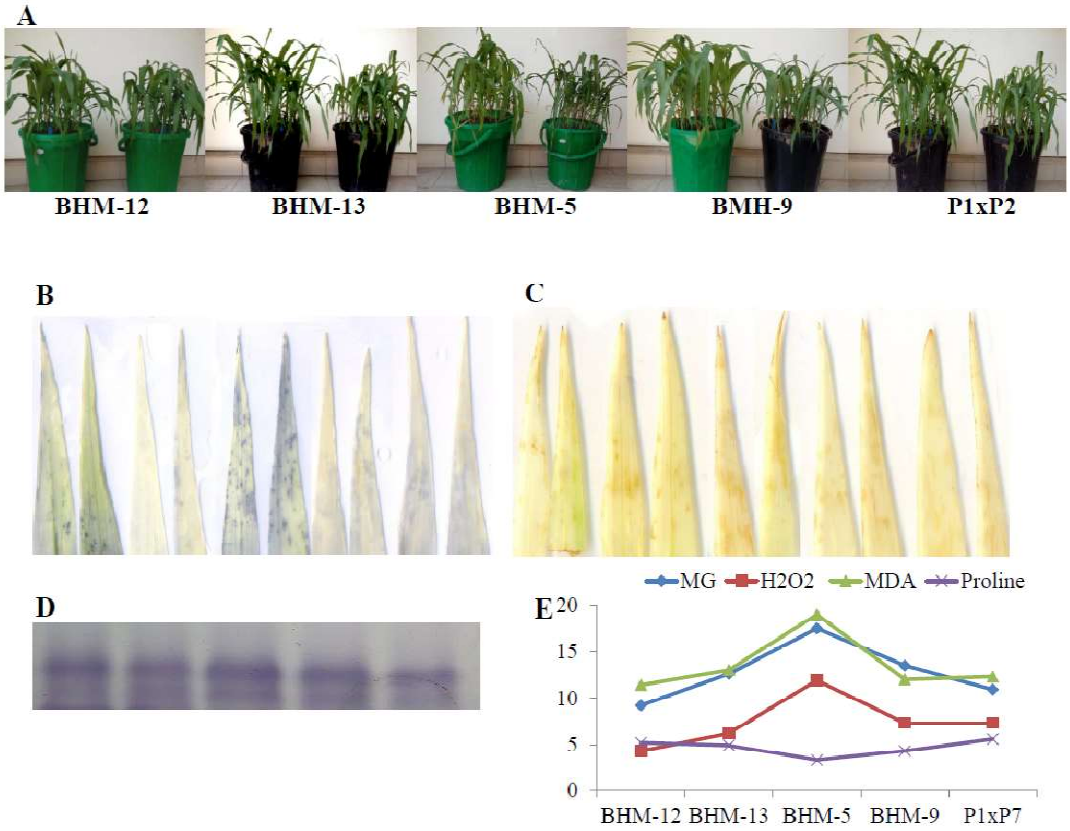

Fig. 2. Comparative phenotypes and stress markers for selecting drought tolerant maize genotypes: A) Selected genotypes for drought related enzymatic study, B) histochemical detection of $O_2^{\bullet-}$, C) histochemical detection of H_2O_2, D) magnitude of expression of NOX and E) Biochemical stress markers, MG, MDA and proline

Protocol development and Improvement

In this subproject, some important protocol like In-gel activity of SOD, POD, CAT, APX and GPX were developed, and important anaerobic enzymatic assay methodlike Alcohol dehydrogenase, Pyruvate decarboxylase and Lactate dehydrogenase were improved. These protocols were much convenient and economic than the existing ones. Importantly, protocols of anaerobic enzymes were economic than existing protocols. Here, we used 700µl reaction mixture whereas other protocols used 3.0 ml reaction mixture. On the other hand, we sued 0.5 unit enzyme instead of 3 unit. Remarkably, in this study, 1.0 ml semi-microcell (cuvette) was used. The methodologies of the protocols are given below.

Native PAGE and In-gel activity staining

Changes in proteins having isoenzymic activity of the ROS scavenging enzymes were studied using PAGE under non-reduced, non-denatured conditions at 4^0C according to Laemmli (1970). Native PAGE analysis was performed for various enzymes involved in the ascorbate-glutathione cycle on a gel (10% for SOD and 8% for others). Specific procedures for running and staining of gels for different enzymes are given below as protocol format.

SOD:

- Prepare staining solution (Potassium phosphate buffer, pH 7.8, containing 0.0068 g l^{-1}, 0.0175 g $l^{-1}Na_2HPO_4$, 0.372 g l^{-1}EDTA, 31% (v/v) TEMED, 7.5 mg l^{-1} riboflavin and 0.2 g l^{-1} NBT)
- Incubate the gel for 30 min in the dark at room temperature
- Expose the gel to white light until SOD activity bands become visible
- Take snap with a photography system

POD:

- Prepare staining solution of 50mM potassium buffer (pH 7.0) containing 10mM guaiacol and 10mM H_2O_2
- Pour down the gel into the staining solution
- POD band will be appeared soon
- Take snapwith a photography system

APX:

- For the analysis of APX activity, add 2 mM Ascorbate to the electrode buffer
- Run the gel in pre-run for 30 min before the samples loading
- Run the gel in running buffer for 2 hours
- Equilibrate the gel with 50mM Sodium Phosphate buffer (pH 7.0) containing 2mM ascorbate for 30 min
- Incubate the gel in a solution composed of 50mM Sodium phosphate buffer (pH 7.0), 4mM ascorbate and 2mM H_2O_2 for 20 min
- Wash the gel in the buffer for 1min and submerge in a solution of 50mM Sodium phosphate buffer (pH 7.8) containing 28mM TEMED and 2.45 mM NBT for 10-20 min with gentle agitation by shaker.
- After band visualized take photograph.

GPX:

- Finish the gel running
- Wash the gel for 15 min in 2.5% Triton-X-100
- Incubated the gel in 100 ddH_2O for 15 minutes
- Immerse the gel in 10 mM K-P buffer (pH 7.2) containing 2mM o-Dianisidine for 1 hour
- After that immerse in 10 mM H_2O_2 until band appear
- Take photograph

GR:

- Finish the gel running
- Incubate the gel in Tris-HCl buffer (10mM, pH 7.9) containing oxidized glutathione (4mM GSSG), NADPH (1.5mM), and 5,5'-dithiobis, 2-nitrobenzoic acid (2mM DTNB) for 20 min
- Incubate the gel Tris-HCl buffer (10mM, pH 7.9) containing 1.2 mM MTT and 1.6

mM PMS for 5-10 minutes

- The band will appear soonest and take photograph

NOX:

- Finish the gel running
- Immerse the gel in 50 mM Tris-HCl (pH 7.4) buffer containing 0.2 mM NBT; 0.1 mM $MgCl_2.6H_2O$ and 1 mM $CaCl_2$ for twenty minutes in dark
- Add 0.2 mM NADPH in the solution
- Shake until the band appear
- Take photograph

GST:

- Finish the gel running
- Incubate the gel to dye solution containing 4.5 mM GSH, 1 mM CDNB and 1 mM NBT in 0.1 M KP buffer pH 6.5
- Stain the GST band with a solution containing 3 mM PMS prepared in 0.1M Tris-HCl pH 9.6
- Take photograph

CAT:

- Finish the gel running
- Soak the gel in DW for 3x15 minutes
- Immerse the gel in 0.03% H_2O_2 for 10 minutes
- Rinse shortly in DW
- Pour 2% FeCl3 and 2% KFeCN solution on the gel at a time
- Rinse with DW after appearing band
- Take photograph

Protocol of enzymes under anaerobic condition

Alcohol Dehydrogenase (EC 1.1.1.1)

Spectrophotometric assay (A_{340}, Light path = 1 cm, path width 3 mm, temperature 25°C)

Principal is based on the following reaction:

$$\text{Aldehyde} + \beta\text{-NADH} \xrightarrow{\text{Alcohol dehydrogenase}} \text{Ethanol} + \beta\text{-NAD}$$

β-NADH = β-nicotinamide adenine dinucleotide phosphate, reduced
β-NAD = β-nicotinamide adenine dinucleotide phosphate, oxidized

Procedure

- Prepare 1.25 mM Tris-HCl buffer (pH 7.5). take 590 µl in cuvette
- Prepare 50 µM NADH in 1.25 mM Tris-HCl buffer (pH 7.5). from here take 87.5 µl in cuvette
- Prepare 1.25 mM Acetaldehyde in 1.25 mM Tris-HCl buffer (pH 7.5). take 17.5 µl in cuvette
- Lastly, add 5 µl sample in cuvette

Unit definition: One unit will convert 1.0 µmole of acetaldehyde to ethanol per minute at pH 7.5 at 25 °C.

Calculation:
Specific activity
$=(\Delta A340 \times reactionmixture \times df)/(6.22 \times Proteinconcentration\ (mg/ml) \times Vol.of enzyme(ml) usedinreactionmixure)$
Where,
$\Delta A340$ = Absorbance changed/min
df=dilution factor
6.22= mM extinction coefficient of NADH at 340nm
Unit: mM/min/mg protein

Pyruvate decarboxylase (EC 4.1.1.1)

Spectrophotometric assay (A_{340}, Light path = 1 cm, path width 3 mm, temperature 25°C)
Principal is based on the following reaction:

$$\text{Pyruvate} \xrightarrow{\text{Pyruvate decarbixylase}} \text{Acetaldehyde} + CO_2$$

$$\text{Acetaldehyde} + \beta\text{-NADH} \xrightarrow{\text{Alcohol dehydrogenase}} \text{Ethanol} + \beta\text{-NAD}$$

β-NADH = β-nicotinamide adenine dinucleotide phosphate, reduced
β-NAD = β-nicotinamide adenine dinucleotide phosphate, oxidized

Procedure

- Prepare 5 mM MES buffer (pH 6.5). Take 543 µl in cuvette
- Prepare 20 µM NADH in 5 mM MES buffer (pH 6.5). Take 50 µl in cuvette
- Prepare 50 µM Thiamine pyrophosphate in 5 mM MES buffer (pH 6.5). Take 10 µl in cuvette
- Prepare 0.1 mM $MgCl_2.6H_2O$ in 5 mM MES buffer (pH 6.5). Take 10 µl in cuvette
- Prepare 2 mM Oxamic acid in 5 mM MES buffer (pH 6.5). Take 70 µl in cuvette
- Add 5 µl of ADH in cuvette (1 unit)
- Take 5 µl sample in cuvette
- Prepare 1 mM Na-pyruvate in 5 mM MES buffer (pH 6.5). Take 7 µl in cuvette

Calculation:
Specific activity
$=(\Delta A340 \times reactionmixture \times df)/(6.22 \times Proteinconcentration\ (mg/ml) \times Vol.of enzyme(ml) usedinreactionmixure)$
Where,
$\Delta A340$ = Absorbance changed/min
6.22= mM extinction coefficient of NADH at 340 nm

df= dilution factor

Unit definition: One unit will convert 1.0 µmole of pyruvate to acetaldehyde per minute at pH 6.5 at 25 °C.

Lactate Dehydrogenase (EC 1.1.1.17)

Spectrophotometric assay (A_{340}, Light path = 1 cm, path width 3 mm, temperature 25°C)

Principal is based on the following reaction:

$$\text{Pyruvate} + \beta\text{-NADH} \xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}$$

Procedure

- Prepare 10 mM KP buffer (pH 7). Take 508 µl in cuvette
- Prepare 50 µM NADH in 10 mM KP buffer (pH 7). Take 175 µl in cuvette
- Prepare 0.75 µM KCN in 10 mM KP buffer (pH 7). Take 2 µl in cuvette
- Prepare 1 mM 4-methyl pyrazolein 10 mM KP buffer (pH 7). Take 5 µl in cuvette
- Prepare 2.5 mM Na-pyruvate in 10 mM KP buffer (pH 7). Take 5 µl in cuvette
- Finally add 5 µl sample in the cuvette.

Calculation:

Specific activity

$=(\Delta A340 \times reactionmixture \times df)/(6.22 \times Proteinconcentration\ (mg/ml) \times Vol.of enzyme(ml) usedinreactionmixure)$

Where,

$\Delta A340$ = Absorbance changed/min

df=dilution factor

6.22= mM extinction coefficient of NADH at 340nm

Unit definition: One unit of lactate dehydrogenase will convert 1.0 µmole of pyruvate to lactate per minute at pH 7 at 25 °C.

Comparative ROS regeneration in selected genotypes under salinity and drought

Before investigating salinity inducible protein, ROS were examined as stress inducible protein are related with ROS metabolism. Superoxide ($O_2^{\bullet-}$) is the first radical that cause substantial damage of DNA, protein, lipid and pigments (Gill and Tujeta, 2010). In this study, $O_2^{\bullet-}$ generation rate increased with the stress duration in all the genotypes under both aerobic and anaerobic salinity stress (Fig. 3A). Importantly, the generation rate was remarkably lower in tolerant genotype P1xP7 than the other genotypes, while it was the highest in susceptible genotype BHM-5. The generation rate of $O_2^{\bullet-}$ in moderately tolerant genotypes, BHM-7 and BHM-9, was amid of tolerant and susceptible genotypes. Interestingly, the generation rate decreased in recovery in all the genotypes in both aerobic and anaerobic conditions. On the other hand, under drought stress, remarkable increment $O_2^{\bullet-}$ generation in all the genotypes as compare to respective control (Fig. 3B). However, the magnitude in susceptible genotype BHM-5 was much higher than the tolerant genotypes, BHM-12 and BHM-13. On the other hand, $O_2^{\bullet-}$ generation in BHM-9 and P1xP7 was almost similar to that in BHM-12 and BHM-13.

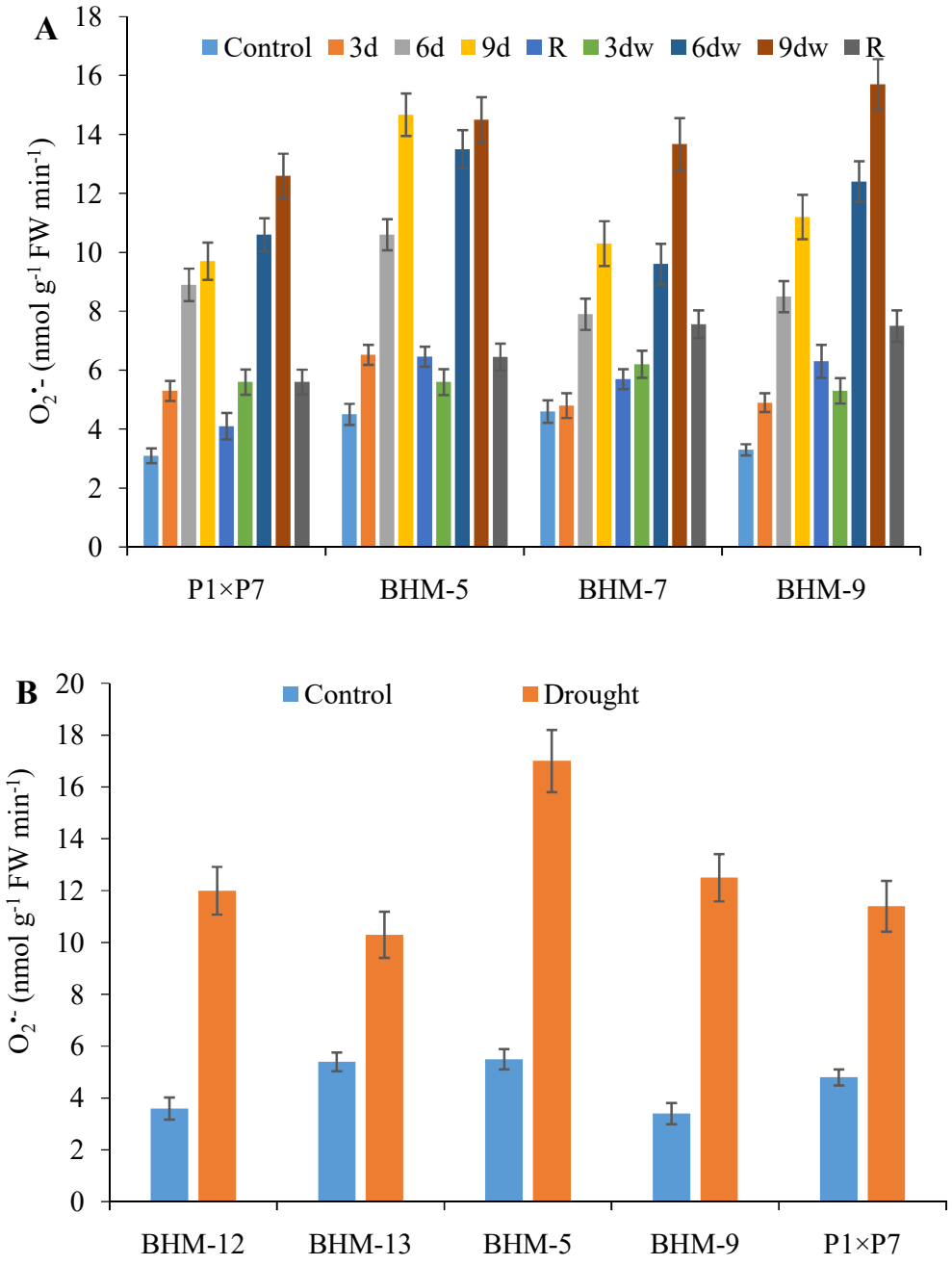

Fig. 3. Comparative $O_2^{\bullet-}$ generation rate in selected genotypes under salinity(A) and drought (B). d: days of stress under aerobic salinity, dw: days of stress under anaerobic salinity and R: recovery

Like $O_2^{\bullet-}$, H_2O_2 accumulated in all the genotypes under salinity and drought stress (Fig. 4). In case of salinity stress, the tolerant genotype P1xP7 produced the least amount of H_2O_2 (Fig. 4). On the other hand, accumulation was comparatively higher in susceptible genotype BHM-5 which was almost similar to BHM-7 and BHM-9. Between the tolerant genotypes, BHM-13 produced the lowest amount of H_2O_2. The highest concentration of H_2O_2 supported the results of histochemical detection (Fig. 2).

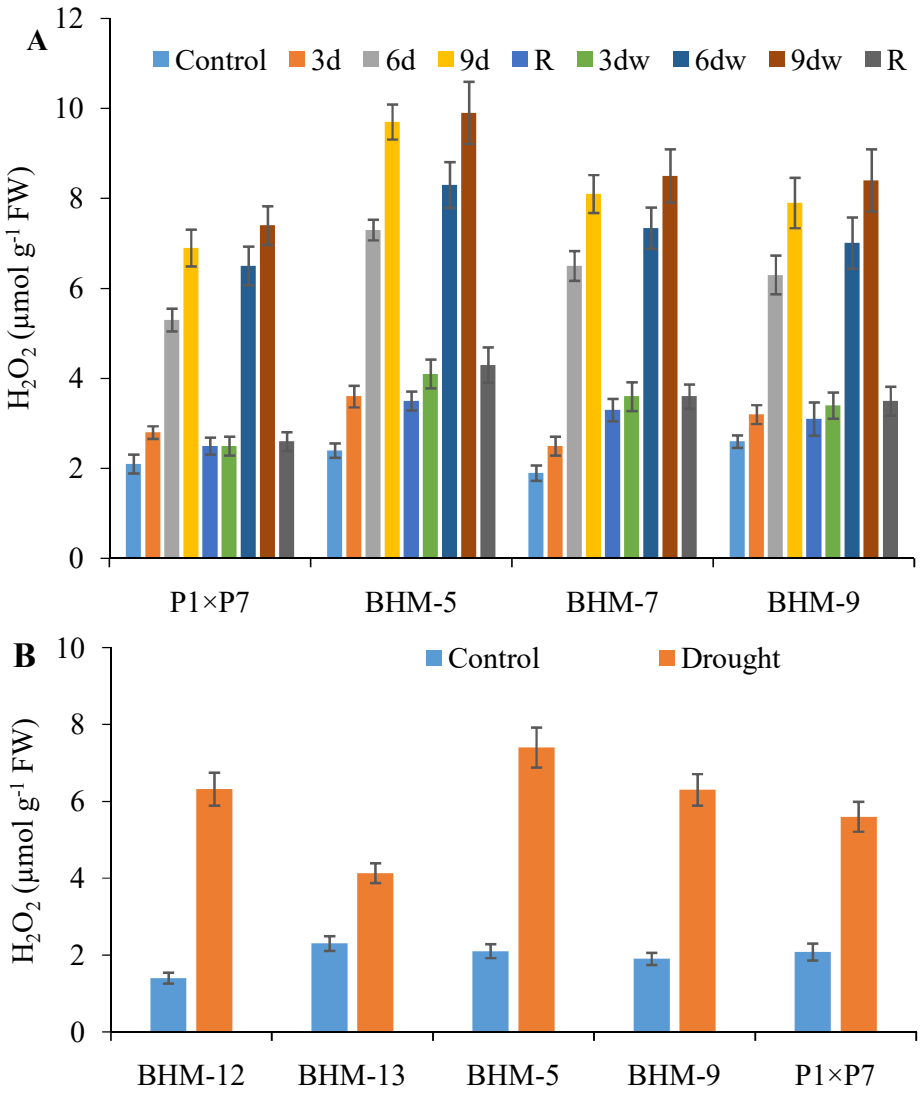

Fig. 4. Comparative H_2O_2 concentration in leaves of genotypes under salinity (A) and drought (B). d: days of stress under aerobic salinity, dw: days of stress under anaerobic salinity and R: recovery

Molecular oxygen is an essential and a relatively stable molecule and non-reactive to living cells. However, when oxygen receives extra energy or electrons under environmental stressful condition, it generates a variety of ROS causing oxidative damage to cellular organs including lipids, proteins and nucleic acids. The most common ROS are singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$). Triplet oxygen has two unpaired electrons with parallel spin located indifferent orbitals. Upon receiving extra energy from a photosensitizer such as chlorophyll, these two electrons show anti-parallel spin increasing the oxidizing power of oxygen (singlet oxygen) (reviewed in Krieger-Liszkay 2004). When triplet oxygen receives an electron, it produces $O_2^{\bullet-}$, which generates H_2O_2 and $OH^{\bullet}$ through a series of chemical conversions (reviewed in Apel and Hirt, 2004). In photosynthesis, light energy is

captured by Photosystem II and I (PSI and PSII) and used to excite electrons which go through a series of electron transport reactions. It is estimated that about 10% of the photosynthetic electrons leak from the photosynthetic electron transport chains to oxygen as a final electron acceptor (Mehler reaction) resulting in the formation of $O_2^{\bullet-}$ (Foyer and Noctor, 2000). When the terminal oxidases-cytochrome c oxidase and the alternative oxidase react with O_2, four electrons are transferred and H_2O is released (Gill and Tujeta, 2010). However, occasional reaction of O_2 with other ETC components and only one electron is transferred resulting in the formation of $O_2^{\bullet-}$. It has been noted that $O_2^{\bullet-}$is the first ROS to be generated in plant tissues @1-2% of O_2 consumption (Puntarulo, 1998). Usually, $O_2^{\bullet-}$ is produced during electron transport upon reduction of O_2 and also the non-cyclic pathway in the ETC of chloroplasts and other compartments of the plant cell. Reduction of O_2 can occur to $O_2^{\bullet-}$ in the ETC at the level of PSI. The $O_2^{\bullet-}$ may produce more reactive ROS like $OH^{\bullet}$ and $^1O_2$ (Elstner, 1987), These ROS is responsible for peroxidation to membrane lipids and cellular leakage. The protonation of generated $O_2^{\bullet-}$ can produce a powerful oxidizing agent, perhydroxy radical ($HO_2^{\bullet}$) in membrane surfaces negatively, which directly attack the polyunsaturated fatty acid (PUFA) (Bielski et al., 1983). Furthermore, $O_2^{\bullet-}$ can produce H_2O_2 and $OH^{\bullet}$ through Habere-Weiss's and Fenton's reactions (Apel and Hirt, 2004).

Under normal conditions, the most common ROS are $O_2^{\bullet-}$ H_2O_2 resulting from electron leakage from the photosynthetic and respiratory electron transport chains to oxygen. H_2O_2 is also produced due to photorespiration resulting from the oxygenase activity of ribulose-1,5-bisphosphatecarboxylase/oxygenase (Rubisco). Rates of photorespiration are basically controlled by the ratio of [CO_2] to [O_2]and temperature. The key feature of C_4 photosynthesis is the operation of a CO_2-concentrating mechanism in the leaves (Hatch, 1987). C_4 plant like maize uses NADP-malic enzyme-type photosynthesis (Omoto et al. 2012), and fix atmospheric CO_2 principally into oxaloacetate through phosphoenolpyruvate carboxylase in mesophyll cells. Oxaloacetate is then transported to mesophyll cell chloroplasts and reduced to malate by the NADP-dependent malate dehydrogenase enzyme. Malate is then shifted to bundle sheath cells of chloroplasts and decarboxylated by NADP-malic enzyme to provide CO_2 and reducing power. This CO_2 is fixed by via the Calvin cycle as per C_3 plants (Reviewed in Farooq et al., 2015). In that way, higher carbon dioxide contents near Rubisco in the bundle sheath cells hold down oxygenase activity and help to improve photosynthesis compared with C_3 plants (Kanai and Edwards, 1999). In this research work, higher ROS in susceptible genotypes can cause higher oxidative damage than tolerant genotypes. The comparatively lower ROS in tolerant genotypes be explained by enzymatic defense.

Regulations of antioxidants under salinity and drought

Superoxide Dismutase: Superoxide dismutase (SOD, 1.15.1.1), a metalloenzyme, plays very important role against oxidative stress in all aerobic organisms and all subcellular organelles sensitive to ROS. The enzyme SOD deployed 1st line protection and catalyzes the dismutation of $O_2^{\bullet-}$to $O_2$and H_2O_2. In the study three SODs were found under salinity (Fig. 5A,B). However, additional SOD isoform was visualized in P1×P7 under drought. Variation was observed in expression of the SODs suggesting their time dependent regulation under salinity. However, the magnitude of difference in SOD expression was not measured due to lack of facility of imaging system.

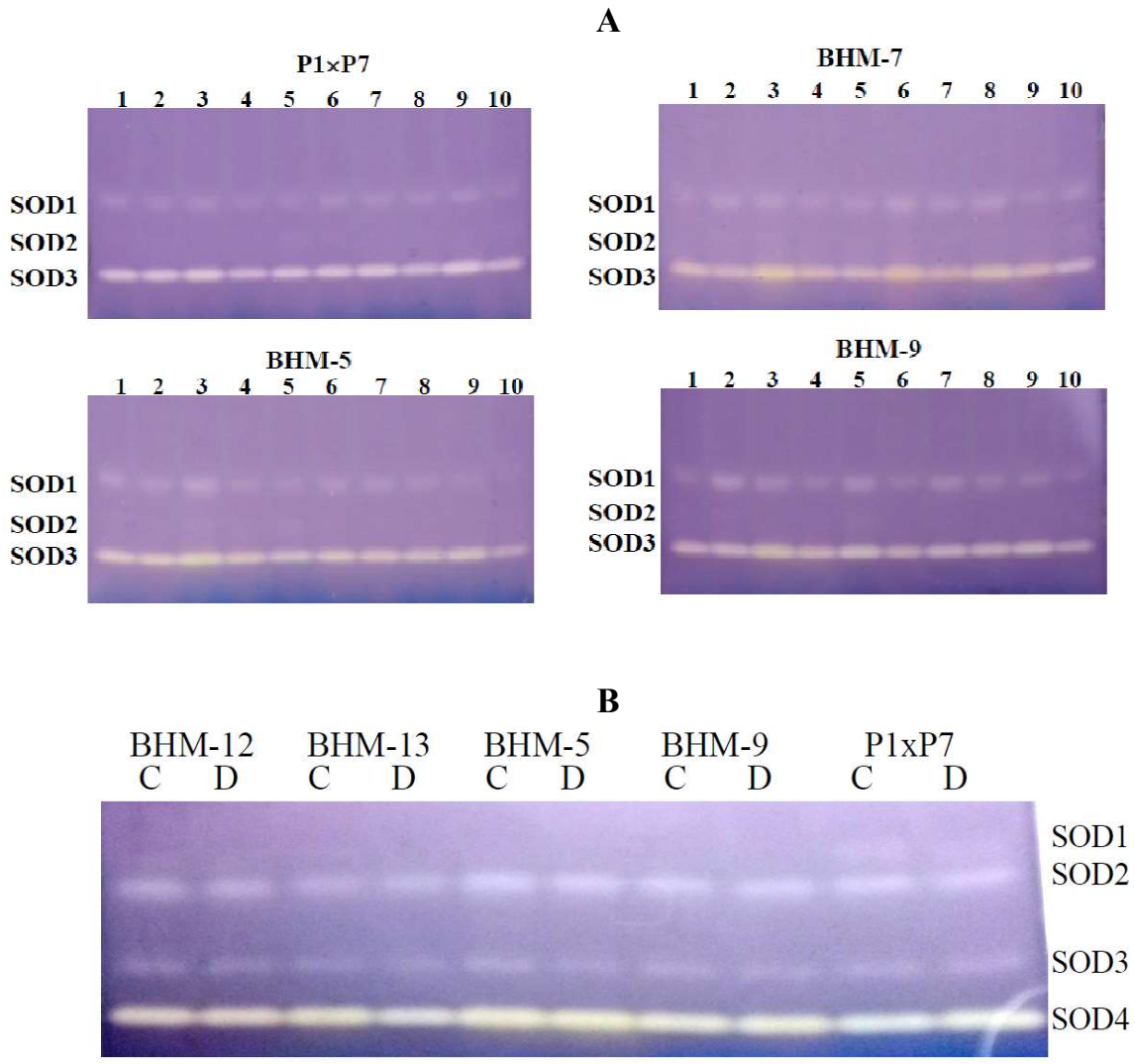

Fig. 5. Expression of SODs in-gel activity under salinity (A) and drought (B). In Fig. A, Lane1-Control, Lane2-3day aerobic salinity, Lane3-6day aerobic salinity, Lane4-9day aerobic salinity, Lane5-Recovery, Lane6-Control, Lane7-3day anaerobic salinity, Lane8-6day anaerobic salinity, Lane9-9day anaerobic salinity, Lane10-Recovery. Seven days old seedlings of different maize genotypes grown on stone were transferred to a hydroponic system with Hoagland nutrition solution containing 12 dSm^{-1} equivalent NaCl induced salinity. In Fig B, C and D indicate control and drought, respectively. Ten days seedlings were subjected to water withdrawal and sampling was done at 12.5% FC of soil in bucket. Proteins were extracted from fully expanded leaves. For each lane 50 µg proteins were loaded.

SODs are localized in different cellular compartments and according to metal cofactor they have three isozymes viz, the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron(Fe-SOD) (Mittler, 2002). The Mn-SOD is localized in the mitochondria of eukaryotic cells and in peroxisomes (del Río et al., 2003); some Cu/Zn-SOD isozymes are present in the cytosolic fractions, and also in chloroplasts of higher plants (del Río et al., 2002). The Fe-SOD isozymes are usually associated with the chloroplast compartment (Alscheret al., 2002). The Mn-SOD and

Fe-SOD are prokaryotic and the Cu/Zn-SOD enzymes are eukaryotic and dimers, whereas Mn-SOD of mitochondria are tetramers. In this study, the types SODs under salinity and drought have not yet been analyzed. Noticeably, isoforms of maize SODs in maize were not reported earlier. However, Maize SOD activity was also found to increase under chilling stress (Fryer et al., 1998). Activity of SOD isozymes have been reported under salinity in olive (Valderrama et al., 2006), drought in rice (Sharma and Dubey, 2005; Mishra et al., 2013), sugar beet (Sayfzadeh and Rashidi, 2011) and wheat (Sgherri et al., 2000).

Catalase showed significant variation in their expression both under salinity and drought (Fig. 6A,B). Total three CATs were found. It is clear that susceptible genotype BHM-5 had weaker expression as compare to other genotypes both under salinity and drought. Therefore, CAT played important role in ROS management in tolerant genotypes. However, in anaerobic salinity, CAT showed better expression in BHM-5.On the other hand, under drought, expression of CATs differed significantly among the genotypes (Fig. 6B) where CAT2 was highly expressed in BHM-7. Importantly, BHM-13, a drought loving hybrid, had highly expressed activity of CAT2.

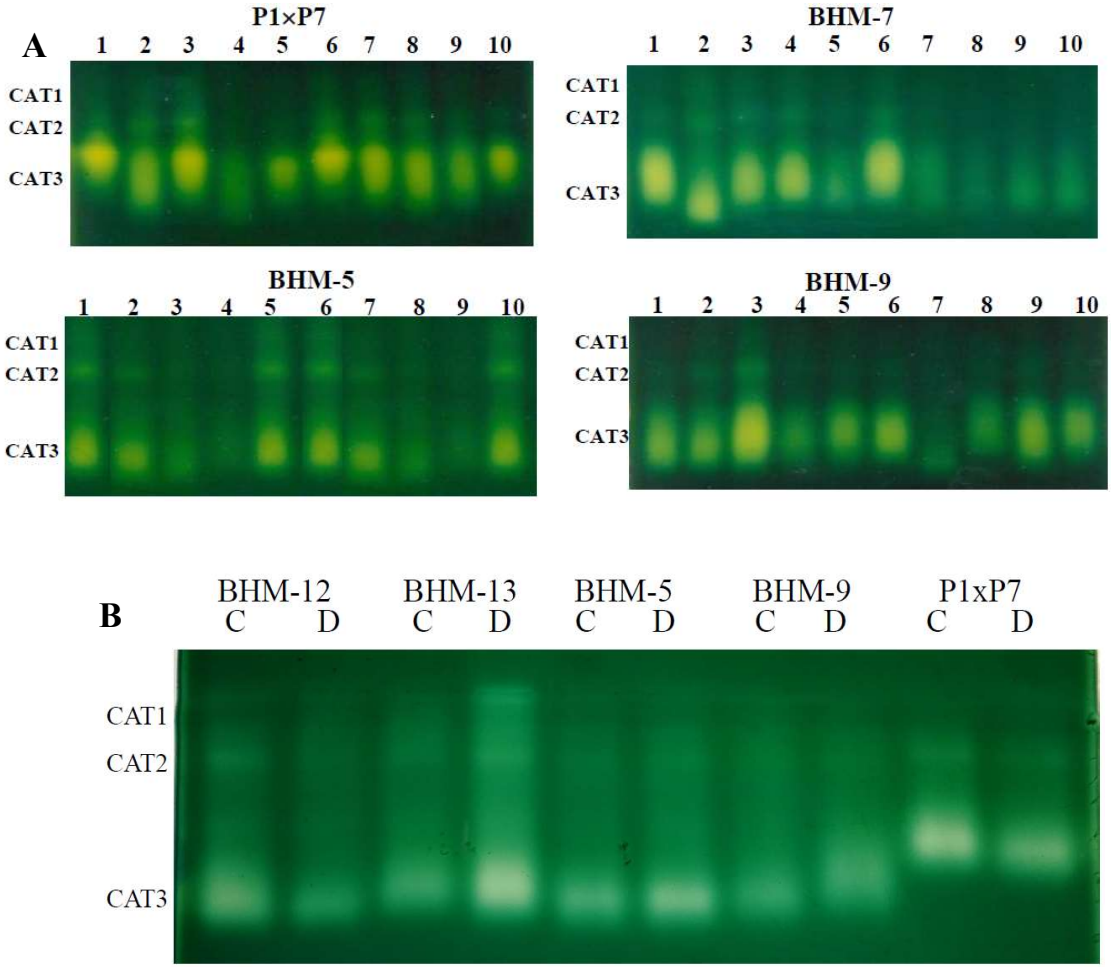

Fig. 6. Expression of CATs in-gel activity under salinity (A) and drought (B). Details are written in Fig. 5.

Catalase is a tetrameric heme-containing enzyme that catalyzes the dismutation of H_2O_2 into water and oxygen. This enzyme is important for scavenging of H_2O_2 generated in peroxisomes during photorespiratory oxidation, β-oxidation of fatty acids and purine catabolism (del Rio et al., 2006). It is considered to have the highest specificity for H_2O_2 with a very fast turnover rate. However, it is reluctant to show low affinity to organic peroxides because in enzymatic reaction, CAT is independent of other cellular reductants for instituting its activity (Scandalios, 2005). Therefore, CATs are unique as they do not require cellular reducing equivalent. As a result, it shows a much lower affinity for H_2O_2compared to APX. Three types of CATs are proposed (Willekens et al., 1995) in which Class I CATs are expressed in photosynthetic tissues and are regulated by light. Class II CATs are expressed at high levels in vascular tissues, whereas Class III CATs are highly abundant in seeds and young seedlings. CATs are reported to be changed by abiotic stresses and the activity is either enhanced or decreased depending on types and intensity of stress (Hasanuzzaman et al., 2014, Rohman et al., 2016a; Sharma et al., 2005). Three isoforms have been reported in maize (CAT1, CAT2 and CAT3) on separate chromosomes and are differentially expressed and independently regulated (Scandalias, 1990). CAT1 and CAT2 are found in peroxisomes and cytosol, whereas, CAT3 is found in mitochondria. As C_4 crop, maize was thought not to increase CAT activity. Recently, CAT activity is reported to increase in tolerant maize genotypes (Rohman et al., 2016b, Chugh et al., 2011). In this study, highly expressed CAT2 activity under drought in tolerant hybrid BHM-13 (Fig. 6)can be an important H_2O_2 scavenger.

Peroxidase

Under salinity, total three isozymes were detected in all the genotypes (Fig. 7A). However, after 6 day the activity of POD drastically decreased in susceptible genotypes BHM-5 as well in moderately tolerantly genotypes BHM-7 and BHM-9. On the other hand, continuous accumulation in P1xP7 can important role in conferring tolerance in this genotype. On the other hand, under drought stress, BHM-13 and BHM-9 had better expression of POD activity (Fig. 7B) which is a indication of scavenging role of POD in maize under drought stress. Peroxidase is a heme containing enzyme. It uses guaiacol and pyragallol at the expense of H_2O_2 (Asada, 1999). It is found in higher organism and microbes. Many POD isoenzymes are localized in vacuoles, cell wall, and cytosol of plant tissue (Asada, 1992). POD is important for biosynthesis of lignin and ethylene, degradation of IAA, , healing of wound as well as for conferring tolerance of plants under environmental stresses (Kobayashi et al., 1994). PODs are important for quencher of reactive intermediary forms of O_2 and peroxy radicals under stressed conditions (Vangronsveld and Clijsters, 1994). Induced activity of POD under salinity was reported in soybean (Weisany et al., 2012), liquorice (Pan et al., 2006) and in *Lepidium sativum* (Manaa et al., 2014) whereas drought stress induced POD activity in rape seed (Abedi Pakniyat et al., 2010) and liquorice (Pan et al., 2006). Recently, Tayefi-Nasrabadi and coworkers (2011) reported increased tolerance through induced POD activity in sunflower. POD had significant role in ROS detoxification in maize (Rohman et al., 2016a,b). The activity of maize POD is organ specified (Rios-Gonzalez et al., 2002).

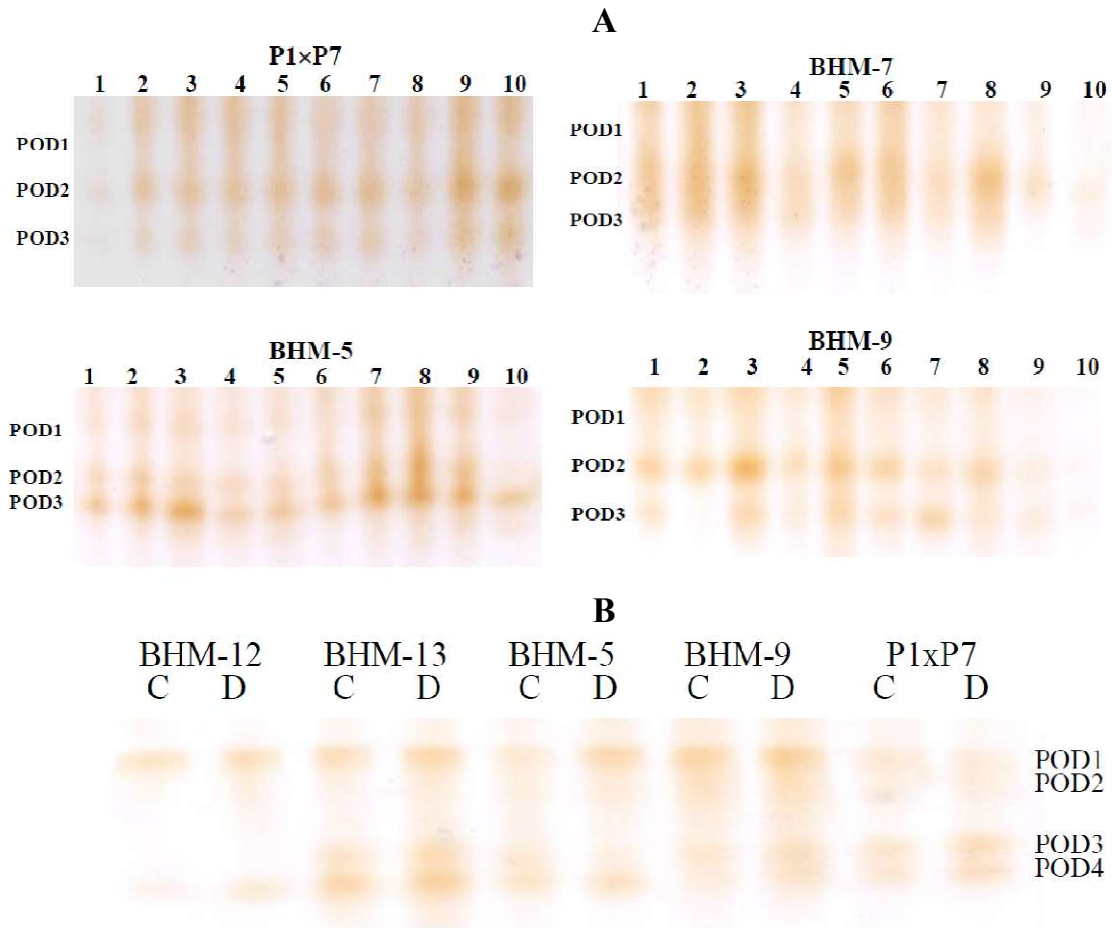

Fig. 7. Expression of PODs in-gel activity under salinity (A) and drought (B). Details are written in Fig. 5.

Ascorbate peroxidase:

Ascorbate peroxidase (APX, EC1.1.11.1) plays the most important role in scavenging ROS and thus protects cells in all living organism. After CAT, it is reported as most important ROS scavenger (Gill and Tujeta, 2010). Under salinity four APXs were found (Fig. 8A). Among which, APX1 and APX2 showed induced activity under stress in tolerant genotypes. On the other hand, Four APX were also found under drought except P1xP7 (Fig. 8B). Genotypes like BHM-13 and P1xP7 had higher activity in APX3 under drought. The APX is multigenic family consisting of at least five different isoforms including thylakoid (tAPX) and glyoxisome membrane form (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Peng et al., 2005). APX scavenges H_2O_2 in water-water and ASA-GSH cycles where ASA act as the electron donor (Apel and Hirt, 2004). Enhanced activity of APX in response to abiotic stresses including drought and salinity have been established (Sharma and Dubey, 2005, 2007; Han et al, 2009; Maheshwari and Dubey, 2009; Hefny and Abdel-Kader, 2009; Hasanuzzaman et al. 2014; Nahar et al. 2015; Ghaderi et al. 2015).

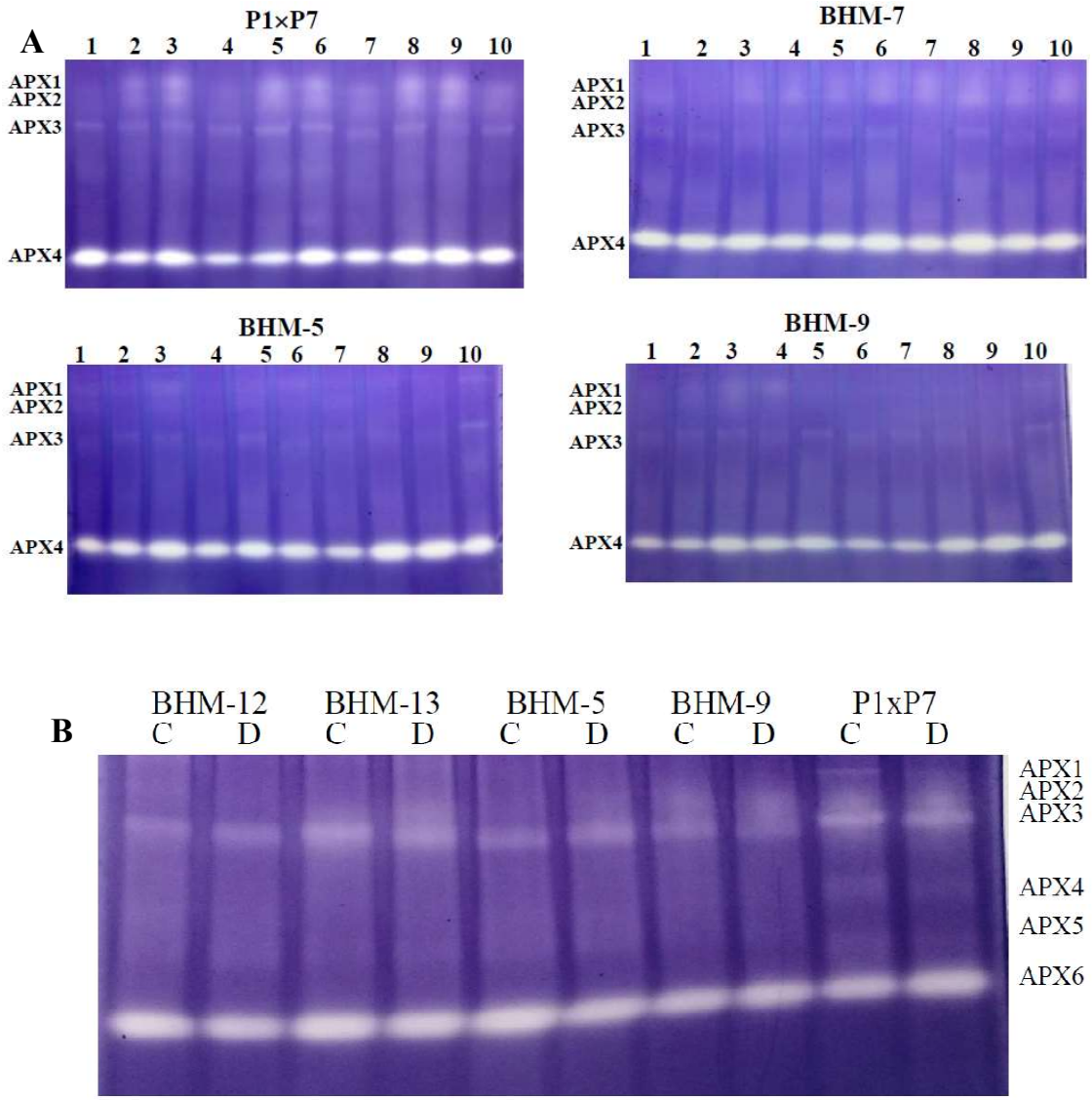

Fig. 8. Expression of APXs in-gel activity under salinity (A) and drought (B). Details are written in Fig. 5.

Regulation of APX in different crop species under various stresses had been reviewed (Gill and Tujeta, 2010). Recently, Anjum et al. (2017) found enhanced APX activity which varied with drought duration and severity where high yielder genotypes exhibited comparatively higher APX activity. Previously, we also found higher APX activity in tolerant inbreds and hybrids under both drought and salinity stress (Rohman et al., 2016a,b). AbdElgawad et al, (2016) reported higher APX activity in root of maize under salinity. Six APX homologs with primary gene/protein features have reported in maize (Ozyigit et al., 2016). In this study, under both aerobic and anaerobic saline condition, four isozymes appeared in SDS-PAGE while six isozymes were found under drought. New isozymes, particularly APX1 and APX2 under salinity in P1×P7 can play important role in H_2O_2 scavenging.

Glutathione peroxidase

Two GPX isoforms were detected in maize seedlings under saline stress (Fig. 9A). In most of the cases, the expression after 6 days decreased in aerobic condition. On the other hand, higher GPX played important role in recovery. In case of drought stress, three GPXs were detected in the selected genotypes under drought (Fig. 9B). The intensification of GPX bands in BHM-12 and BHM-13 indicated in higher accumulation under drought.

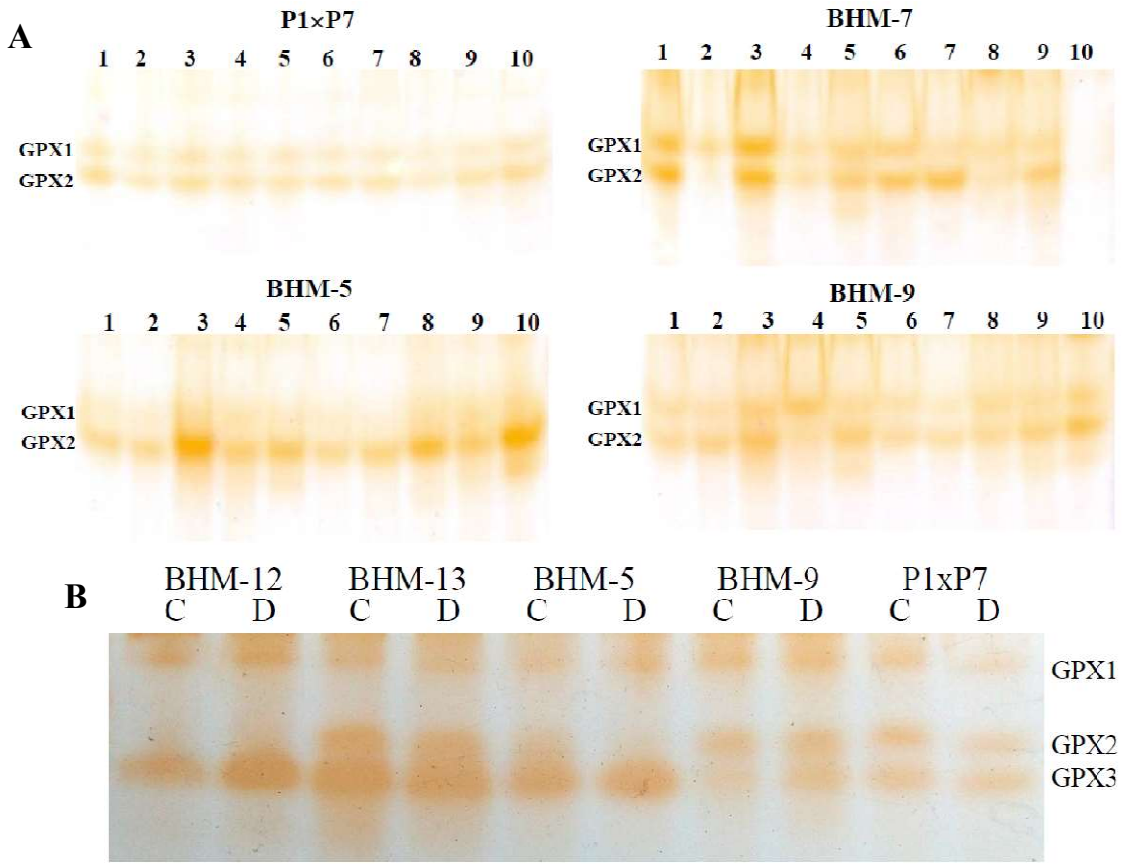

Fig. 9. Expression of GPX in-gel activity under salinity (A) and drought (B). Details are given in Fig. 5.

Glutathione peroxidase (GPXs, EC 1.11.1.9) are a large and diverse isozyme family that use GSH to reduce H_2O_2 and organic and lipid hydroperoxides to escape oxidative (Noctor et al., 2002) stress. Ozyigit et al. (2016) identified different GPX homologs in eighteen plant species most of them are localized in chloroplast, mitochondria, cytosol, and endoplasmic reticulum, where three GPX were reported in maize. Plant GPXs have cysteine residue in their active site (Koua et al., 2009), which is functional in both GSH and thiol peroxidase classes of the non-heme family. GPXs were also reported to be involved in many studies demonstrating the significant stress mitigating role under various abiotic/abiotic stress conditions such as oxidative stress, pathogen attack, metal, cold, drought and salt (Navrot et al., 2006; Diao et al., 2014; Fu, 2014; Gao et al., 2014). Overexpression of GPX in soybean, tomato showed higher tolerance against abiotic stress (Ferreira Neto et al., 2013; Herbette et al., 2011). In addition to stress response, GPXs are also thought to regulate cellular redox

homeostasis by modulating the thiol-disulfide balance (Bela et al., 2015). Upregulated GPX expression was reported to maintain redox homeostasis in *Brassica rapa* under oxidative stress (Sugimoto et al., 2014). Introduction of radish phospholipid hydroperoxide GPX gene (RsPHGPx) into yeast protected membrane damage (Yang et al. (2005). The information of regulation of GPX activity in maize under drought is limited. Rohman et al. (2016a,b) reported the importance of GPX in both inbred and hybrid under drought and salinity.

Glutathione reductase

The GR activity increased up to six days of salinity stress which decreased later (Fig. 10A). Higher activity was found in tolerant genotypes as compared with susceptible genotype BHM-5. However, the activity was almost similar under anaerobic condition. On the other hand, three GRs were detected in drought stress related genotypes (Fig. 10B). In most of the cases, GR activity was increased in in-gel activity.

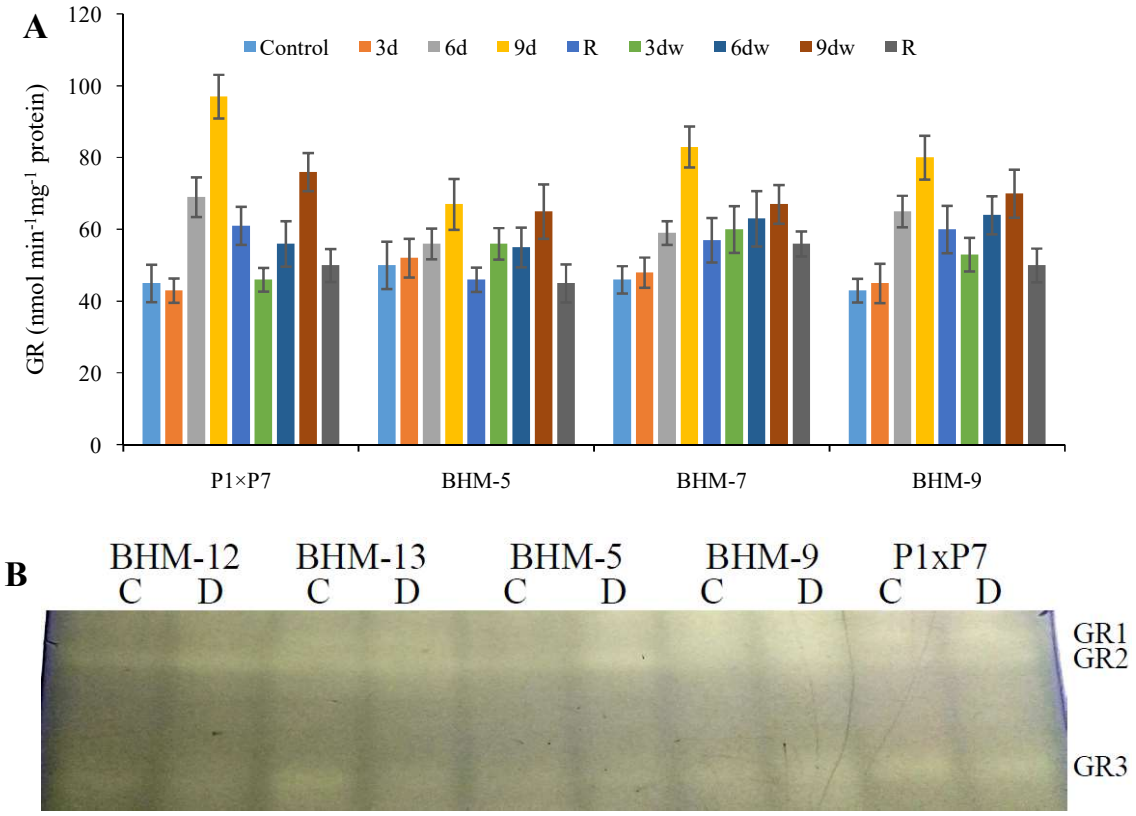

Fig. 10. Activity of GR under salinity (A) and expression of GRs in-gel activity under drought (B). Details are written in Fig. d: days of stress under aerobic salinity, dw: days of stress under anaerobic salinity and R: recovery

The changes in the ratio of GSH to GSSG and ASA to DHA and are essential for maintaining most important non-enzymatic antioxidant GSH and ASA. GR is very crucial for catalyzing the reduction of GSH, involved in many metabolic regulatory and antioxidative processes in plants in presence of NADPH (Apel and Hirt, 2004). It is located in the chloroplasts, cytosol, mitochondria, and peroxisomes, but about 80% of GR activity in photosynthetic tissues occurred as chloroplastic

isoforms (Edwards et al., 1990). Both GSH and GR in chloroplast are involved in detoxification of H_2O_2 generated by Mehler reaction. GSH participate in maintaining sulfhydryl (-SH) group, a substrate for GSTs (Noctor et al., 2012). Both GR and GSH play a vital role in maintaining the tolerance of plants under various stresses. The genotypes showing higher GR activity suggested its importance in maintaining GSH as well as ASA. The importance of GR in maintaining of GSH and as antioxidant has been reported by many studies (Noctor et al., 2012; Apel and Hirt, 2004, Hasanuzzaman et al., 2014). Caverzan et al. (2016) reported up-regulation of GR in different wheat genotypes. GR also provides tolerance in transgenic plants (Yousuf et al., 2012). Sometimes, the activity was tissue specific (Rohman et al., 2016a,b; AbdElgawad et al., 2016). Alhasan et al. (2017) studied three species of *Juncus* viz. *J. maritimus*, *J. acutus* (both halophytes) and *J. articulates* (salt-sensitive) where the GR activity was significantly higher in halophyte species over salt sensitive species.

Monodehydroascorbate Reductase (MDHAR)

The activity of MDHAR increased in all the genotypes under both salinity and drought stresses (Fig. 11). Maximum activity was observed at 6 day in tolerant and moderate tolerant genotypes in aerobic saline stress (Fig. 11A). Continual increase of the activity was found in susceptible genotypes in both aerobic and anaerobic saline stresses. In case drought stress, all the tolerant genotypes showed higher activity than the other genotypes (Fig. 11B). For ROS scavenging a high ratio of reduced ascorbate (ASA) to oxidized ascorbate and GSH to GSSG are essential in cells. In ASA-GSH cycle and dehydroascorbate reductase (DHAR) are important for maintaining ASA (Apel and Hirt, 2004). MDHAR is a flavin adenin dinucleotide (FAD) enzyme that is present as chloroplastic and cytosolic isozymes. MDHAR shows a high specificity for monodehydroasorbate (MDHA) as the electron acceptor, using NAD(P)H as the electron donor (Hossain and Asada, 1985). Hydrogen peroxide is also converted into water by the ascorbate-glutathione cycle. The reducing agent in the first reaction catalyzed by ascorbate peroxidase (APX) is ascorbate, which oxidizes into MDHA. MHDAR reduces MDA into ascorbate with the help of NAD(P)H. Dehydroascorbate (DHA) is produced spontaneously by MHDA and can be reduced to ascorbate by DHAR using of GSH to oxidize into GSSG. Sudan et al. (2015) reported stress tolerance role in finger millet under different abiotic stresses (drought, salt and UV radiation). In this study, MDHAR increased in all the genotypes in both stress (Fig. 11) and thus, the induced MDHAR activity in maize under drought and salinity can play in maintain ASA in maize. However, comparatively higher activity in tolerant genotypes under drought might be important for better tolerance of the genotypes. The role of MDHAR gene is crucial for for a mutualistic interaction between *Piriformospora indica* and *Arabidopsis* (Vadassery et al., 2009). Co-expression of MDHAR and DHAR gene confers tolerance in *Brassica rapa* (Shin et al., 2013). Leterrier et al. (2005) that the peroxisomal *MDAR1* in pea has a differential regulation that could be indicative of its specific function in peroxisomes. High MDHAR activity has been reported in rice (Hasanuzzaman et al., 2014). Maize MDHAR activity were reported earlier to be regulated under drought and salinity (AbdElgawad et al., 2016; Rohman et al., 2016a,b).

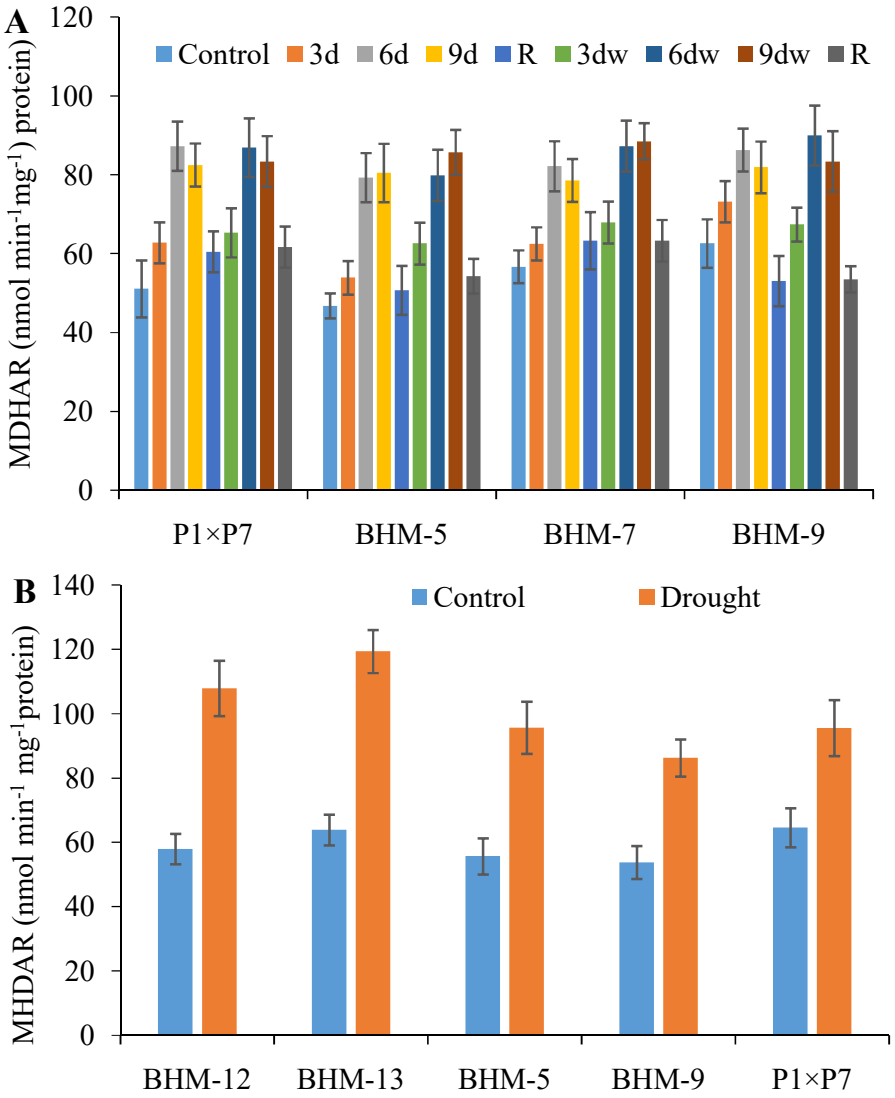

Fig. 11. Activity of MDHAR under salinity (A) and drought (B). Details are written in Fig 5. D: days of stress under aerobic salinity, dw: days of stress under anaerobic salinity and R: recovery

Dehydroascorbate reductase (DHAR)

Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity increased all the genotypes under salinity, and its increment gradually increased with increasing duration of salinity stress (Fig. 12A). It was remarkable that the susceptible genotype had the highest activity. However, the activity decreased drastically at recovery. The activity also increased in all the genotypes under drought, and the susceptible and moderate susceptible genotypes maintained higher activity as compared to tolerant genotypes (12B). DHAR maintains ASA by catalyzing the reduction of DHA by using GSH as the reducing substrate (Apel and Hirt, 2004). Thus, it is very important to maintain ASA in plant under environmental stress. Higher DHAR activity in susceptible genotypes suggested higher recover of ASA content in maize under both stress (Fig. 12A, B). In past, overexpression of DHAR increases the ASA content in tobacco, maize and potato (Chen et al., 2003; Qin et al., 2011). The role, purification and overexpression DHAR under different stresses have been reported in different crops by several reporters (Hossain and Asada, 1984; Dipierro and Borraccino, 1991; Yoshida et al., 2006;

Rubio et al., 2009; Wang et al., 1999; Hernandez et al., 2001; Sharma and Dubey 2007; Maheshwari and Dubey, 2009; Sharma and Dubey, 2005; Rubi et al., 2009; Eltayeb et al., 2011; Lu et al., 2007; Chang et al., 2017). DHAR gene expression along with MDHAR gene reported better tolerance in *Brassica rapa* (Shin et al., 2013), although they did not examine the isozymes.

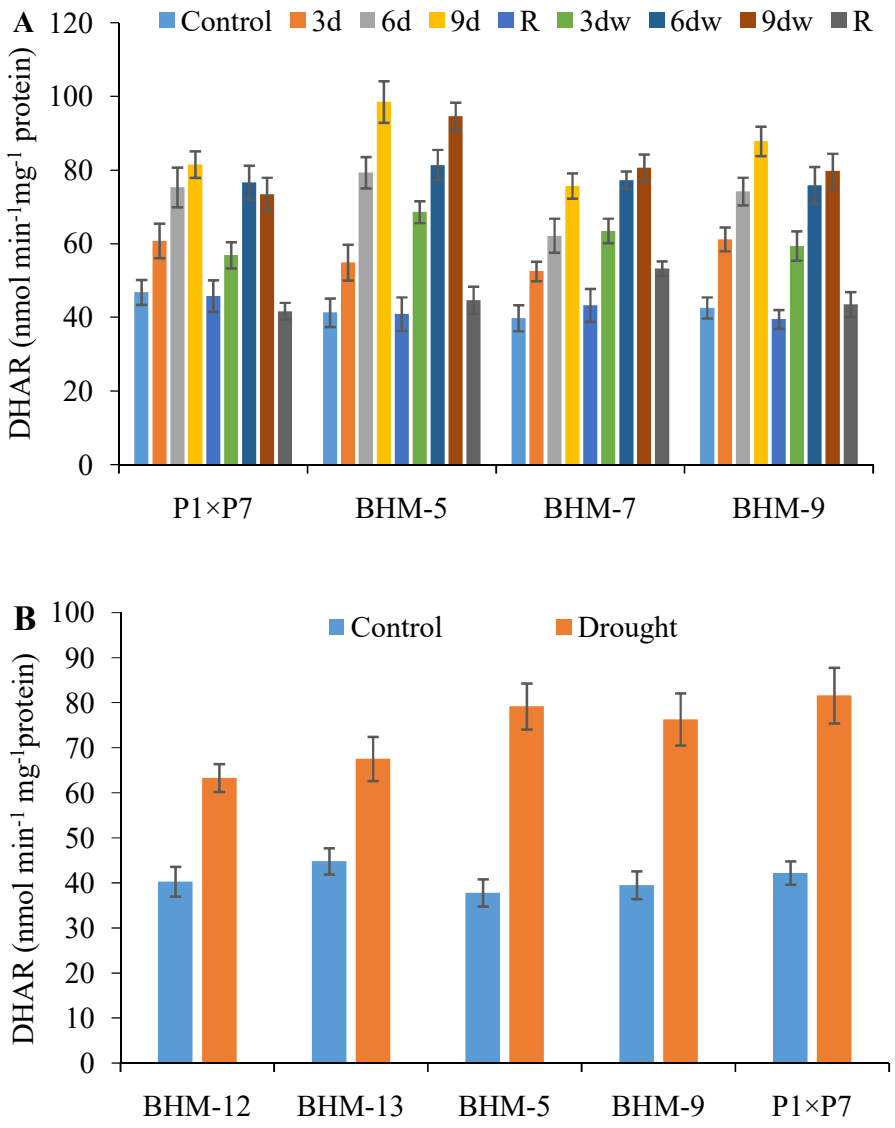

Fig. 12. Activity of DHAR under salinity (A)and drought (B). Details are written in Fig. 5. D: days of stress under aerobic salinity, dw: days of stress under anaerobic salinity and R: recovery

Glutathione *S*-transferases

The GST activity increased in all the genotypes under salinity (Fig. 13A). Importantly, susceptible genotypes showed comparatively higher activity than tolerant genotype. The in-gel activity of GST isozymes also implicated to be accumulated under drought, stress, being varied with genotypes (Fig. 13B). Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are multigenic family enzymes which catalyze

the conjugation of reduced tripeptide glutathione (GSH) to electrophilic substrates. GSTs can metabolize various toxic exogenous compounds (xenobiotics) by GSH conjugation (Cummins et al., 2011). Plant GSTs are commonly known for their role in herbicide detoxification and also considered as glutathione peroxidases. Furthermore, GSTs function as non-enzymatic carriers (ligandins) in intracellular transport and catalyze anthocyanin-GSH conjugates, thereby allowing transport into vacuoles via a glutathione pump (Marrs, 1996, Rohman et al., 2009). The GST activity increased in all the genotypes under salinity (Fig. 13A). Importantly, susceptible genotypes showed comparatively higher activity than tolerant genotype. The higher induced GST can involve in leaf senescence to reduced photosynthetic loss under the stress condition. It might also be involved in detoxify secondary toxicity of MDA (Rohman et al., 2018) or can act as glutathione peroxidases. The Higher GST activity is associated with detoxification of hydroperoxides as well as direct scavenging of H_2O_2 by showing glutathione peroxidase activity produced under stresses (Noctor et al., 2012). Remme et al. (2013) reported three homologs of maize GST, among which a GST showing higher activity was upregulated under salinity stress. Increased GST activity in maize under salinity and drought might be involved in leaf senescence (Rohman et al., 2016a,b).

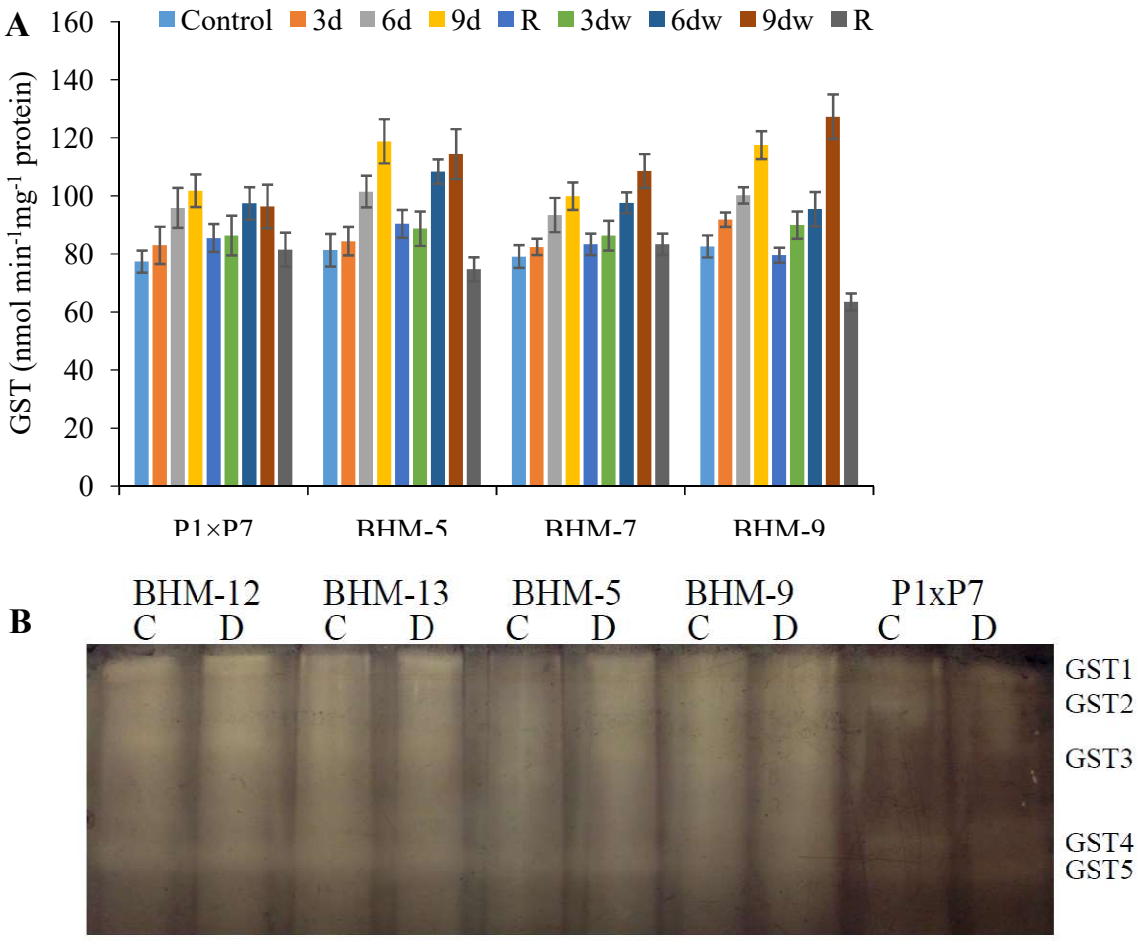

Fig. 13. Activity of GST under salinity (A) and In-gel activity under drought (B). Details are written in Fig. 5. D: days of stress under aerobic salinity, dw: days of stress under anaerobic salinity and R: recovery

Nonenzymic antioxidants like ASA and GSH play crucial roles in defense interacting with numerous cellular components. They are abundant in plants and play important role in activation of enzymatic antioxidants. Their regulations under drought salinity are reported below.

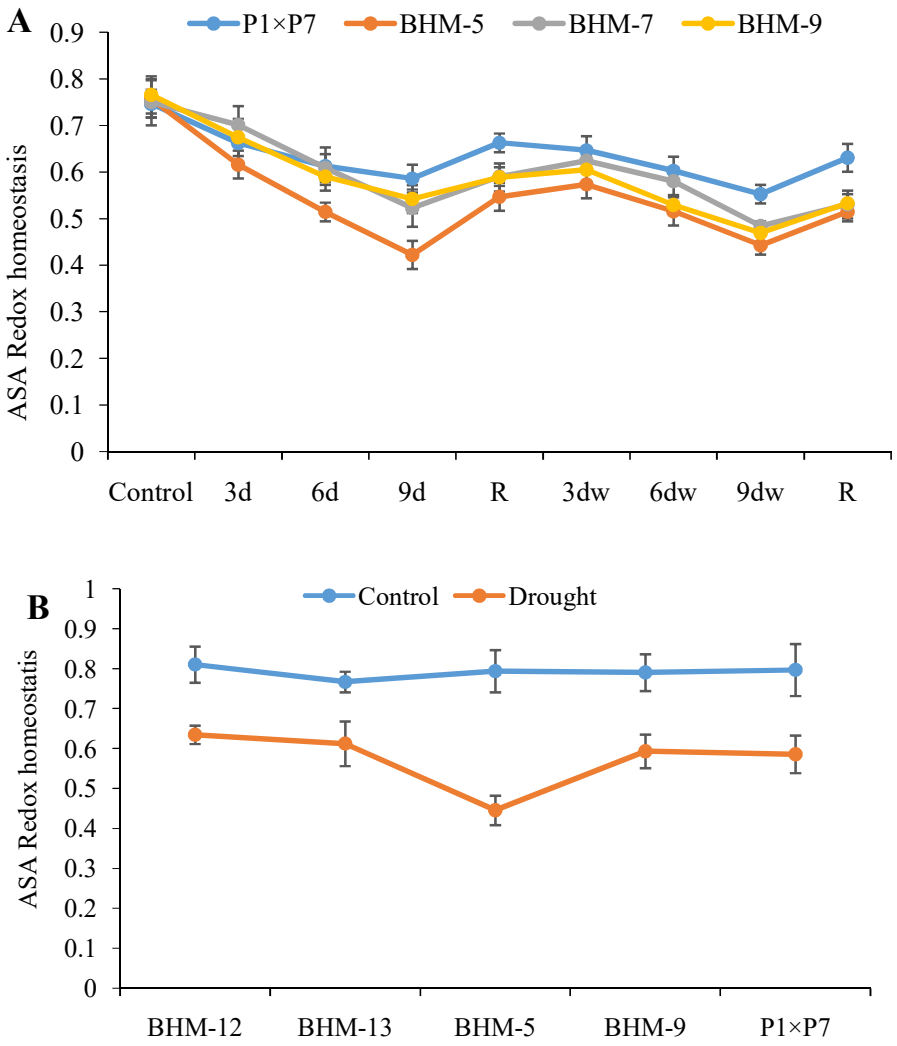

Fig. 14. ASA Redox homeostatis in maize under salinity (A) and drought (B)

Ascorbic acid Redox homeostatis [ASA/(ASA+DHA)]

Ascorbic acid Redox homeostasis is the ration of ASA to total ascobate. Fig. 14A showed that the value of homeostasis decreased with duration of saline stress where the susceptible genotype (BHM-5) had the lowest record. Similarly, the drought sensitive genotypes exhibited also lowest redox homeostasis under drought stress (Cig. 14A). Ascorbic acid is the most abundance and powerful ROS scavenger due to its ability to donate electrons in several number of enzymatic and non-enzymatic reactions. It is found in all plant tissues and be highest in mature leaves with fully developed chloroplast and highest chlorophyll. Under normal physiological condition, ASA mostly remains as reduced form in leaves and chloroplast (Szarka et al., 2007). It protects cells and organelles from ROS, over-accumulate under environmental stress including salinity and drought (Latif et al., 2016; Mukhtar et al., 2016; Naz et al., 2016). It plays important role in cell division and

expansion, photosynthesis, hormone biosynthesis and regeneration of antioxidants (Gallie, 2012; Lisko et al., 2014). It provides protection to membranes by directly scavenge the $O_2^{\bullet-}$ and $OH^{\bullet}$ and by regenerate a-tocopherol from tocopheroxyl radical (Gill and Tujeta, 2010). ASA acts as a cofactor of violaxantin de-epoxidase thus sustaining dissipation of excess excitation energy in chloroplast (Smirnoff (2000). In ASA-GSH cycle, the ASA redox system consists of reduced ASA and oxidized MDHA and DHA, both oxidized forms of ASH being relatively unstable. MDHA is very transitional and disappropriates to either ASA or DHA (Apel and Hirt, 2004). Therefore, it is very import to convert DHA into ASA to maintenance cellular redox. MDHA is reduced to ASA in presence of NADPH while DHA can be chemically reduced by GSH to ASA (Foyer and Halliwell 1976; Apel and Hirt, 2004; Akram et al., 2017). ASA in the ASH-GSH cycle, ASA not only preserves the activity of APX but also preserves the activities of enzymes containing prosthetic transition metal ions (Noctor and Foyer 1998). Under salinity stress we found gradual decrease in ASA redox homeostasis in all the genotypes (Fig. 14A). However, higher loss of homeostasis might be due to higher oxidation of ASA to DHA. Similarly, drought stress caused higher oxidation in BHM-5 (Fig. 14B) which might can make it more susceptible under the stressful condition. Chug et al. (2013) reported higher ASA contents in drought tolerant maize as compared to sensitive one at reproductive stage.

Glutathione (GSH): Redox homeostasis [GSH/(GSH+GSSG)] indicated that GSH reduced in saline and drought treated seedlings (Fig. 15). The redox homeostasis of GSH was also the lowest in susceptible genotype BHM-5 among the genotypes under both stress. Glutathione, a tripeotide of γ-Glutamyl-cysteinyl-glycine, is the most important non-enzymatic antioxidants in ASA-GSH cycle which plays central role in antioxidant defense through scavenging of ROS and maintaining of redox homeostasis in plant tissue in stressful environment (Noctor et al., 2012). It is localized in cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts and in apoplast. In physiological processes, it plays important role in detoxification of xenobiotics, transport, conjugation of metabolites, signal transduction and stress-responsive genes (Mullineaux and Rausch, 2005, Noctor et al., 2012). Besides, it is important for growth and development of tissue, cell death and senescence and pathogen resistance enzymatic regulation (Sharma et al., 2012). Plants maintain a high cellular ratio of reduced GSH to its oxidized form GSSG (about 20:1 in unstressed conditions) and balance between the GSH and GSSG is a central component in maintaining cellular redox state (Labudda and Azam, 2014; Foyer and Noctor, 2005). GSH necessary active the functions GPXs, GSTs and glyoxalases where GR plays central role in recycling of GSH from GSSG in presence of NADPH (Fig. 6). In ASA-GSH cycle, regeneration of ASA is extremely important because fully oxidized DHA has a short half-life and would be lost unless it is reduced back. GSH is crucial important to maintain ASA (Apel and Hirt, 2004). Therefore, GSH plays key role in ROS in stress tolerance under oxidative stress in association of other ROS metabolizing enzymes. In this study, salinity stress caused gradual decrease in GSH redox homeostasis in all the genotypes (Fig. 15A). However, higher loss of homeostasis might be due to higher oxidation of GSH to GSSG where susceptible genotype BHM-5 had the lowest redox capacity. Similarly, drought stress caused higher oxidation in BHM-5 (Fig. 15B) which might can make it more susceptible under the stressful condition. Drought Tolerance was correlated with higher GSH in tolerant maize genotypes (Chug et al., 2013). Rohman et al. (2016a,b) found higher GSH and its redox in tolerant maize genotypes at seedling stage.

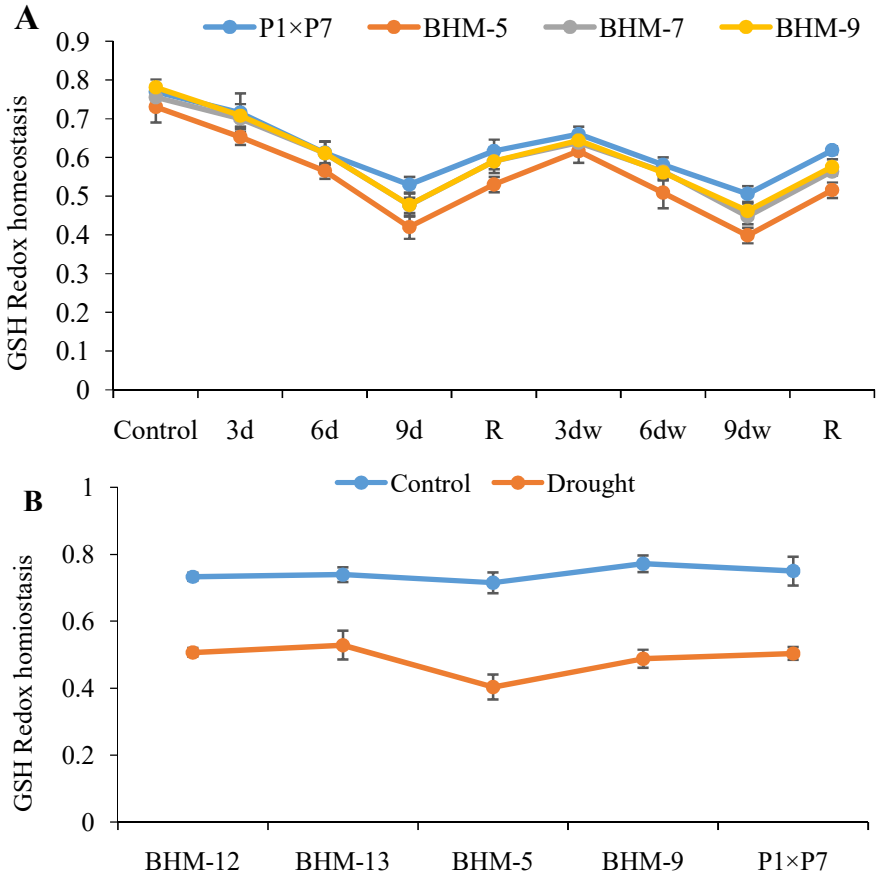

Fig. 15. GSH Redox homeostatis in maize under salinity (A) and drought (B)

Methylglyoxal detoxification system

Methylglyoxal (MG), a highly reactive a-ketoaldehyde, accumulated in all the genotypes under both saline and drought stress (Fig. 16A, B)). Importantly, the synthesis of NG was the highest in susceptible genotypes followed by moderate tolerant genotypes. Comparatively lower MG contents in tolerant genotype might be important cause of showing tolerant. MG interacts with proteins and nucleic acids. It is produced primarily as a byproduct of several metabolic pathways, like glycolysis, lipid peroxidation and oxidative degradation of glucose. Therefore, it must be detoxified or eliminate from cell through biological system. In this study, As compared to other genotypes, accumulation of MG was higher in susceptible genotype under both stresses suggesting alteration of normal cellular metabolic pathway.

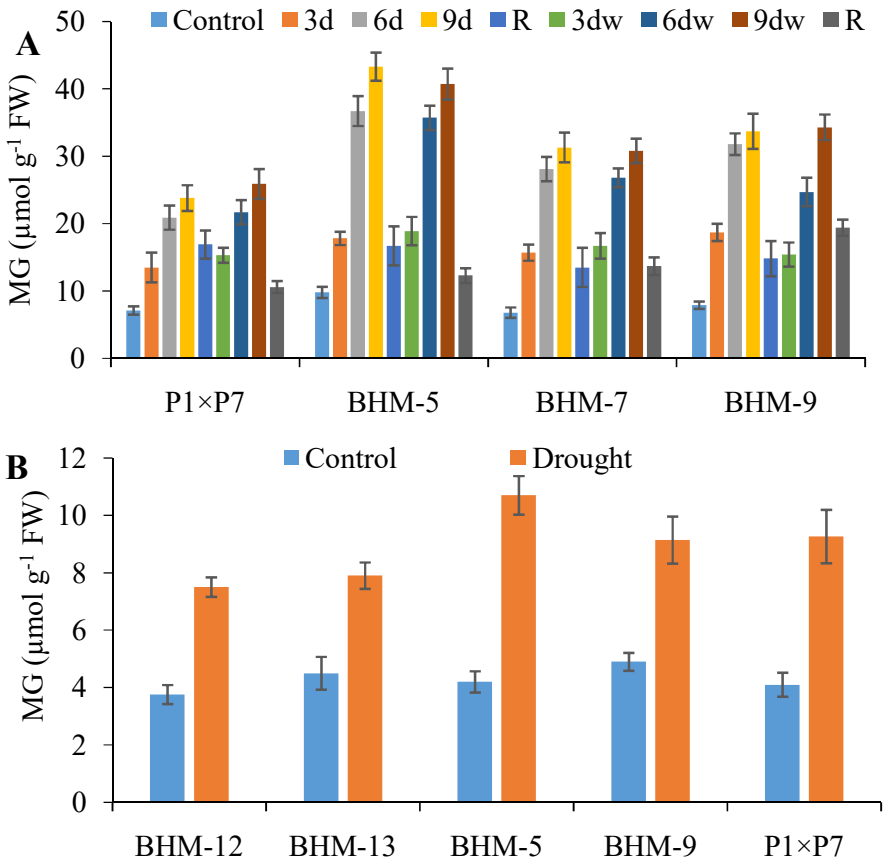

Fig. 16. Comparative MG content in maize seedlings under salinity (A) and drought stress (B)

Comparatively higher induction in activities of Gly-I and Gly-II were observed under both saline and drought stresses (Fig. 17A, B, C, D). Comparatively lower induction in Gly-II activity was observed in salinity, and almost unchanged in drought stress (Fig. 17C, D).

In plants, MG is detoxified mainly via the glyoxalase system, which comprises two enzymes, glyoxalase I (Gly I: EC, 4.4.1.5)) and glyoxalase II (Gly-II: EC 3.1.2.6). Gly I converts MG to *S*-D-lactoyl glutathione (SLG), utilizing GSH, while Gly-II converts SLG to D-lactic acid (Yadav et al., 2005a,b). During the latter reaction, GSH is regenerated. In this study, Both Gly-I and Gly-II activities were higher in tolerant genotypes (Fig. 17). Therefore, lower MG content in tolerant genotypes resulted due to detoxification of MG by the glyoxalases. Overexpression of the glyoxalase enzymes in plants has been found to limit increases in ROS and MG levels under stress conditions, by maintaining GSH homeostasis and antioxidant enzyme levels (Sigla-Pareek et al., 2006, 2008). Increased activities of glyoxalases have also been reported under drought in other plant species (Hasanuzzaman and Fujita, 2011; Alam et al., 2014) Transgenic tomato with highly expressed glyoxalase pathway decreased oxidative stress and enhanced salinity tolerance (Viveros et al., 2013). Upregulation or overexpression of these enzymes increases tolerance to abiotic stresses in tobacco (Singla-Pareek et al., 2008; Saxena et al., 2011).

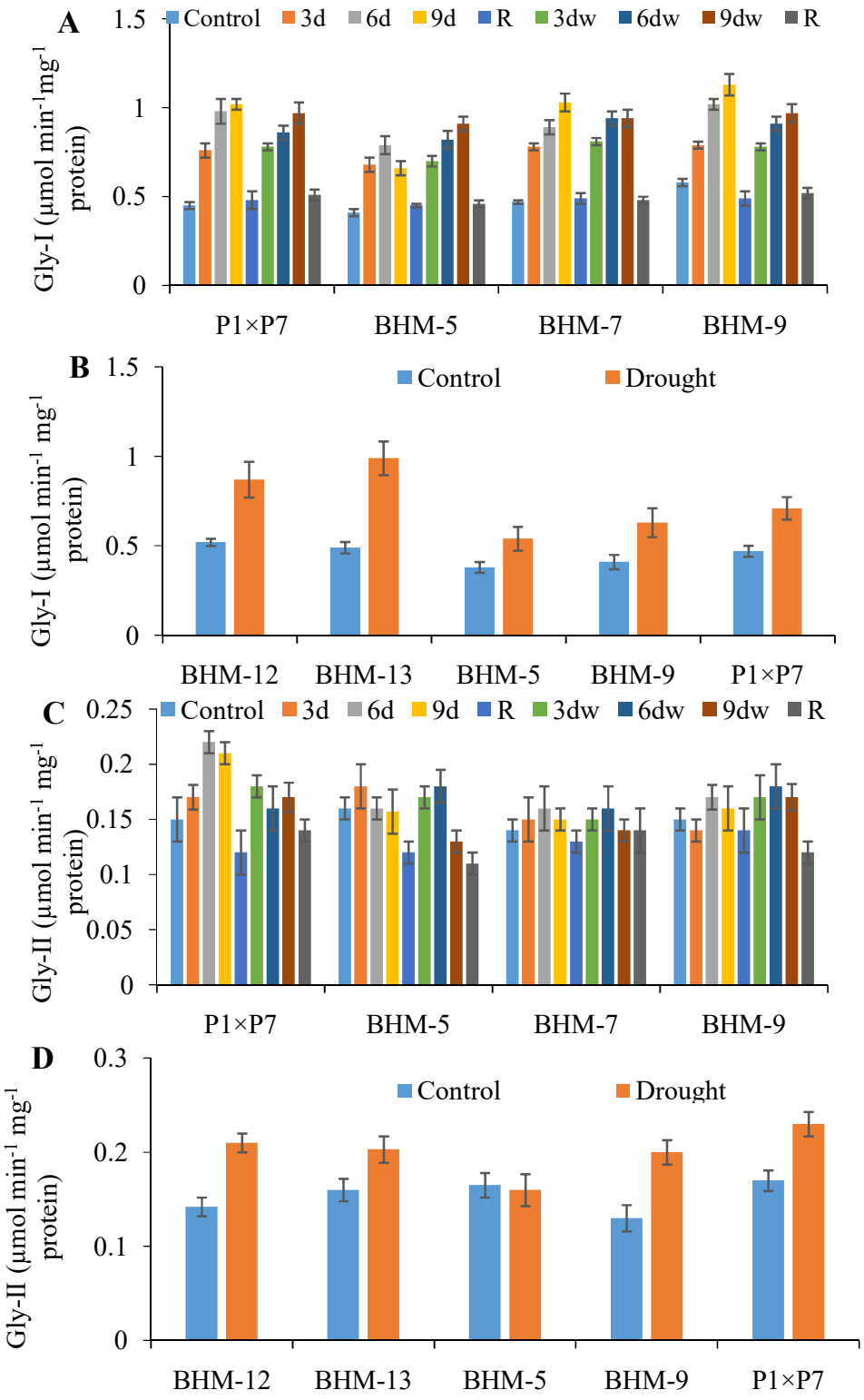

Fig.17. Comparative glyoxalases activities in maize seedlings under salinity (A, C) and drought stresses (B, D).

Coordinated response of antioxidants and glyoxalase in increasing salinity tolerance was reported in rice (Hasanuzzaman et al., 2014), in mungbean (Nahar et al., 2016). Rohman et al. (2016a) reported higher activities of Gly-I and Gly-II under drought in tolerant maize genotypes only (Rohman et al.,

2016b). However, Mir et al., (2018) found decreased activity of both enzymes under salinity. Therefore, response of glyoxalase in maize in genotypes depended. Since Gly-I increment was better than Gly-II in susceptible genotypes, MG detoxification as well GSH refunding can be hampered in susceptible genotypes as Gly-II induction was very low or static.

Regulation of anerobic enzymes

Activities of alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC) and lactate dehydrogenase (LDH) were assayed in leaves of seedlings under anaerobic saline and drought stress (Fig. 18 and 19). In both cases the activities increased in all the genotypes. Under salinity the activities increased with stress duration and in recovery, the activity drastically decreased (Fig. 18A, B, C). On the other hand, all the activities increased under drought, but the tolerant genotype maintained higher activities than susceptible and moderate tolerant genotypes (Fig. (19A, B, C).

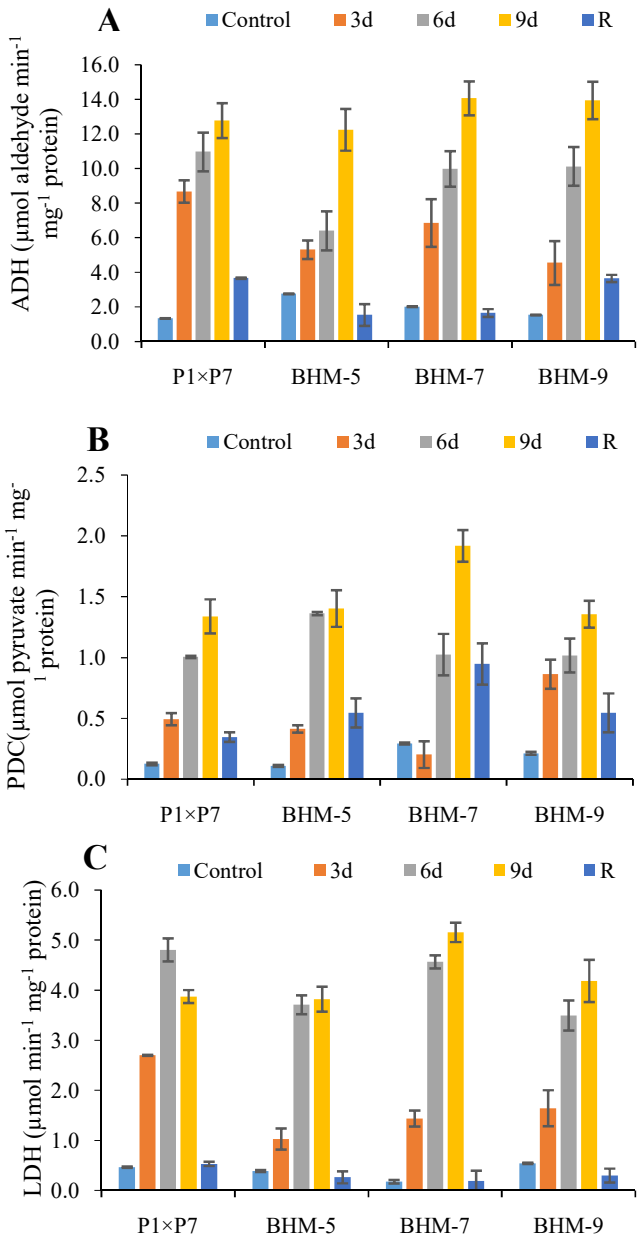

Fig. 18. Activities of ADH, PDC and LDH in maize under water logging with saline water

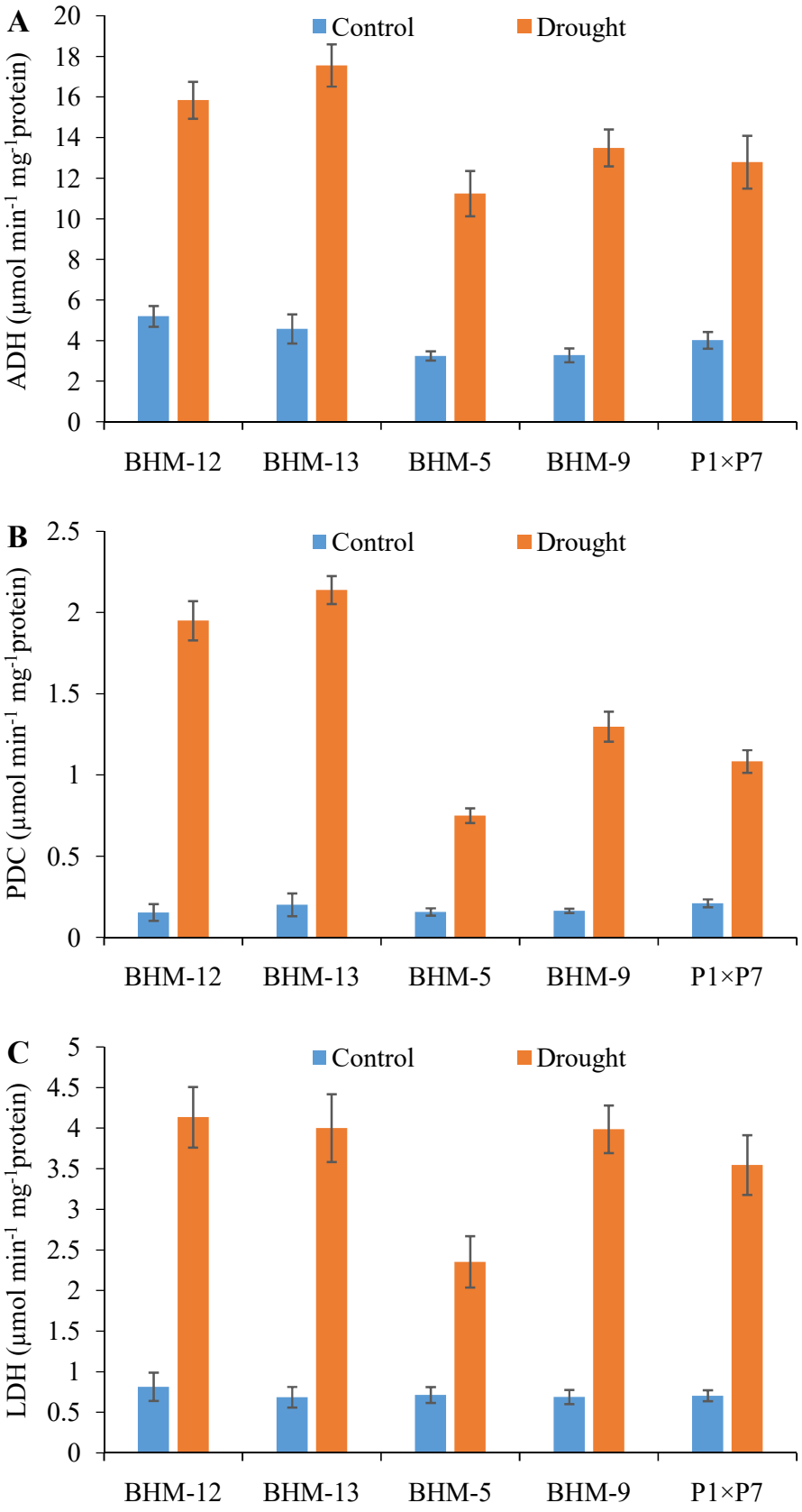

Fig. 19. Regulation of anaerobic enzymes in maize under drought stress

Enzymes like alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC) and lactate dehydrogenase (LDH) have been reported to play important role under hypoxia and anoxia condition as well as other abiotic stress like salinity and drought (Welchen et al., 2012, 2016). We estimated the activities of the enzymes in salinity in anaerobic condition (Fig. 18A, B, C). Continuous and significant increase of the activities was observed under stress condition in all the maize genotypes. However, LDH activity was comparatively lower in BHM-5 (Fig. 18C).Since ADH, PDC and LDH have important metabolic role under drought condition (Thawda Myint et al., 2015; Shi et al., 2017), the induced activities in the maize genotypes under drought conditionalso might have biological role in cell. Decrease in the oxygen level under water logging is the main factor that causes stress, leading to a chain signaling that unleashes a series of metabolic changes (Horchani et al., 2009), such as N metabolism and interconversion of amino acids (Puiatti and Sodek, 1999; Oliveira et al., 2013), changes in carbohydrates levels and energetic metabolism (Sousa and Sodek, 2002), in an effort to secure plant survival and growth when exposed to hypoxic stress (Geigenberger, 2003). Anaerobic metabolism is activated under low oxygen concentration and as a consequence, there is a significant decrease in energy production that is derived mainly from glycolysis in contrast to oxidative phosphorylation (Kumutha et al., 2008; Horchani et al., 2009; Sairam et al., 2009; Zabalza et al., 2009). Plant survival under these conditions depends almost exclusively on anaerobic metabolism (Sousa and Sodek, 2002). Due to the lack of O_2as the final electron acceptor, there is an accumulation of intermediates of Krebs cycle, $NAD(P)^+$ levels decrease, pyruvate accumulate and ATP levels decrease. All these modifications act as a signal for further adaptive responses (Horchani et al., 2009). Enzymes of two important pathways are induced under anaerobic conditions: lactic fermentation is a one-step reaction from pyruvate to lactate, catalyzed LDH with the regeneration of NAD^+. Ethanolic fermentation is a two-step process regenerating NAD^+in which pyruvate is first decarboxylated to acetaldehyde by (PDC), and acetaldehyde is subsequently converted to ethanol by ADH (Tadege et al., 1999; Zabalza et al., 2009). Thus, the significant increases in the activities under anaerobic condition claimed their role in maize under anaerobic saline stress (Fig. 18A, B, C).

In plants, the activities of the ADH enzymes were up-regulated not only in low oxygen conditions (Ismond et al., 2003; Mustroph et al., 2003; Tesniere et al., 2006; Kato-Noguchi 2006, 2010; and Kumutha et al., 2008) but also in other environmental stresses like dehydration, low temperature, or chemical treatments in different plant species (Matton et al., 1990; Dolferus et al., 1994; deBruxelles et al., 1996; Kato-Noguchi ,2001; Peters and Frenkel, 2004 and Kato-Noguchi et al., 2007). The involvement of ADH enzymes in plant adaptation to abiotic stress was also evident at the gene level in which the *ADH1* gene were up regulated in plants grown under low oxygen levels (Shiao et al., 2002). In addition, the expression of *ADH1 gene* was induced by many abiotic stresses including cold and osmotic stresses (Christie et al., 1991 and Conley et al., 1999), wounding (Kato-Noguchi, 2001), dehydration and water deficit (Dolferus et al., 1994 and Senthil-kumar et al., 2010), low temperature (Peters et al., 2004 and Kato-Noguchi et al., 2007), and abscisic acid treatment (de Bruxelles et al., 1996). These observations along with our findings supported that the higher ADH enzyme engaged in plant adaptation to drought stress. Moreover, both Gly-I and LDH are reported to detoxify MG for cellular survival (Jain et al., 2018; Mostofa et al., 2018). D-lactate is metabolized by D-lactate dehydrogenase (D-LDH; At5g06580; Engqvist et al., 2009; Wienstroer et al., 2012). D-LDH is inactive in the presence of NAD+, NADP+, or oxygen as electron acceptor in presence of cytochrome-c (CYTc) or an artificial, small molecular electron acceptor such as

dichlorophenolindophenol (Engqvist et al., 2009).Therefore, the higher LDH can play important role in MG detoxification in maize under drought as well as saline condition.

Conclusion

Considering all, the susceptible genotypes produced higher ROS and MG including MDA and NOX. Based on the antioxidant analysis, tolerant genotypes exhibited higher activities of CAT3, APX1 and APX2. On the other hand, SOD had almost equal role in $O_2^{\bullet-}$ metabolism to H_2O_2. However, MDHAR and DHAR had equal role in ASA maintenance in all the genotypes under stresses. POD, GR and GPX activity varied with genotypes, but in some cases, they have better role in tolerant genotypes under both stresses. The higher activity of GST in susceptible genotypes can involve in leaf senescence under stress. The lower MG in tolerant genotype under both stresses resulting from higher glyoxalases activities. The ASA and GSH redox homeostasis suggested higher oxidation of susceptible genotype under stress. Therefore, both antioxidants and glyoxalase system can improve the tolerance in maize. Taken together, The research under project provided strong prove to express some enzymatic antioxidant that claim further development of use for biotechnological and molecular approaches for improving tolerance in maize under drought and saline stress. In this study, some important protocols have been developed for in-gel separation and anaerobic enzyme measurement. Importantly, anaerobic enzyme measurement protocols are highly efficient that the existing ones. Highly expressed enzymes like CAT, APX1, and APX2 need more research for delineating their role in stress tolerance.

12. Research highlight/findings (Bullet point – max 10 nos.):

- Developed in-gel activity protocol of SOD, CAT, POD, APX, GPX and GR
- Activity assay protocol of enzymes under anaerobic condition like ADH, PDC and LDH which economic than the existing protocols
- CAT3, APX1 and APX2 are identified as responsive proteins under salinity
- CAT1, CAT2, CAT3 and GPX2 are important for drought tolerance
- The results indicated important antioxidant enzymes (CAT3, APX1 and GPX2) for further development and use by biotechnological and molecular approaches.

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	01	60,000	01	60,000	
(b) Lab &field equipment	08	529,000	08	529,000	
(c) Other capital items					

2. Establishment/renovation facilities: N/A

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: N/A

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	272785	255908	255908	0	100%	Delay approval of project
B. Field research/lab expenses and supplies	1490715	1453687	1453687	0	100%	
C. Operating expenses	75000	72861	70861	2000	100%	Bank account maintenance
D. Vehicle hire and fuel, oil & maintenance	150000	147886	147886	0	100%	
E. Training/workshop/seminar etc.	0	0	0	0	100%	
F. Publications and printing	140000	0	0	0	-	
G. Miscellaneous	82500	76646	76646	0	100%	
H. Capital expenses	589000	589000	589000	0	100%	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	**Major technical activities performed in respect of the set objectives**	**Output(i.e. product obtained, visible, measurable)**	**Outcome(short term effect of the research)**
Understanding oxidative stress tolerance in maize at physiological, biochemical and molecular level in	1. Screening of maize genotypes for obtaining tolerant and sensitive genotypes to drought and salinity 2. Analysis of enzymatic and non-enzymatic antioxidants in relation to ROS and MG	Oxidative stress tolerance mechanism in maize under drought and salinity	Metabolism of ROS and MG by antioxidants and glyoxalases

contrast maize genotypes in relation to tolerance to drought and salinity.	metabolism		
Protocol development for expression of inducible proteins	In-gel separation of ROS metabolizing enzymatic antioxidants like SOD, CAT, POD, APX and GPX, and spectrophotometric activity assay of anaerobic enzymes.	In-gel separation of ROS metabolizing proteins and assay activity of anaerobic enzymes	Regulation of ROS by modulating antioxidants
Identification of important inducible protein under drought and salinity tolerance for further biotechnological work.	Development SOD, CAT, POD, APX and GPX protein staining solution for developing the protein bands	Identification of stress inducible proteins	Expression of RIOS scavenging proteins

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.			
Journal publication	01		
Information development			
Other publications, if any		Some results have published in a Book chapter	Maize production under salinity and drought condition: Oxidative stress regulation by antioxidant defense and glyoxalase systems in *Plant abiotic stress tolerance-Agronomic, Molecular and Biotechnological Approaches. Springer, Cham, Switzerland. https://doi.org/10.1007/978-3-030-06118-0_1*

F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

ii. **Generation of new knowledge that help in developing more technology in future**

Identified highly stress inducible proteins like APX1, APX2, CAT3 under salinity and GPX3under drought which claims their further development and use through biotechnological and molecular approach.

iii. Technology transferred that help increased agricultural productivity and farmers' income

iv. Policy Support

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring[description & output of consultation meeting, monitoring workshops/seminars etc.):

ii) Field Monitoring (time& No. of visit, Team visit and output):

1. Internal Monitoring team of BARI (29.01.18)
2. PIU-BARC, NATP-2 (14.03.18)

I. Lesson Learned/Challenges (if any)

i) How to manage and execute a project.
ii) Laboratory management

J. Challenges (if any)

1. Hampering of experimentation due to sudden interruption of electricity
2. High prices of brand chemicals
3. Management of laboratory waste, particularly used chlorinated and other toxic solvent

Signature of the Principal Investigator
Date ……..

Seal

Counter signature of the Head of the organization/authorized representative
Date ……..
Seal

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