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Comparison of Antioxidant System and Anaerobic Metabolism in Seedlings of Contrasting Maize Genotypes under Short Term Waterlogging

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AKG and NK designed the study and experiments. Author VC performed the experiments. Authors VC and AKG analyzed and discussed the data. Author VC wrote the manuscript. Author NK provided overall supervisory support during the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of the study was to understand the biochemical mechanism of tolerance against waterlogging stress in two contrasting maize genotypes *viz*. Parkash (waterlogging tolerant) and Paras (waterlogging sensitive).
Methodology: Both the genotypes were subjected to short term waterlogging stress treatment (18 h) after fifteen days of germination. Two major biochemical defense systems under hypoxia conditions, namely antioxidant and anaerobic metabolism, were compared in leaf and root tissues of tolerant and sensitive maize genotypes.
Results: Both the genotypes efficiently mitigate the oxidative stress generated due to waterlogging, as shown by increased activities of SOD (superoxide dismutase), POX (peroxidase), CAT (catalase) and APX (ascorbate peroxidase) and constant level of H_2O_2 (hydrogen peroxide) and MDA (malondialdehyde) in plant tissues. ADH activity was also significantly enhanced in the roots of both

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the genotypes but ALDH activity was significantly induced in tolerant genotype only, whereas activity remained unchanged in sensitive genotype.
Conclusion: The present results suggest that under sudden short term waterlogging shocks, plant survival depends upon the simultaneous activation of ADH and ALDH activity for continuous energy supply and removal of toxic end products of anaerobic respiration.

Keywords: Waterlogging; Zea mays; antioxidants; anaerobic respiration.

1. INTRODUCTION

Flooding and submergence are major abiotic stresses and rank alongside water shortage, salinity and extreme temperatures as major determinants of species distribution worldwide. It causes huge crop loss worldwide reducing average yields for major crops including maize by more than 50% [1]. In South-East Asia alone, over 15% of the total maize growing areas are frequently affected by floods and waterlogging problems [2]. In India, out of total 6.6 million ha area under maize over 2.5 million ha is prone to excessive soil moisture/waterlogging conditions, which cause on an average 25-30% loss of national maize production almost every year [3]. It has been shown that the early stages of maize development, especially from the second leaf stage (V2) to the seventh leaf stage (V7) are most sensitive to waterlogging [4]. In waterlogging-sensitive maize cultivars, waterlogging for more than 24 h can kill the plants, while waterlogging can be tolerated for periods of up to 1 week for waterlogging-tolerant maize cultivars [5].

All higher plants require access to free water but excess water in the root environment of land plants can be injurious or even lethal because it blocks the transfer of oxygen and other gases between the soil and the atmosphere. Under oxygen deficiency, injury and death of plant tissues have been attributed to the accumulation of toxic end products of anaerobic metabolism, the lowering of energy charge, the lack of substrates for respiration and generation of free radicals. As aerobic respiration ceases, levels of energy rich adenylates drops rapidly, causing a dramatic decline in uptake and transport of ions [6].

Excessive generation of reactive oxygen species (ROS) or oxidative stress is an integral part of many stress situations, including waterlogging. Hypoxic tissues exhibit enhanced mitochondria-dependent ROS generation and main cellular components susceptible to damage by these free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), sugars and nucleic acids. To cope with the high reactivity of ROS, plants possess antioxidative mechanisms, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POX) [7,8,9]. When plant roots are subjected to waterlogging conditions, SOD activity increases in barley roots [10] and remains unaffected in tomato [11] and wheat roots [12]. It has also been reported that higher peroxidase (POX) and lower malondialdehyde (MDA) concentrations can be used as a marker for screening waterlogging tolerant maize genotypes at seedling stage [13].

Metabolic adjustments occur as an adaptive strategy to facilitate tolerance of the plants to waterlogged or flooded soils, which plays an important role in producing energy for the short-term survival of plants in anaerobic environments [14,15]. Oxygen deprivation under waterlogging stress causes a rapid repression of normal protein synthesis and induces the synthesis of specific polypeptides termed as anaerobic polypeptides first described in maize roots [16]. The identified anaerobic proteins include sucrose synthase, phosphohexoseisomerase, fructose-1,6-diphosphate aldolase, pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) which supplies uninterrupted supply of ATP necessary for plant survival [17]. Also the simultaneous detoxification of fermentation products (ethanol and acetaldehyde) also becomes crucial for plant survival under waterlogging conditions [18].

Maize can tolerate heavy rains provided fields are not subjected to excessive soil moisture or waterlogging for longer periods. Waterlogging particularly at young vegetative stage causes great damage to the crop. In most of the regions of India due to sudden rainfall at young vegetative stage and also in the absence of proper drainage system, waterlogging is very common for 18 to 24 hours. Hence it is very useful to understand the metabolic activities

occurring in waterlogging tolerant and sensitive maize genotypes at these initial hours of short term waterlogging shock. For this objective, two important aspects i.e. the detoxification of harmful free radicals and energy production during anoxic conditions were taken into consideration while designing the experiment. Under such stress conditions, it is equally important to avoid oxidative stress through proper scavenging of free radicals as well as to meet the energy requirements of the cell through uninterrupted ATP production which will eventually determine the survival of the plant cells. Also simultaneous detoxification of toxic end products of ethanolic metabolism is essential. Hence this study provides us the insight to improve the waterlogging tolerance of agronomically important maize cultivars so that they can survive these kinds of sudden short stress shocks.

2. MATERIALS AND METHODS

In the present study, two maize genotypes, Parkash (waterlogging tolerant) and Paras (waterlogging sensitive) differing in their tolerance towards waterlogging stress were selected. Fifteen days old seedlings were subjected to short term waterlogging treatment under laboratory. Activities of different enzymes were determined in root and leaf tissues.

2.1 Plant Material, Growth Conditions and Waterlogging Treatment

The germplasm of maize was provided by the Maize Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana (30.91℃N 75.85℃E), India. For conducting lab experiments, the seeds were surface sterilized with 0.1 % mercuric chloride for 5 min and then washed with double distilled water before use. Experiment was conducted in plant growth chamber (NSW-193 CALTAN) under controlled temperature of 30 ± 2℃ in the dark and continuous illumination of 200 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Waterlogging stress was imposed by cup method under lab conditions. Disposable plastic cups (250 cm^3) were used to grow maize seedlings. Cups were filled with 220 cm^3 of its volume with mixture of farmyard manure (FYM) and siphoned soil. Filled cups were placed in plastic trays (40 × 28 × 6 cm). At the 6-leaf stage, the seedlings were subjected to 18 h waterlogging treatment by filling the cups with water 3 cm above the surface of the soil and this water level was maintained throughout the experiment. On the other hand, control plants were provided by normal moisture throughout the experiment. Root and leaf tissues were washed free of soil and used for the study of different biochemical parameters.

2.2 Extraction and Assay of Antioxidant Enzyme Activities

All extractions were made in three replicates at 4℃. For enzyme extraction, seedlings from three different cups were taken separately. Each cup constituted one independent replicate. Enzymes were extracted at 4℃. Superoxide dismutase (SOD), peroxidase (POX) and glutathione reductase (GR) were extracted by homogenizing the samples in 0.1 M phosphate buffer (pH 7.5) containing 1% polyvinylpyrrolidone (PVP), 1 mM EDTA and 10 mM β-mercaptoethanol and catalase (CAT) and ascorbate peroxidase (APX) were extracted with 0.05 M phosphate buffer (pH 7.5) containing 1% PVP and 1mM ascorbic acid [19]. The homogenates were centrifuged at 10,000 g for 20 min and the supernatant was used for assaying.

Assay system of SOD contained 1.4 ml of 100 mM Tris HCl buffer (pH 8.2), 0.5 ml of 6 mM EDTA, 1 ml of 6 mM pyrogallol solution and 0.1 ml of enzyme extract was added [20]. Change in absorbance was recorded at 420 nm after an interval of 30 sec upto 3 min. A unit of enzyme activity has been defined as the amount of enzyme causing 50% inhibition of auto-oxidation of pyrogallol observed in blank.

Assay of GR contained 0.2 ml of 200 mM potassium phosphate buffer (pH 7.5), 0.1 ml $MgCl_2$ (1.5 mM), 0.1 ml EDTA (0.2 mM), 0.2 ml NADPH (0.025 mM), 0.2 ml of enzyme extract followed by 0.2 ml of oxidized glutathione (0.25 mM) in a quartz cuvette [21]. Decrease in absorbance at 340 nm after an interval of 30 sec upto 3 min was recorded. The molar extinction coefficient for NADPH is 6.22 mM^{-1} cm^{-1}. GR activity was expressed as nmoles of $NADP^+$ formed $min^{-1}g^{-1}$ of FW.

Assay system of POX contained 3 ml of 0.05M guaiacol in 100 mM phosphate buffer (pH 6.5), 0.1 ml of enzyme extract and 0.1 ml of 0.8 M H_2O_2 [22]. The reaction mixture without H_2O_2 was taken as a blank. The reaction was initiated by adding H_2O_2 and rate of change in absorbance was recorded at 470 nm for 3 min at an interval of 30 sec. POX activity has been defined as change in absorbance $min^{-1}g^{-1}$ of FW.

Activity of APX was assayed by taking 1 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.8 ml of 0.5 mM ascorbic acid, 0.2 ml of enzyme extract and 1 ml of H_2O_2 solution in total volume of 3 ml [23]. Absorbance was recorded at 290 nm in a spectrophotometer after an interval of 30 sec upto 3 min. Extinction coefficient of monodehydroascorbic acid (MDAA) has the value of 2.8 $mM^{-1}cm^{-1}$. APX activity was expressed as nmoles of MDAA formed $min^{-1}g^{-1}$ of FW.

Activity of CAT was determined by taking 1.8 ml of 50 mM sodium phosphate buffer (pH 7.5) to which 0.2 ml of enzyme extract was added. The reaction was initiated by adding 1 ml H_2O_2 and utilization of H_2O_2 was recorded at an interval of 30 sec for 3 min by measuring the decrease in absorbance at 240 nm [24]. Extinction coefficient for H_2O_2 has the value of 0.0394 $mM^{-1}cm^{-1}$. CAT activity was expressed as µmoles of H_2O_2 decomposed $min^{-1}g^{-1}$ of FW.

2.3 Extraction and Assay of H_2O_2 and MDA

For the extraction of H_2O_2, tissue (0.3 g) was homogenized with 2 mL ice cold sodium phosphate buffer (pH 7.0) using liquid nitrogen. Homogenate was centrifuged at 10,000*g* for 20 min and H_2O_2 concentration was estimated in supernatant [7]. Malondialdehyde (MDA) was extracted and estimated by using a thiobarbituric acid reaction [7].

2.4 Extraction and Assay of Anaerobic Enzyme Activities

For the extraction of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), tissue (0.1 g) was crushed with 2 ml of 100 mM HEPES buffer containing 2 mM dithiothreitol (pH 6.5) to fine powder using liquid nitrogen in pre-chilled pestle and mortar. After centrifugation at 10,000 g for 15 min at 4℃, supernatant was taken for assay. Methods of Ke et al. [25] and Liu et al. [26] were followed for the determination of the activities of ADH and ALDH. Protein concentration was estimated by the method of Lowry et al. [27].

2.5 Statistical Analysis

Statistical analyses were carried out by student's *t*-test using GSTAT statistical software tool. The results presented are the means of three replicates. The statistical significance of differences between the obtained experimental values was assessed at $P < 0.05$. Data shown in the graphs are means ± standard deviation (SD). Standard deviations are represented as vertical bars.

3. RESULTS AND DISCUSSION

Not many studies have been reported to anlayze the effect of short term waterlogging conditions on the antioxidant and anaerobic enzyme activities in maize at early vegetative stage. When subjected to waterlogging treatment, maize seedlings respond through alterations in physiological and biochemical processes [28]. Additionally, waterlogging leads to oxidative stress through an increase in ROS. Therefore, waterlogging stress resistance may depend on the enhancement of the antioxidant defense system which includes antioxidant enzymes as well as other antioxidant compounds. SOD, POX and CAT are the most important detoxifying enzymes, which work together with APX and GR of the ascorbate-glutathione cycle to promote the scavenging of ROS [29]. In the present study it was observed that both the tolerant and sensitive genotypes showed an overall increase in the activities of antioxidant enzymes, hence exhibited less injury on exposing to short term waterlogging. This result is in agreement with the reports on the dynamics of these enzymes under chilling [30], waterlogging [31] and drought [32]. The short term waterlogging of 18 hours enhanced SOD activity in the leaves of Paras by 77% whereas in Parkash, the corresponding increase was 92% (Fig. 1a). SOD activity in the leaves of Parkash was found 44% higher than the Paras under stress conditions. The SOD activity in roots remained unchanged in Paras under waterlogging but a slight increase was observed in roots of Parkash only. SOD is the ubiquitous enzyme in aerobic organisms and plays a key role in cellular defense mechanism against ROS. In antioxidative systems of plants, SOD can remove O_2^-. As SOD may control other activated species (H_2O_2, OH^-), it is defined as a key antioxidative enzyme in the system [33]. When O_2^- levels are elevated, the activities of SOD and other protective enzymes also increased during early waterlogging [34]. In the present investigation also under short term waterlogging conditions, SOD activity increased in the leaves of tolerant as well as sensitive genotype. One probable explanation could be that SOD is an enzyme induced by substrate O_2^-, hence it is possible that increased levels of active

oxygen has stimulated the cellular protective mechanism to mitigate damage. Hence, short term waterlogging did not have harmful effects on tolerant as well as sensitive maize plants.

Waterlogging significantly enhanced GR activity in leaf tissues of both the genotypes, but the upregulation was more pronounced in Parkash, i.e. 24% as compared to controls (Fig. 1b). Root GR activity of both the genotypes was also increased under waterlogging in comparison to control. Paras showed about 53% increase in GR activity respectively, while in case of Parkash, the corresponding increase was of about 17%. Overall roots of Parkash maintain higher GR activity under non-stressed (75%) and stressed conditions (34%) as compared to the Paras (Fig. 1b). Hence the results imply that GR showed differential response under stress conditions but overall trend was increasing. GR plays key role in oxidative metabolism by converting oxidized glutathione (GSSG) to reduced glutathione (GSH) and maintaining a high GSH/GSSG ratio [35].

Parkash showed higher POX activity (53%) than Paras under waterlogging conditions (Fig. 1c). Activity of POX could not be detected in roots of both the genotypes. Waterlogging stress increased POX activity significantly in the leaves of both the genotypes (73% in Paras and about 190% in Parkash). An overall higher POX activity was observed in the leaves of Parkash genotype under stress condition. Peroxidases are involved not only in scavenging of H_2O_2 produced in chloroplasts but also in growth and developmental processes [36]. POX is among the enzymes that scavenge H_2O_2 produced through dismutation of $O_2^{\cdot}$ catalyzed by SOD. POX activity was found to be increased in leaf tissues of both genotypes under stress conditions. Present study indicates that the tolerant genotype exhibited higher POX activity as compared to sensitive genotype under control as well waterlogged conditions. Another researcher also observed higher induction in POX activity in anoxia tolerant barley cultivar as compared to sensitive one under waterlogging conditions [37]. Increase in POX activity in shoots of maize seedlings has been reported under flooding stress [38]. Higher POX activity has also been suggested as a reference index for material screening for waterlogging tolerance [13].

Differential responses were observed for APX activity in waterlogged leaf and root tissues. APX activity of Paras genotype remained almost unchanged but waterlogging caused an increase of 29% in Parkash leaves (Fig. 1d). APX activity in the roots of Paras increased its activity by about 39%. Similarly significant increase in APX activity was also observed in the roots of Parkash under waterlogging stress. Similar to GR, APX also scavenges H_2O_2 and uses ascorbate as an electron donor in plants [39]. The early rise of enzyme activities in present study was considered to be the response against increased generation of ROS caused by sudden hypoxia. Possibly, increased ROS levels stimulate the cellular protective mechanism to mitigate damages. Our results are also in agreement with the recent findings of Chiang et al. [40] who established that over expression of the ascorbate peroxidase gene from eggplant and sponge gourd enhances flood tolerance in transgenic *Arabidopsis.*

SOD enzyme action results in H_2O_2 and O_2 formation. H_2O_2 is toxic in plants and must be converted to H_2O by subsequent reactions. Although there are a lot of enzymes to regulate H_2O_2 intracellular levels, CAT and APX are considered the most important. In present study, like SOD, CAT activity was also found to be slightly increased under stress conditions in roots of both the genotypes. Waterlogging caused an increase in CAT activity in root tissue but no clear pattern in activity could be observed in leaf tissues of both the genotypes (Fig. 1e). Waterlogging increased the CAT activity in the roots of both the genotypes (41% for Paras and 23% for Parkash). Stress strongly induced the CAT activity in leaves of Parkash (by 74%) whereas no change was observed in the activity in Paras leaves. CAT, which is localized in the peroxisomes of higher plants, functions in the decomposition of H_2O_2, which is also produced outside the chloroplasts by H_2O_2-generating oxidases in the peroxisomes [41]. Despite its restricted localization, it may play a significant role in removing H_2O_2. Present results also indicate that CAT and APX activities coordinated with SOD activity play a central protective role in the $O_2^{\cdot}$ and H_2O_2 scavenging process and the activity of these enzymes is related, at least in part to short term waterlogging-induced oxidative stress tolerance in maize seedlings. These results are also supported by some previous studies [5]. Present findings are also supported by a recent study in which 20 days old maize seedlings of the waterlogging tolerant (HUZM-26) and susceptible (HUZM-55) genotypes were subjected to waterlogging stress and found that

in waterlogged plants of resistant genotype there was significant increase in the levels of reactive oxygen species (ROS) scavenging enzymes viz., catalase (CAT), peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidise (APX) [42].

Results in the present study also showed that both the genotypes maintained the concentration of H_2O_2 and MDA in their tissues comparable to controls in response to waterlogging stress (Fig. 2a and 2b). However, tolerant genotype (Parkash) exhibited lower concentration as compared to sensitive genotype (Paras) under control as well as waterlogging conditions. In the leaf tissue, waterlogging increased H_2O_2 concentration of Paras by 27% whereas in Parkash, it remained comparable to controls (Fig. 2a). In root tissues also, the concentration of H_2O_2 and MDA was lower in Parkash as compared to Paras under controlled (50% and 31%) as well as stressed conditions (24% and 17%) (Fig. 2a and 2b). Only a slight increase in H_2O_2 concentration of leaves of sensitive genotype was observed. This behavior is in complete accordance with increased activities of SOD, CAT APX and POX. These results can be compared with reported lower concentration of H_2O_2 in drought resistant cultivars than drought susceptible cultivars when exposed to such stresses [41,43,44].

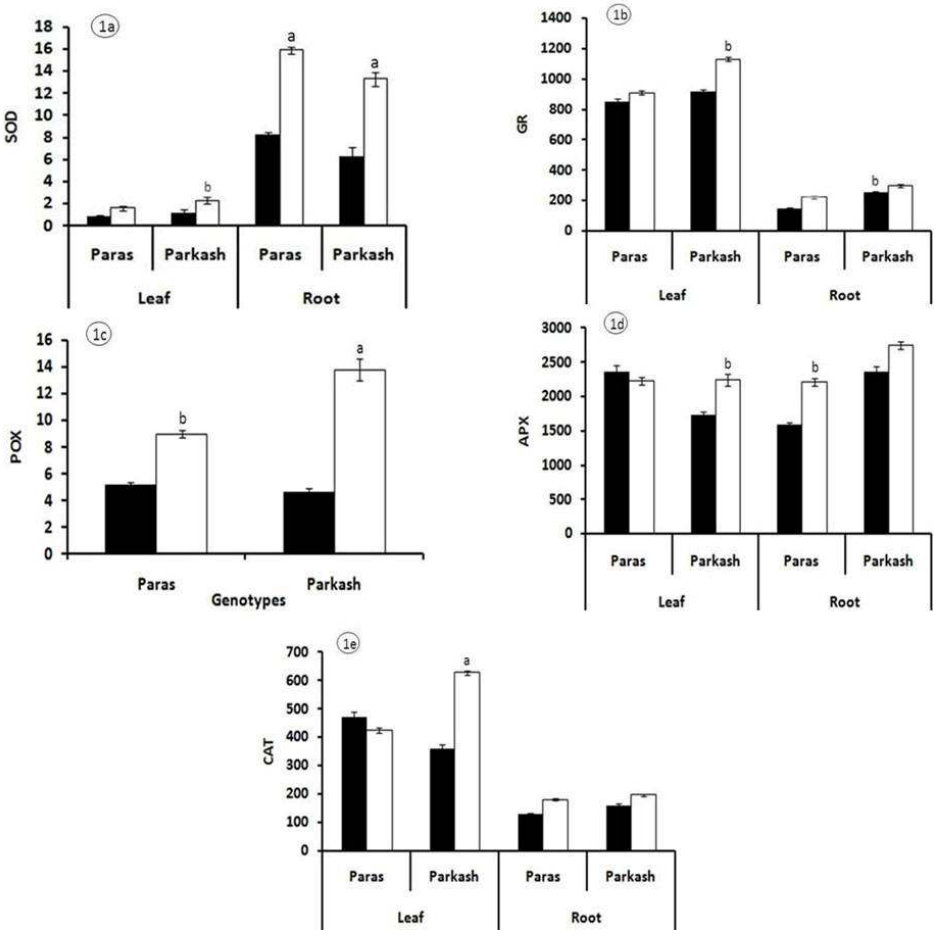

Fig. 1. Changes in the activities of (a) SOD (number of units/min/mg protein), (b) GR (nmoles of NADP+ formed/min/mg protein), (c) POX activity (change in absorbance/min/mg protein), (d) APX (nmoles of MDAA formed/min/mg protein) and (e) CAT (µmoles of H_2O_2 decomposed/min/mg protein), in controlled (■) and waterlogged (□) leaf and root tissue in sensitive (Paras) and tolerant (Parkash) maize genotype [Vertical bars showed SD from mean of three replicates. Significant at a- 1% level, b- 5% level as compared to control (Student's t-test)]

Plants react to an absence of oxygen by switching from an oxidative to a solely substrate-level phosphorylation of ADP to ATP, the latter reactions predominantly involve glycolysis and fermentation. [45]. In response to short term waterlogging treatment, both the maize genotypes showed significant changes in ADH and ALDH activity in the roots only while no direct inference could be drawn with respect to leaf tissue. However tolerant plants exhibited much higher increase in enzyme activities as compared to sensitive plants. Waterlogging stress caused strong induction in ADH activity in the roots of both Paras and Parkash (Fig. 3a). Roots of Parkash showed 95% higher ADH activity than the roots of Paras under stressed conditions. Previously also ADH activity has been reported as essential component for the extended survival of maize during waterlogging [46]. Activity of ADH remained unaltered in leaves under short term waterlogging. However significant differences among genotypes were observed for ALDH activity. ALDH activity in Parkash was significantly enhanced in leaf (31%) and root (157%) tissues as compared to controls while in Paras, ALDH activity remained unaltered in both the tissues under waterlogging stress. These results give the possible explanation for the tolerance behavior of Parkash. Increase in ADH activity is helping the tolerant genotype to survive under the hypoxia condition by supplying necessary ATP and reducing equivalents and simultaneously its increased ALDH activity is neutralizing toxic acetaldehyde produced during ethanolic fermentation and avoiding cell toxicity efficiently. While sensitive genotype is trying to maintain its ATP supply by increasing ADH activity effectively but unchanged ALDH activity is causing the accumulation of toxic end product (acetaldehyde) in the cells which ultimately leads to cell death. Higher increase in ADH and ALDH activities in tolerant plants might reduce the detrimental effects of the accumulation of toxic acetaldehyde under waterlogging conditions [47] and help to maintain ATP production in the absence of O_2 [48,49].

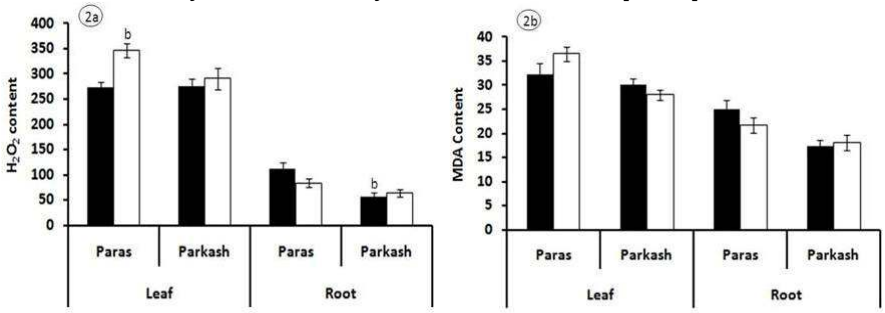

Fig. 2. Changes in (a) H_2O_2 concentration (mmoles/g of dry weight) and (b) MDA concentration (µmoles/g of dry weight), in controlled (■) and waterlogged (□) leaf and root tissue in sensitive (Paras) and tolerant (Parkash) maize genotype [Vertical bars showed SD from mean of three replicates. Significant at a- 1% level, b- 5% level as compared to control (Student's t-test)]

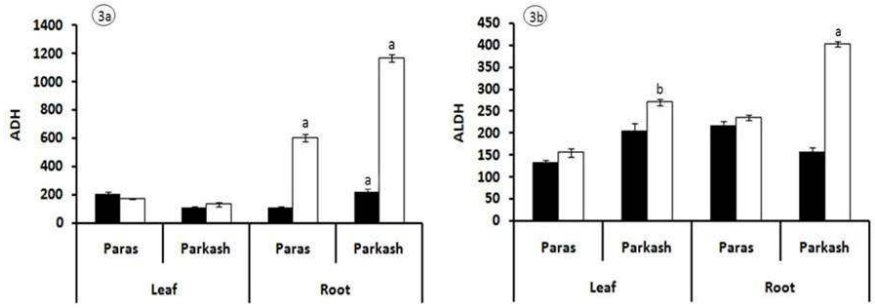

Fig. 3. Changes the activities of (a) ADH activity (nmoles of NAD formed/min/mg protein) and (b) ALDH activity (nmoles of NADH formed/min/mg protein), in controlled (■) and waterlogged (□) leaf and root tissue in sensitive (Paras) and tolerant (Parkash) maize genotype [Vertical bars showed SD from mean of three replicates. Significant at a- 1% level, b- 5% level as compared to control (Student's t-test)]

4. CONCLUSION

It may be concluded that in the initial hour of waterlogging stress both tolerant and sensitive genotype activates their antioxidant system and control the resulting oxidative damage. Also sensitive genotype attempts to continue required ATP supply through ethanolic ferementation. But fails to detoxify the acetaldehyde produced as end product of anaerobic respiration which might be potential cause its susceptible behavior. Hence in present investigation, at early waterlogging stress, the scavenging capacity of ROS in tolerant and sensitive maize plants exceeds free radical generation rate and results in less damage to the plants. However both genotypes showed increase in ADH and ALDH activity in roots but the increase was much pronounced in tolerant genotype as compared to sensitive. Also tolerant genotype maintained higher antioxidant and ethanolic enzymatic activities in controlled conditions which might be the possible explanation for the tolerant behavior of Parkash. Hence, improved tolerance to waterlogging stress may be accomplished by increased capacity of antioxidative and ethanolic fermentation system simultaneously.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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