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NITRIC OXIDE MITIGATES WATERLOGGING STRESS BY REGULATING ANTIOXIDATIVE DEFENSE MECHANISM IN MAIZE *(ZEA MAYS* L.) ROOTS

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Key words: *Zea mays*, Waterlogging, Antioxidative enzymes, Nitric oxide, Sodium nitroprusside

Abstract

Twenty days old pot grown seedlings of two genotypes of maize *viz.*, HUZM-265 (waterlogging resistant) and HUZM-55 (waterlogging susceptible) were subjected to root zone waterlogging supplemented with 50, 500 and 2000 µmol/l sodium nitroprusside (SNP) as a donor of NO. Levels of reactive oxygen scavenging enzymes and non-enzymes were quantified in terminal root portions at 0, 1, 3, 5 and 7 days after waterlogging. As 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 peroxidase (APX). Malondialdehyde (MDA) and H_20_2 levels also increased in waterlogged plants, but increment was more in susceptible genotype. Levels of ascorbic acid and phenols decreased in waterlogged plants. Supplementing root zone solution of waterlogged plants with SNP was effective in ameliorating activities of ROS scavenging enzymes at relatively lower concentration (50 µmol/l) in resistant genotype than in susceptible genotype. Higher concentration of SNP (2000 µmol/l) was deleterious for plants. Results indicated that NO played a role in enhancing tolerance to waterlogging stress at its lower concentration.

Introduction

Waterlogging lowers production and productivity of maize (*Zea mays* L.) in Asian regions (Lone and Warsi 2009). Excess soil moisture stress in maize is caused by waterlogging, high water table or heavy soil texture. Crop is more susceptible to waterlogging during early growth stages (Shah *et al*. 2012). Waterlogging leads to hypoxia i.e., reduced oxygen concentration, in soil and it progressively leads to anoxia (absence of oxygen). Since O_2 acts as terminal electron acceptor in the mitochondrial electron transport, therefore, O_2 depletion leads to reduction in the ratio of ATP/ADP and shifting of cells metabolism to anaerobic fermentation to produce ATP and $NADP^+$ for maintaining their energy requirements and longer survival. Production of reactive oxygen species (ROS) is enhanced under various stresses. To protect cells from detrimental effects caused by ROS under stress conditions, plant tissues possess ROS scavenging enzymes *viz*., superoxide dismutase (SOD), catalase (CAT), peroxidases (POX) and polyphenol oxidase (PPO) and a network of low molecular mass non-enzymatic antioxidants such as ascorbate (AA), glutathione (GSH), phenolic compounds and tocopherols (Noctor and Foyer 1998). To regenerate active forms of the antioxidants enzymes such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) are further required. NO is a highly reactive, membrane-permeable free radical and a key signaling molecule in different intracellular processes in plants including induction of seed germination and reduction of seed dormancy, regulation of plant metabolism and senescence (Crawford and Guo 2005). Radi *et al*. (1991) postulated two mechanisms by which NO might abate stress by forming peroxynitrite which is less toxic than

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peroxides by functioning as an antioxidant and directly scavenging the reactive oxygen species (ROS) such as superoxide radicals (O_2^-) and by functioning as a signaling molecule in the cascade of events leading to changes of gene expression. When present at low amounts, NO acts as signals for the activation of defense responses, however its higher concentrations produced by uncontrolled ROS generation cause severe cellular injuries.

Materials and Methods

Pot culture experiment was conducted during rainy season *(kharif)* at the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. Seeds of two genotypes of maize HUZM-265 (waterlogging resistant) and HUZM-55 (waterlogging susceptible) identified at this centre (Jaiswal and Srivastava 2015), were sown in plastic pots containing 750 g sand. After one week of growth each pot was supplied with 100 ml normal Hoagland's nutrient solution. Twenty days after sowing plants were subjected to waterlogging stress by putting pots in plastic containers containing water or 50, 500 and 2000 µmol/l SNP (as a donor of NO) solutions. Water/SNP solutions in containers were maintained 4 - 5 cm above the sand surface in the pots. Normal plants were maintained at optimum supply of soil moisture. Plants were uprooted carefully at 0, 1, 3, 5 and 7 days after imposing stress. Terminal 2.5 cm root segments were cut and saved. One hundred mg such root segments were immediately dipped in liquid N_2. After 24 hrs, samples were withdrawn from liquid N_2 and stored in deep freezer (–20°C) for further analysis. Lipid peroxidation in terms of TBARS content was estimated by the method given by Heath and Packer (1968). Hydrogen peroxide content was estimated by method of Jana and Choudhuri (1981). Standard protocols were followed to measure activities of catalase (EC1.11.1.6) (Aebi 1983), peroxidase (EC1.11.1.7) (Kar and Mishra 1976), superoxide dismutase (EC1.15.1.1) (Dhindsa *et al*. 1981) and ascorbate peroxidase (EC1.11.1.11) (Nakano and Asada 1981). Enzymes activities were expressed on the basis of per g fresh weight and per mg protein (specific activity). Total soluble protein content was determined according to the method of Bradford (1976) with bovine serum albumin as standard. Ascorbic acid was measured by method of Mukherjee and Choudhary (1983) and total phenol content was measured by the method of Mahadevan and Sridhar (1982). All experiments were performed in triplicates. Results were subjected to statistical analysis by adopting method of "Analysis of Variance" for completely randomized design factorial. Critical differences were calculated at 1% level of significance for comparing treatment means (Gomez and Gomez 1984).

Results and Discussion

Activity of CAT enzyme increased under stress in both genotypes and it was found to be more in HUZM-265 than in HUZM-55. Generally 50 µmol/l SNP treatment of waterlogged plants induced CAT activity significantly in resistant genotype and 2000 µmol/l in susceptible genotype (Fig. 1a, b). CAT is involved in detoxification of H_2O_2. Immediately after waterlogging increased CAT activity in both genotypes indicated that it might be the initial enzyme in detoxification of H_2O_2 and reducing cellular damage caused by H_2O_2 in waterlogged maize plants. Wang *et al*. (2011) also reported that treatment of maize seedlings at 50 and 500 µmol/l SNP kept the enzyme activity to higher levels. POX is also involved in the detoxification of H_2O_2. Activity of peroxidase increased in waterlogged plants of both genotypes (Fig. 2). Its higher activity was seen in resistant genotype HUZM-265. In both genotypes POX activity increased with increased waterlogging duration and in HUZM-265 the maximum activity was at day 5 and in HUZM-55 at day 3 after imposing waterlogging stress. Fifty and 500 µmol/l SNP treatment of waterlogged plants appeared to be effective in elevating POX activity in waterlogged plants in resistant and

susceptible genotypes respectively (Fig. 2). SOD is an important antioxidant enzyme. Enzyme acts as the first line of defense against oxidative stress in plants. SOD catalyses the dismutation of O_2^- to molecular oxygen (O_2) and H_2O_2. Increment in activity of superoxide dismutase enzyme was observed in waterlogged plants of both genotypes (Fig. 3). Resistant genotype HUZM-265 had higher superoxide dismutase (SOD) activity as compared to susceptible genotype HUZM-55 (Fig. 3). Maximum SOD activity was seen at day 5 of stress which declined further till 7

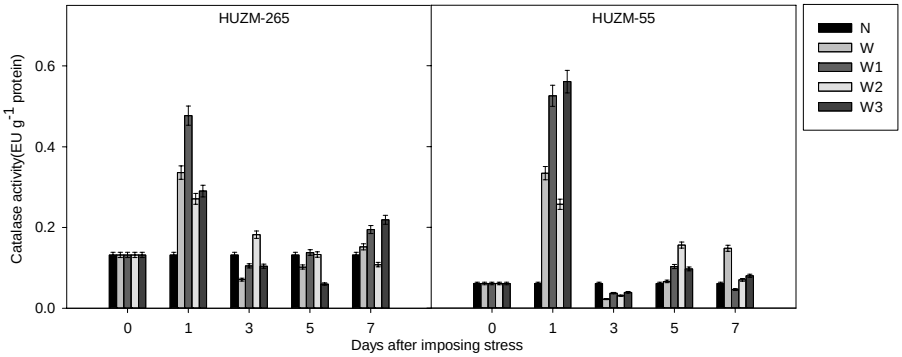

Fig. 1. Changes in CAT (Units/mg protein) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. N = Normal, W = Waterlogged, W1 = 50 µmol/l, W2 = 500 µmol/l, W3 = 2000 µmol/l SNP.

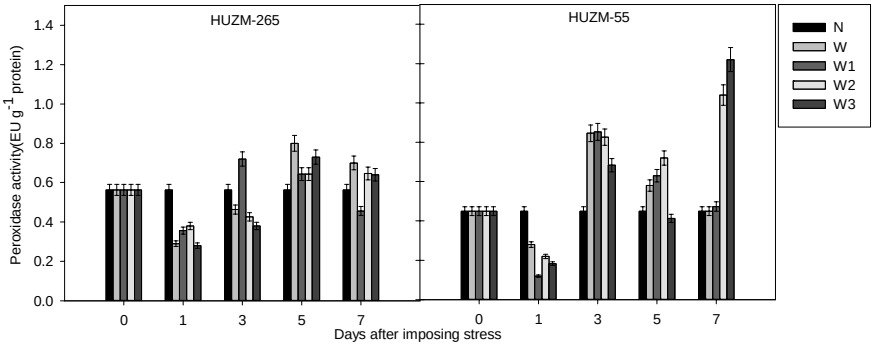

Fig. 2. Changes in POX (Units/mg protein) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. Otherwise as Fig.1.

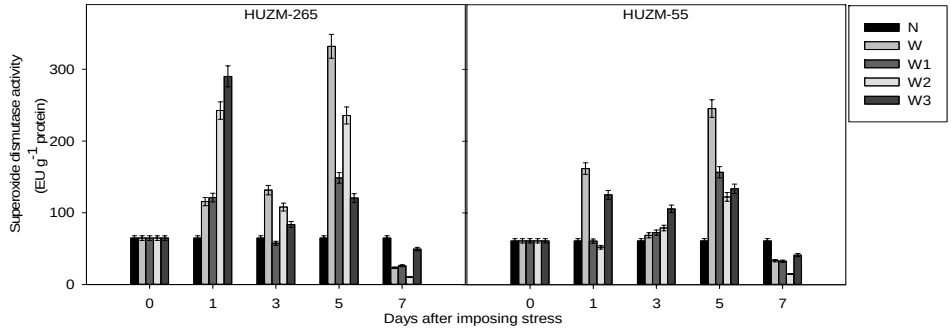

Fig. 3. Changes in SOD (Units/mg protein) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. Otherwise as Fig.1.

days of stress in both genotypes. SNP at concentration of 500 and 2000 µmol/l (depending upon the genotype) enhanced SOD activity in roots of waterlogged plants in HUZM-265 and HUZM-55, respectively. Increased SOD activity causes increased production of H_2O_2 which is also highly damaging to cells but lesser than O_2^-. Therefore, enhanced stress tolerance associated with high SOD activity in plants is possible only when other important antioxidant enzymes *viz*. APX, DHAR, monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), GSH and non enzyme viz. ascorbic acid (AA) are also present in higher levels to scavenge H_2O_2. NO increased the SOD activity and also H_2O_2 degrading enzymes in waterlogged plants to maintain its level and to confer waterlogging resistance. Scavenging of H_2O_2 by APX is the first step of the reduced ascorbate-reduced glutathione (AA- GSH) cycle. In the AA-GSH cycle, APX catalyses the reduction of H_2O_2 into H_2O with AA serving as an electron donor. On the other hand, it is known that APX is more efficient than CAT to detoxify H_2O_2, since it is widely distributed inside the cell and has high substrate affinities in the presence of AA as reductant. Under waterlogging, increased APX activity was seen in both genotypes (Fig. 4). Resistant genotype had more activity than susceptible one. Maximum activity was seen at day 5 of stress which declined further till 7 days of stress. Fifty µmol/l SNP appeared to be more effective in alleviating deleterious effect of

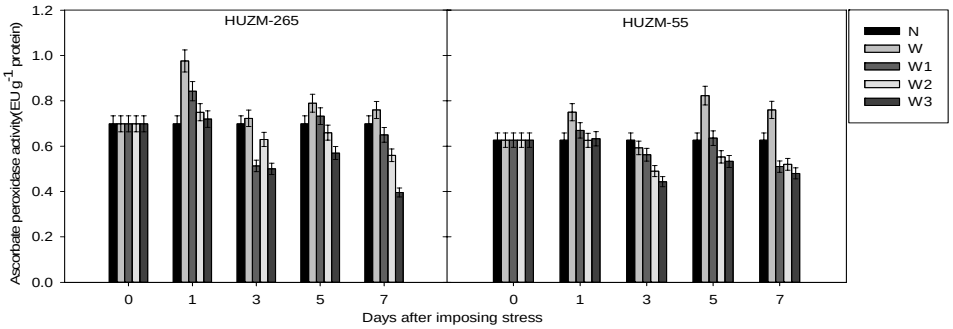

Fig. 4. Changes in APX (Units/mg protein) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. Otherwise as Fig. 1.

waterlogging in both genotypes. Present study indicated that increased level of APX in HUZM-265 enabled this genotype in detoxification of H_2O_2 as well as in regeneration of $NADP^+$ efficiently in response to stress. Linn *et al*. (2004) indicated that root APX activity under waterlogged condition could be taken as criterion to evaluate waterlogging resistance in plants. These results are in conformity with that of Fan *et al*. (2014) who also reported an increase in APX activity in waterlogged cucumber plants and exogenous application of SNP conferred resistance to waterlogging. It is given that NO is involved in a series of reactions in plants conferring resistance by adjusting activities of APX and other relative enzymes containing hemachrome iron or by restraining aconitase which does not contain hemachrome iron. Levels of non-enzyme antioxidants *viz*. AA and phenols decreased in waterlogged plants of both genotypes (Figs 5 and 6). However, their levels were higher in resistant genotype. In waterlogging resistant and susceptible genotypes the maximum ascorbic acid content was seen after 5 and 3 days of waterlogging, respectively. While, phenol content was the maximum after 5 days of waterlogging in both genotypes and there after it declined. SNP treatments of waterlogged plants tend to ameliorate the adverse effect of waterlogging on root ascorbic acid and phenol contents. Application of 2000 and 500 µmol/l SNP was effective in maintaining both ascorbic acid and

phenol content in waterlogged plants of resistant and susceptible genotype, respectively (Figs 5 and 6). It is reported that ascorbic acid is an important agent for mitigating damage caused by ROS produced in hypoxic and anoxic roots (Inglett *et al*. 2005). The origins of NO in hypoxic plant cells are still unrevealed but significant NO concentrations are produced in such environments and it can directly scavenge reactive oxygen radicals thus providing protection to membrane. However, not much literature is cited to explain the role of SNP on phenol content in waterlogged plants. Waterlogging caused an increase.

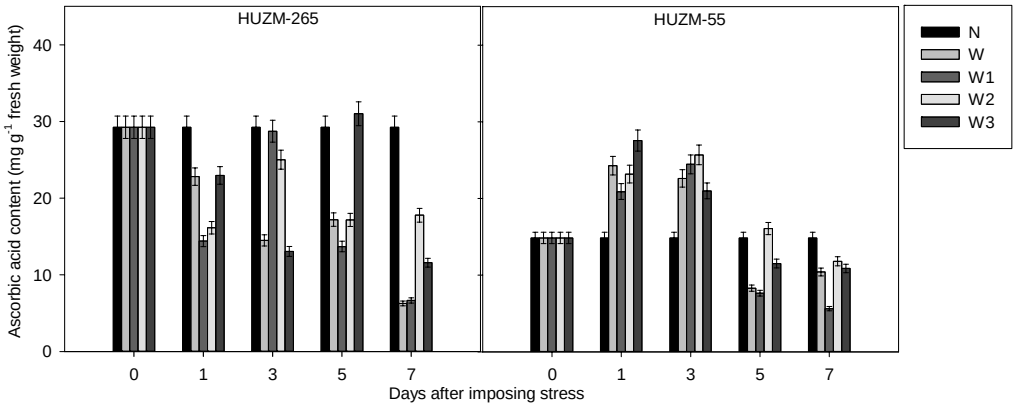

Fig. 5. Changes in ascorbic acid content (mg g^{-1} fresh weight) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. Otherwise as Fig. 1.

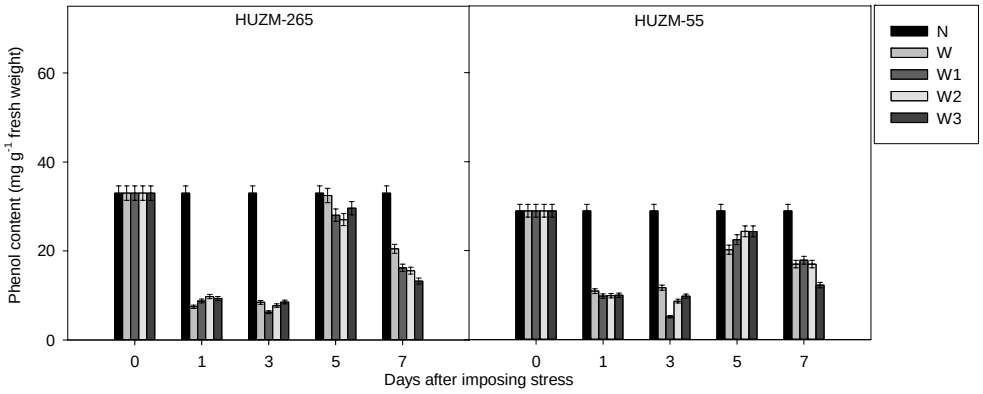

Fig. 6. Changes in phenol content (mg g^{-1} fresh weight) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. Otherwise as Fig. 1.

in the MDA content in both genotypes. Increment was more in waterlogging susceptible genotype HUZM-55 than in resistant genotype HUZM-265 (Fig. 7). The maximum MDA content was seen after 3 days of waterlogging in resistant genotype and after 7 days of waterlogging in susceptible genotype. MDA content is an indicative of lipid peroxidation of cell membranes (Wang *et al.* 2011). Therefore, higher MDA content is linked with more damage in cell membrane. Present investigation indicated that waterlogging induced damage in cell membrane and the magnitude of damage was more in susceptible genotype. Five hundred and 2000 µmol/l SNP treatment to waterlogged plants tend to ameliorate damaging effect of waterlogging in cell membrane in HUZM-265 and HUZM-55 respectively. Prior studies also support increased lipid peroxidation in

waterlogging susceptible genotypes of pigeonpea (Bansal and Srivastava 2012). As a consequence of increased ROS level under stress, there was increased malondialdehyde (MDA) accumulation. Exogenous NO effectively reduces the membrane lipid peroxidation and possesses the functions of repairing and protecting the cell membrane to alleviate the injury in the cell membrane system. Modulation of NO by superoxide formation and inhibition of lipid peroxidation shows its potential antioxidant role mainly due to its ability to maintain the cellular redox homeostasis and to regulate ROS toxicity. Results were in accordance with that of Wang *et al.* (2011) who also reported an increase in MDA content under waterlogging stress in maize. SNP at concentration of 2000 µmol/l resulted in higher MDA content. Also, similar results were obtained by Fan *et al*. (2014) when cucumber leaves were sprayed with 100 µM SNP. It resulted in alleviated accumulation of MDA under waterlogging stress. Waterlogging caused an increase in hydrogen peroxide (H_2O_2),

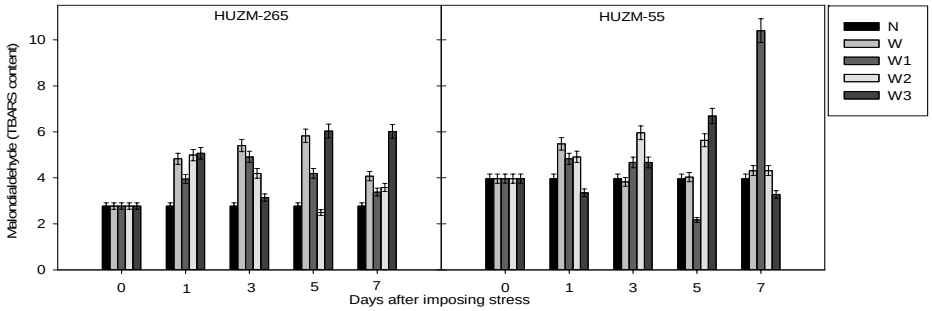

Fig. 7. Changes in MDA (TBARS content) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. Otherwise as Fig. 1.

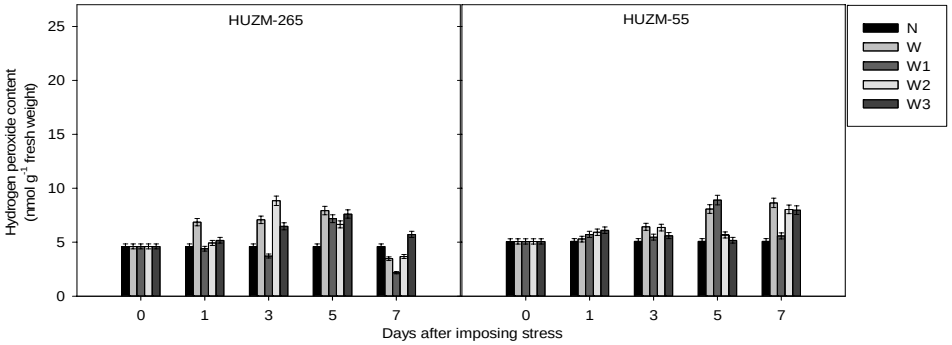

Fig. 8. Changes in hydrogen peroxide content (nmol/g fresh weight) in roots of HUZM-265 and HUZM-55 under waterlogging stress with different concentrations of SNP. Otherwise as Fig. 1.

a reactive oxygen species in both the genotypes. However, its content was observed more in HUZM-55 than the HUZM-265. At 5 days of stress imposition its content was seen maximum in HUZM-265 while it was maximum at day 7 of stress in HUZM-55. Two thousand and 500 µmol/l SNP treatment was found to be more efficient in reducing root H_2O_2 content under waterlogged condition in resistant and susceptible genotypes, respectively (Fig. 8). As H_2O_2 is potentially damaging ROS, its accumulation under stress may lead to significant cell injury. Similar results were obtained in pigeon pea (Singh 2010). In ROS-mediated signal transduction, H_2O_2 is involved because it has ability to penetrate the plasma membrane as an uncharged molecule and hence can be transported to the site of action.

It is inferred that there is an increase in levels of ROS scavenging enzymes *viz.* CAT, POX, SOD and APX and decline in levels of non-enzyme antioxidants *viz*. ascorbic acid and phenol in roots of waterlogged maize plants. More H_2O_2 content in roots of waterlogged plants indicated that rate of synthesis of H_2O_2 is higher in such plants than its rate of scavenging. Relatively lower amount of H_2O_2 in waterlogging resistant genotype indicated that waterlogging resistance in maize is linked with efficient detoxification of ROS. Higher MDA content in roots of waterlogged plants further proved that cell membranes are severely damaged during waterlogging stress and the extent of damage is more in sensitive genotype. SNP (donor of NO) may alleviate deleterious effects of waterlogging in maize. Nevertheless, SNP is effective in its lower concentrations which vary with genotypes. Higher SNP concentrations are lethal to plants. However, more work is needed to investigate the mechanism and action of NO in waterlogged plants and optimizing SNP concentration to efficiently mitigate waterlogging stress in maize.

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