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Research Article

Protein Oxidation in Response to Nitrogen Stress in Wheat seedlings

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Abstract
Ammonium as the sole source of N is stressful for the wheat plants. Wheat seedlings were grown at varied N forms and supply. Increased ROS generation, increased lipid peroxidation, free amino acid levels and protease activities were observed in wheat seedlings grown under zero-N and NH_4^+ -N. Presence of carbonylated proteins, in plants is indicative of oxidative stress damage. Conditions that promote formation of reactive oxygen species (ROS) enhance protein carbonylation, and protein degradation is required to reverse the damage. In our study, we show that in spite of high ROS, ammonium and zero-N grown wheat plants had low levels of protein carbonyls indicating increased degradation of total soluble proteins in these treatments. Loss of carbonylated proteins corresponded to a loss of soluble protein and accumulation of free amino acids. However, it is not clear how the degradation of carbonylated proteins is controlled *in planta*.

Keywords: Reactive Oxygen Species (ROS), Protein carbonylation

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Introduction

Nitrogen is the major mineral nutrient required by the plant for its proper growth and development. Maintenance of nitrogen status of plants plays a critical role in increasing both crop productivity and resistance to environmental stress [1]. Nitrogen deprivation triggers distinct redox changes and induce oxidative stress with a rather defined pattern in the context of nutrient-specific alterations in metabolism [2]. Deficiency of nitrogen results in nutrient imbalance affecting several metabolic pathways [3] including increased production of ROS [4]. N deficiency implies an oxidative stress because of an increased Mehler reaction and because of an energetic imbalance related to a reduction in N assimilation [5].

ROS are produced continuously as by-products of various metabolic pathways that are localized in different cellular compartments such as chloroplast, mitochondria and peroxisomes [6]]. Under steady state conditions, the ROS molecules are scavenged by various antioxidative defense mechanisms [7]. The equilibrium between the production and the scavenging of ROS may be perturbed by various biotic and abiotic stress factors such as salinity, UV radiation, drought, heavy metals, temperature extremes, nutrient deficiency, air pollution, herbicides and pathogen attacks.

These disturbances in equilibrium lead to sudden increase in intracellular levels of ROS which can cause significant damage to cell structures and it has been estimated that 1-2% of O_2 consumption leads to the formation of ROS in plant tissues [8]. Through a variety of reactions, O_2 leads to the formation of H_2O_2, OH^- and other ROS. The ROS comprising O_2^-, H_2O_2, O_2^-, HO_2^-, OH^-, ROOH, ROO^-, and RO^- are highly reactive and toxic and causes damage to proteins, lipids, carbohydrates, DNA which ultimately results in cell death. Accumulation of ROS as a result of various environmental stresses is a major cause of loss of crop productivity worldwide. ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid per oxidation (LPO). It is important to note that whether ROS will act as damaging, protective or signalling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and time [9].

Reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide and hydroxyl radical can cause lipid peroxidation and consequently membrane injury which leads to leakage of cellular content, protein degrading, enzyme inactivation, pigment bleaching and disruption of DNA strands and thus cell death [10]. Enhanced production of oxygen free radicals is responsible for peroxidation of membrane lipids and the degree of peroxides damage of cell was controlled by the potency of peroxidase enzyme system [11]. Plants have developed a series of both enzymatic and non-enzymatic detoxification systems to counteract AOS, thereby protecting cells from oxidative damage [11]. Tolerance to wide varieties of environmental stress conditions has been correlated with increased activity of antioxidant enzymes and levels of antioxidant metabolites [12]. Plants protect the cellular and sub-cellular systems from the cytotoxic effects of these ROS in the form of enzymes such as superoxide dismutase, ascorbate peroxidase, peroxidase, glutathione reductase and catalase and metabolites such as glutathione, ascorbic acid, α- tocopherol and carotenoids [13]. Modulation of the activities of these enzymes may be important in the resistance of plant to environmental stresses [14].

The carbonylation of proteins is a well recognized marker of oxidative stress and damage. Assumption is that protein carbonylation leads to loss of function, and hence those proteins must be degraded, as there are no repair mechanisms known to replace the damaged side chains.

Protein oxidation is defined as covalent modification of a protein induced by ROS or by-products of oxidative stress. Most types of protein oxidations are essentially irreversible, whereas, a few involving sulphur-containing amino acids are reversible. Protein carbonylation is widely used marker of protein oxidation [15]. The oxidation of a number of protein amino acids particularly Arg, His, Lys, Pro, Thr and Trp give free carbonyl groups which may inhibit or alter their activities and increase susceptibility towards proteolytic attack. Protein carbonylation may occur due to direct oxidation of amino acid side chains (e.g. proline and arginine to g-glutamyl semialdehyde, lysine to amino adipic semialdehyde, and threonine to aminoketobutyrate). Whatever the location of ROS synthesis and action, ROS are likely to target proteins that contain sulfur-containing amino acids and thiol groups. Cys and Met are quite reactive especially with O_2^- and OH^-. Activated oxygen can extract an H atom from cysteine residues to form a thiol radical that will cross-link to a second thiol radical to form disulphide bridges [16]. Alternatively, oxygen can add on to a methionine residue to form methionine sulphoxide derivatives. It has been found that various stresses lead to the carbonylation of proteins in tissues. Carbonylation of storage proteins has been noted in dry Arabidopsis seeds but carbonylation of a number of other proteins increased strongly during seed germination [15]. Bartoli *et al*. found that protein carbonylation was higher in the mitochondria than in chloroplasts and peroxisomes in wheat leaves which suggest that the mitochondria are more susceptible to oxidative damage.

Protein carbonylation is a widely used marker of protein oxidation and sensitive methods for its detection have been developed [17]. Direct oxidative attack on Lys, Pro and Thr by secondary reactions on a reactive carbohydrate and lipid on Cys , His and Lys residues can lead to formation of protein carbonyl derivatives [18] . Carbonylation of protein inhibits or alters the activities and may increase the susceptibility of the protein to the proteolytic attack [19]. In some animals like in rats and in yeast and bacteria nearing senescence, the oxidation has been found to be specific for the respiratory and the stress proteins [20]. Quantitative and qualitative pattern of protein carbonylation has been studied in *Arabidopsis thaliana* during the progression of the life cycle of the plants. The carbonylation increases with the age of the plants similar to that of the animals / microbes but then drops steeply prior to translation of reproductive phase and again increases sharply during the senescence [21].

Experimental

Materials and Methods

Plant material and growth conditions

Wheat variety i.e. PBW-343 (*Triticum aestivum*, 2n=42, AABBDD) was procured from Division of Genetics, Indian Agricultural Research Institute, New Delhi. Seeds were grown on germination paper after surface sterilization with 0.1% $HgCl_2$. After emergence of coleoptiles (5-6 days after sowing), seedlings were transferred into Hoagland solution (low N- 50 µM KNO_3, high N- 5 mM KNO_3, ammonium salt-5 mM $NH_4(SO_4)_2$ and without N-zero N). The composition of nutrient solution used was macronutrient in mM: $Ca(NO_3)_2$ 1.5, KNO_3 5.0, $NH_4(NO_3)_2$ 1.0, $MgSO_4$ 2.0 and micronutrient in µM: H_3BO_3 1.0, $MnCl_2$.$4H_2O$ 0.5, $ZnSO_4$.$7H_2O$ 1.0, $CuSO_4$.$5H_2O$ 0.2, $(NH_4)_6Mo_7O_{24}$.$4H_2O$ 0.075 and $FeCl_3$ + EDTA.

The plants (**Figure 1**) were supported on Styrofoam (2" thickness) and grown in plastic containers of 4 L capacity. The solution was aerated continuously through aquarium pumps and PVC tubings. The solution was changed every alternate day. The whole set up was maintained in controlled environment chambers at National Phytotron Facility, IARI, New Delhi. In these chambers, the growth conditions were maintained as: 22°C/12°C day/night temperature, 10 h photoperiod with photon flux density of 450 µmol m^{-2} s^{-1} (PAR) and the relative humidity (RH) was 90%.

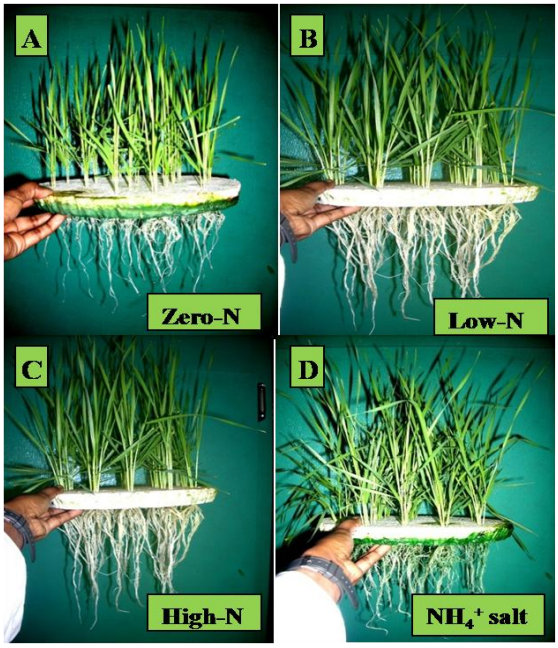


Figure 1 Wheat seedlings raised in Hoagland solution under controlled conditions with different levels and forms of N supply (15 d). (A) Without N (Zero-N), (B) Low (50 µM KNO_3), (C) High N (5 mM KNO_3), (D) Ammonium salt [5 mM $(NH_4)_2SO_4$]

ROS levels in wheat seedlings in response to N

The spectrophotometric assay of total superoxide radical content in the fresh leaf tissues is based on the principle of formation of blue coloured formazone by nitroblue tetrazolium chloride with superoxide radicals (O_2^-) by inhibiting total superoxide dismutase (SOD) activity, as described by Chaitanya and Naithani [22]. Hydrogen peroxide was estimated by forming titanium-hydro peroxide complex [23]. Supernatant was read at 415 nm against blank in UV-visible spectrophotometer (model Specord Bio-200, Analytik Jena, Germany). Hydrogen peroxide contents were calculated by comparing with a standard curve drawn with known hydrogen peroxide concentrations [24]. Lipid peroxidation was estimated as the thiobarbuturic acid reactive substances, according to the method of Heath and Packer [25]. Protease activity and total free amino acid were measured by the method of Moore and Stein [26]. Estimation for both the above parameters was done by the method of Lee and Takahashi [27].

Estimation of protein carbonyls

Total soluble proteins were extracted and derivatized by DNPH [28]. SDS-PAGE was performed [29] and resolved proteins were transferred to PVDF membranes and protein carbonyls were detected using anti-DNP antibodies and area of bands was analyzed using infra red imager (LICOR Odyssey).

Results and Discussion

Results

Wheat seedlings responded differently to the type of the N nutrition as well as to the level of N supply.

Free radicals levels

Maximum level of Superoxide radicals and H_2O_2 (**Table 1**) was observed in the wheat seedlings grown with ammonium salt followed by Zero-N and minimum in the wheat seedlings grown under 5mM NO_3^--N. Increase in the nitrate N dose resulted in the decline in the content of both the ROS in the wheat seedlings.

TBARS levels
There was significant difference of TBARS (**Table 1**) concentration among various N treatments. Maximum level of TBARS 509.64 nmol/g DW was observed in the wheat seedlings grown without N and minimum in the wheat seedlings grown under 5mM NO_3^--N.

Protease activity Maximum Protease activity (**Table 1**) was observed in the wheat seedlings grown without N and minimum in the wheat seedlings grown under 5mM NO_3^--N. Increase in the N dose resulted in the decline in the level of protease activity in the wheat seedlings.

Total free amino acids
There was significant difference of total free amino acids (**Table 1**) concentration among various N treatments. Maximum level of total free amino acids was observed in the wheat seedlings grown without N and minimum in the wheat seedlings grown under 5mM NO_3^--N.

Protein carbonyl/Oxidized protein levels
The 15d old wheat seedlings grown under various levels of N supply and nitrate and ammonium salts were analyzed for the level of protein carbonyls (**Figure 1**). It was observed that wheat seedlings grown without N and ammonium salts had minimum levels of carbonyls followed by 5 mM NO_3^--N. The seedlings from 50 µM NO_3^--N treatment had maximum levels of protein carbonyls. The level of protein carbonyls (**Figure 1c**) was 4 fold more in low N grown wheat seedlings as compared to the level of protein carbonyls in wheat seedlings grown without N. The minimum level of protein carbonyls was in ammonium fed plants wherein the content was 17 % lower as compared to the wheat plants grown without N. The protein carbonyls in the low N grown wheat seedlings were 2 fold higher as compared to the level of protein carbonyls in the high N grown wheat plants. In the wheat plants grown in 5 mM $NH4^+$-N the level of carbonyls was 60 % less as compared to the level of carbonyls in the seedlings from 5 mM NO_3^--N fed plants.

Discussion

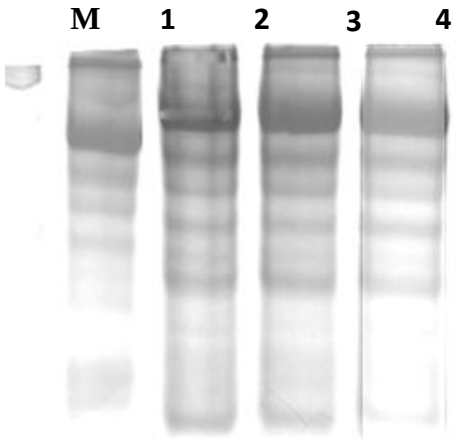
Reactive oxygen species (ROS) are produced continuously as by products of various metabolic pathways. Under steady state conditions these are scavenged by various antioxidative defense mechanisms [30]. The equilibrium between the production and scavenging of ROS may be perturbed by various biotic and abiotic stress factors, such as salinity, drought, UV radiation, nutrient deficiency, leading to sudden increase in ROS, causing significant damage to cell structures. ROS are highly reactive and causes damage to the macromolemolecules of the cells, such as lipids, proteins, DNA etc. the changes in the concentration of ROS can also lead to changes in cell signaling by modulating gene expression.

The implication of reactive oxygen species (ROS) during nitrogen and specifically ammonium stress has not yet been well characterized. Studies that combine ammonium and salt stress in sunflower and corn suggest that ammonium or its assimilation molecules, glutamate or glutamine, may serve as a stress signal to activate antioxidant enzymes, which play a key role in adaptation to stress situation [31]. Polesskaya *et al*. [5] indicated that NH_4^+-N induced antioxidant enzyme activities, and during N deficiency implies an oxidative stress because of an increased Mehler reaction and because of an energetic imbalance related to a reduction in N assimilation. However, they did not observe low-molecular weight antioxidant status or markers of oxidative damage to demonstrate that an oxidative stress was taking place. More recently, Skopelitis *et al*. [32] have shown that ammonium ions induce generation of ROS in a study with cell suspensions of *Vitis vinifera*. However, the cell suspension model may behave differently to the whole plant model, and the analysis of ROS production and its effects in the whole plant deserve attention. Plants have evolved various protective mechanisms to eliminate or reduce ROS, which includes enzymes such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), guiacol peroxidase (GPX) and catalase (CAT). In biological systems, reduced glutathione (GSH) and ascorbate (ASC) appear to be the most important soluble low molecular antioxidants, which can react directly with superoxide and hydroxyl radicals or participate in enzymatic reactions that scavenge ROS [7, 33]. Plant phenolic compounds may also act as antioxidants in different reactions [34]. The production of superoxide radicals and H_2O_2 was maximum in ammonium fed wheat seedlings followed by zero N seedlings. Parallel to this, we observed that lipid peroxidation was enhanced in nutrient stressed and ammonium fed wheat seedlings. Enhanced production of oxygen free radicals is responsible for peroxidation of membrane lipids and the degree of peroxides damage of cell is controlled by the potency of peroxidase enzyme system [35]. Very low

levels of protein carbonyls in low N and ammonium grown wheat seedlings indicate degradation. This correlates well with the low content of the total soluble proteins and high levels of total free amino acids in these treatments. Ammonium being toxic for the wheat plants as seen in this study led to high levels of ROS in the medium. But surprisingly the levels of protein carbonyls were low in wheat seedlings grown in NH_4^+-N and zero-N grown plants. This indicated high turnover rates of protein in NH_4^+-N fed wheat seedlings as the level of free amino acids was high, protease activity was high and level of soluble proteins was low.

Table 1 ROS, TBARS, Total soluble proteins, free aminoacids and Protease levels in various N-treated seedlings

Parameters	Treatments					
	Zero-N	Low N	High N	Ammonium salt	CD at 5%	SE(m)
Total soluble proteins (mg g^{-1} FW)	1.875 ±0.103937	4.803 ±0.341744	10.36 ±0.534206	2.136 ±0.175021	1.103	±0.333
Superoxide radicals (n mol $min^{-1}g^{-1}$ FW^{-1})	16.64806 ±0.910364	13.47696 ±1.107043	12.74683 ±0.436438	18.01695 ±0.523695	2.629	± 0.794
H_2O_2 (µ mol g^{-1} FW^{-1})	0.113 ± 0.003489	0.111 ±0.002946	0.068 ±0.001092	0.132 ±0.001387	0.008	± 0.003
TBARS (n mol g^{-1} DW^{-1})	509.64 ±5.902306	310.63 ±10.74369	191.71 ±4.572007	381.96 ±13.28439	30.847	±9.324
Protease activity (µ mol g^{-1} FW^{-1})	67.62 ±4.528	50.20 ±3.460	27.27 ±3.216	63.62 ±1.290	11.044	± 3.335
free amino acids (µ mol g^{-1} FW^{-1})	45.3 ±1.75	33.34 ±0.604	24.16 ±0.898	40.63 ±1.12	3.894	± 1.176



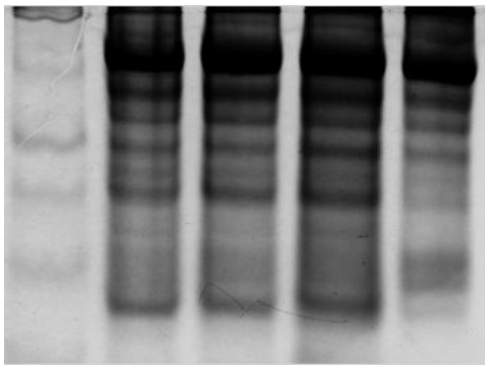


Figure 1a Protein oxidation in different N treated seedlings **Figure 1b** CBB Stain

Lane1: Marker
Lane2: Zero-N
Lane3: Low N-50 µM KNO_3
Lane4: High N-5 mM KNO_3
Lane5: Ammonium salt-5 mM $NH_4(SO_4)$

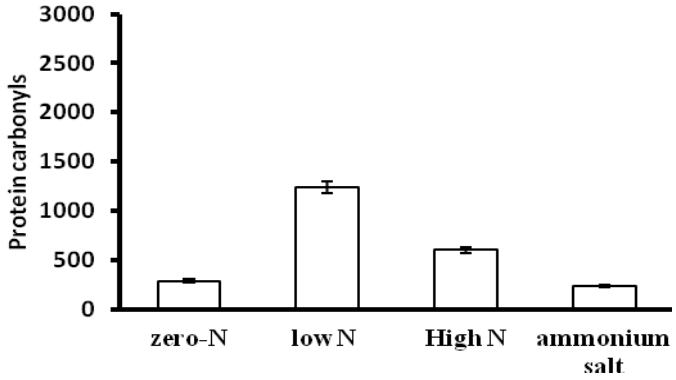


Figure 1c Protein carbonyl levels in various N-treated seedlings

Conclusion

Our results suggest that the wheat plants are sensitive to limiting N supply and form of N provided in the medium. Low N and Ammonium stress led to high levels of ROS in wheat plants and these ROS were responsible for oxidization/carbonylation of proteins. But interestingly the levels of protein carbonyls were low in wheat seedlings fed with NH_4^+-N and zero-N indicating high turnover of protein in these plants which was also seen to correspond with high level of free amino acid, high level of protease activity and low level of total soluble proteins in these plants. However, how these carbonylated proteins are degraded in the plants, which pathway is involved and many such other questions are still needed to be answered.

References

[1] Cakmak I and Marschner H (1992). Magnesium deficiency and high light intensity enhance activity of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. Plant Physiol. 98: 1222-1227.
[2] Kandlbinder A, Finkemeier I, Wormuth D, Hanitzsch M and Dietz KJ (2004). The antioxidant status of photosynthesising leaves under nutrient deficiency: redox regulation, gene expression and antioxidant activity in Arabidopsis thaliana. Physiologia Plantarum. 120: 63-73.
[3] Abrol YP, Chatterjee SR, Kumar PA and Jain V (1999). Improvement in nitrogen use efficiency: physiological and molecular approaches. Curr. Sci. 76:1357-1364.
[4] Dominguez-Valdivi MD, Aparicio-Tejo PM, Lamsfus C, Cruz C, Martins-Loucao MA and Moran JF (2007). Nitrogen nutrition and antioxidant metabolism in ammonium tolerant and sensitive plants. Physiol. Plant. 132: 359-369.
[5] Polesskaya OG, Kashirina EI and Alekhina ND (2004). Changes in the activity of antioxidant enzymes in wheat leaves and roots as a function of nitrogen source and supply. Russ. J. Plant Physiol. 51: 615–620.
[6] Del Rio LA, Sandalio LM, Corpas FJ, Palma JM and Barros JB (2006). Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signalling. Plant Physiol. 141: 330-335.
[7] Foyer CH and Noctor G (2005). Redox homeostis and antioxidant signalling: a metabolic interface between stress perception and physiological responses. Plant Cell. 17: 1866-1875.
[8] Bhattacharjee S (2005). Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plant. Curr. Sci. 89: 1113-1121.
[9] Gratao PL, Polle A, Lea PJ, Azevedo RA (2005). Making the life of heavy metal stressed plants a little easier. Funct. Pl. Biol. 32: 481-494.
[10] Scandalios JG (1993). Oxygen Stress and Superoxide Dismutases Plant Physiol. 101: 7-12.
[11] Sairam RK and Tyagi A (2004). Physiology and molecular biology of salinity stress tolerance in plants. Current Science. 86(3): 407-421.
[12] Davis K J A (1987). Protein damage and degradations by oxygen radicals. J. Biol. Chem. 262: 9895-9901.
[13] Liebler DC, Kling DS, Reed DJ (1986). Antioxidant protection of phospholipid bilayers by a tocopherol. Control of a-tocopherol status and lipid peroxidation by ascorbic acid and glutathione. J Biol Chem. 261:12114–12119.
[14] Allen RD (1995). Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol. 107: 1049-1054.

[15] Job C, Rajjou L, Lovigny L, Belghazi M and Job D (2005). Patterns of protein oxidation in Arabidopsis seeds and during germination. Pl. Physiol. 138: 790-802.
[16] Shringarpure R, Grune T, Mehlhase J, Davies KJ (2003). Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. J Biol Chem. 278: 311–318.
[17] Levine RL, Wehr N, Williams JA, Stadtman ER and Shacter E (2000). Determination of carbonyl groups in oxidized proteins. Methods Mol. Biol. 99: 15–24.
[18] Nystro¨m T (2005). Role of oxidative carbonylation in protein quality control and senescence. The EMBO Journal. 24:1311–1317.
[19] Berlett BS, Stadtman ER (1997). Protein oxidation in aging, disease, and oxidative stress. J Biol Chem. 272:20313– 20316.
[20] Dan I, Watanabe NM, Kusumi A (2001). The Ste20 group kinases as regulators of MAP kinase cascades. Trends Cell Biol. 11:220 –230.
[21] Johansson E, Olsson O, Nystro¨ m T (2004). Progression and specificity of protein oxidation in the life cycle of Arabidopsis thaliana. J. Biol. Chem. 279: 22204–22208.
[22] Chaitanya KSK and Naithani SC(1994). Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of Shorea robusta Gaertn.f. New Phytol.126:623-627.
[23] Rao MV, Paliyath G, Ormrod DP, Murr DP and Watkins CB (1997). Influence of salicylic acid on H_2O_2 production, oxidative stress and H_2O_2 metabolizing enzymes. Plant Physiol. 115: 137-149.
[24] Teranishi Y, Tanaka A, Osumi M and Fukui S (1974). Catalase activity of hydrocarbon utilizing candida yeast. Agric. Biol. Chem. 38: 1213-1216.
[25] Heath RL and Packer L (1968). Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch. Biochem. Biophys. 125: 189-198.
[26] Moore and Stein WH (1954). A modified ninhydrin reagent for photometric determination of amino acids and related compounds. J. Biol. Chem. 211: 907-913.
[27] Lee YP and Takahashi T (1966). An improved colorimetric determination of amino acids with use of ninhydrin. Anal. Biochem. 14: 71-77.
[28] Jain V, Kaiser W and Huber SC (2008). Cytokinin inhibits the proteasome-mediated degradation of carbonylated proteins in Arabidopsis leaves. Plant Cell Physiol, 49(5): 843-8
[29] Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature. 227: 680-685.
[30] Gill SS and Tuteja N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry. 48(12): 909-930.
[31] Rios-Gonzalez K, Erdei L and Lips SH (2002). The activity of antioxidant enzymes in maize and sunflower seedlings as affected by salinity and different nitrogen sources. Plant Sci. 162: 923–930.
[32] Skopelitis DS, Paranychianakis NV, Paschalidis KA., Pliakonis ED, Delis ID, Yakoumakis DI, Kouvarakis A, Papadakis AK, Stephanou EG and Roubelakis-Angelakis K.A (2006). Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell. 18:2767–2781.
[33] Smirnoff N (2000). Ascorbate, tocopherol and carotenoids: metabolism, pathway engineering and functions. In: Smirnoff N (ed) Antioxidants and Reactive Oxygen Species in Plants. Blackwell Publishing, Oxford, pp 53–78.
[34] Kahkonen MP, Hopia AI., Vuorela HJ, Rauka JP, Pihlaja K, Kujala TS and Heinonen M (1999). Antioxidant activity of plant extracts containing phenolic compounds. J. Agric. Food Chem. 47: 3954–3962.
[35] Gomathi R and Rakkiyapan P (2011). Comparative lipid peroxidation, leaf membranethermostability, and antioxidant system in four sugarcane genotypes differing in salt tolerance. International Journal of Plant Physiology and Biochemistry. 3(4): 67-74.

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