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Antioxidant Defences During Heat Stress in Leaves of Two Varieties of *Sorghum bicolor* (L.) Moench

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ABSTRACT. *The activities of different enzymes involved in oxidation-reduction reactions were affected greatly to protect the membranes from damage. Using physiological and biochemical indices, two sorghum varieties were screened for heat tolerance (a heat tolerant CK 60A and a heat susceptible IS 84). Micro level scrutiny on antioxidant activity was done to study their behaviour under heat stress. Peroxidase (POD) and ascorbic acid oxidase (AAO) enzyme activities increased upon heat stress and decreased after recovery. In contrast, catalase (CAT) activity declined on heat shock and the decline was more severe in IS 84. On recovery, there was a slight increase in CAT activity in the susceptible genotype. Superoxide dismutase (SOD) activity increased on heat stress in both varieties and there was decline in SOD activity on recovery. The activity of glutathione reductase increased on heat shock in both varieties. CK 60A was evaluated as a more heat tolerant type by way of having increased antioxidant defence mechanisms than IS 84.*

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) ranks 4th in terms of world cereal production and is the major staple food for rural poor. Where water, nutrients, diseases and insect pests are not limiting, crop growth is determined by environmental factors. In semi-arids where sorghum is grown, the air temperature often exceeds 40°C and a leaf temperature as high as 55°C has been recorded (Peacock, 1982). Drought is commonly associated with high temperature making it difficult to separate the effect of these 2 factors on crop yield under drought conditions. Response to heat is based ultimately on cell function and structure. In addition, it is important to have an account on reversible effects. Ability to recover rapidly is crucial, since functional disturbances such as depression of photosynthetic or protein metabolism reduces the productivity, growth and competitive ability of a plant. Therefore, the need for laboratory techniques were emphasized to identify heat tolerant genotypes since conditions which allow selection for heat tolerance often do not occur naturally in the field. Under heat stress, activities of different enzymes involved in oxidation-reduction reactions are affected greatly to protect the membranes from damage.

Plants subjected to water stress undergo increased exposure to activated forms of oxygen and accumulation of free radicals associated with damage to membranes and build up of lipid peroxides (Smirnoff, 1993). Plant cells normally are protected against such effects by a complex antioxidant system, which includes lipid soluble, membrane associated antioxidants, water soluble reductants and enzymatic antioxidants, *e.g.*,

superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6), peroxidase (POD) (EC 1.11.1.7) and enzymes of the ascorbate-glutathione cycle.

Many biochemical changes are associated with the exposure of plants to harmful conditions. Among them, increased peroxidase activity has been cited as an indicator of physiological stress (Levitt, 1972). Ascorbic acid oxidase is widespread in plant tissues. The role of this enzyme is to regulate the levels of oxidised and reduced glutathione and nicotinamide adenine dinucleotide phosphate (NADPH). The activity of this enzyme is increased during infection. Upadhayaya *et al.* (1991) studied the heat shock tolerance and anti oxidant activity in moth bean in the presence and absence of epibrassinolide (EBL) and observed that at 48°C, EBL increased ascorbic acid oxidase activity. Catalase catalyses the destruction of hydrogen peroxide radicals. This is expected to limit the concentration of free radicals, which cause membrane damage through lipid peroxidation. Catalase activity was found to be involved in mediating the response of plant cells to various stresses (Larson, 1988).

CAT reacts directly with H_2O_2 to form water and oxygen (Smirnoff, 1993). Catalase activities showed an increase or maintenance in the early phase of drought and then a decrease with further increase in magnitude of water stress in seven Triticum species (Jingxian Zhang and Kirkham, 1994). A variety of toxic oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen are generated in chloroplasts during photosynthesis. The mechanisms evolved by explants to scavenge these toxic oxygen species in the chloroplast are superoxide dismutase, ascorbate peroxidase and glutathione reductase with the possible addition of DHA reductase. These systems protect membranes from the deleterious effects of toxic oxygen species, which are produced at elevated rates when plants are exposed to environmental and xenobiotic stress conditions. The active oxygen species are all very reactive and cause a cascade of oxidative reactions resulting in chlorophyll bleaching and membrane destruction. H_2O_2 generated through the action of SOD is removed by catalases and peroxidases. The SOD protects cells and tissues from damaging effects of oxidative stress by removing O_2 before it reacts with cellular H_2O_2 to form highly reactive hydroxyl (OH) radicals. Elevated activity of SOD was reported during flooding stress in maize leaves (Yan Bin *et al.*, 1996).

Glutathione reductase plays an important role in the protection of plants from both high and low temperature stresses by preventing the oxidation of enzymes and membranes. Mahan *et al.* (1990) determined the thermal dependencies of the apparent K_m of the glutathione reductases from spinach, corn and cucumber. They proposed the term "thermal kinetic window" to describe the range of temperature over which the apparent K_m of the glutathione reductase was within 200% of its minimum and suggested that it might be an useful indicator of the limits of thermal stress for a given species. Mahan (1994) used a computer programme to develop estimates of the thermal dependence of reaction velocity at a given temperature and substrate concentration. It is suggested that the predicted thermal dependence of velocity may be a useful indicator for the optimal thermal range of plant enzymes.

Therefore, this study was initiated to determine the defence mechanisms of different sorghum varieties using physiological and biochemical indices during heat stress and recovery. This study will facilitate better understanding of the molecular mechanisms

of heat tolerance. Investigations of such functional disturbances may also lead to the finding of significant indicators of plant vitality, which could be used to predict the limits of heat tolerance.

MATERIALS AND METHODS

Investigations of this work were carried out in the Centre for Plant Molecular Biology, TNAU, Coimbatore. Five varieties of Sorghum (CK 60A, 2219A, CO 25, CO 26 and IS 84) were used in the screening procedure, which were based on few physiological indices like relative water content, chlorophyll stability index, membrane thermostability and some biochemical indices like proline content, chlorophyll fluorescence, chlorophyll a, b and carotenoid content. One heat tolerant (CK 60A) and one heat susceptible (IS 84) variety were finally chosen, and were subjected to micro level scrutiny to study the role of different antioxidants in protecting plants during heat stress as the above 2 varieties were distinctly different in their behaviour to heat stress. The optimum temperature for heat shock was fixed as 42°C based on germination studies. For all enzyme assays, samples from heat shocked (42°C for an hour), heat shocked and recovered (42°C for an hour, and an hour recovery at room temperature) and control (room temperature) plants were taken. All enzyme assays were repeated 4 times and tables were subjected to statistical analysis.

Peroxidase (POD)

Leaf samples (2.0 g) were homogenised in cold phosphate buffer (0.05 M, pH 6.5). The homogenate was centrifuged at 10,000 rpm in a Beckman centrifuge J2–21 (rotor JA 20.1) at 4°C for 10 min. The filtrate was used as the enzyme source, assayed following the method of Kar and Mishra (1976) with slight modifications. Five mL of the assay mixture contained 300 μM phosphate buffer (pH 6.8), 50 μM catechol, 50 μM H_2O_2 and 1.0 mL of crude enzyme extract. After incubation at 25°C for 5 min the reaction was stopped with the addition of 1.0 mL of 10% H_2SO_4. The colour was read at 430 nm. For enzyme assay, value at zero time was taken as blank and the activity of enzyme was expressed as unit g^{-1} fresh weight of tissue following the formula.

$$Enzyme\ Activity = \frac{(A \times TV)}{t \times v}$$

Where,

- A - Absorbance of sample after incubation minus the absorbance at zero time
- TV - Total volume of the filtrate
- t - The time (minutes) of incubation with substrate and
- v - The total volume of the filtrate taken for incubation

Ascorbic acid oxidase (AAO)

Leaf samples (2.0 g) were homogenised in cold phosphate buffer (0.05 M, pH 6.5). The homogenate was centrifuged at 10,000 rpm in a Beckman centrifuge J2–21 (rotor

JA 20.1) at 4°C for 10 min. The filtrate was used as the enzyme source, assayed using the combined method of Oliver (1967) and Chinoy *et al.* (1976). The reaction mixture for this enzyme assay consisted of 1.0 mL of 100 μM ascorbic acid, 1.0 mL of 500 μM phosphate buffer (pH 6.8) and 2.0 mL of the enzyme extract. This was incubated at 37°C for 25 min. After incubation, 0.2 mL of 2,6-dichlorophenol indophenol was added and the reading was taken at 620 nm in Spectrochem Spectrophotometer. The enzyme activity was calculated using the formula for peroxidase.

Catalase (CAT)

Leaf tissues (100 mg) were crushed in 0.1 M phosphate buffer (pH 7.0) containing 1.0% PVP and centrifuged at 4°C. The enzyme assay was carried out according to the method described by Biswas and Choudhuri (1976). Enzyme activity was calculated as given for peroxidase.

Superoxide dismutase (SOD)

Assay of superoxide dismutase enzyme was done with slight modification of the original procedure described by Beacuchamp and Fridovich (1971). Treated and control leaves (500 mg) were ground in 50 mM potassium phosphate buffer (pH 7.8) and centrifuged at 10,000 rpm in a Beckman centrifuge J2–21 (rotor JA 20.1) at 4°C for 10 min. The supernatant was used for estimation. A 3.0 mL reaction mixture consisting of 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 0.1 mM EDTA, 75 μM nitroblue tetrazolium (NBT) and 50 μl of crude enzyme was set up with 4 replications. Volume was made up with double distilled water. A blank without enzyme and NBT was used to calibrate the spectrophotometer (Beckman DU 64). Another control (having NBT but no enzyme) served as a reference blank. All the tubes were exposed to fluorescent light (two 42 cm and two 60 cm fluorescent tubes) for 15 min. Immediately the absorbance was read at 560 nm in a Beckman DU 64 Spectrophotometer. The protein in the sample was also measured simultaneously with the available enzyme samples. Fifty per cent inhibition is an unit of SOD enzyme. The percentage inhibition was calculated and expressed as number of units of enzyme mg ml^{-1} min^{-1}.

Glutathione reductase (GR)

The activity of glutathione reductase was estimated as described by Smith *et al.* (1988). Tissues (200 mg) were ground in 500 μl of ice cold 0.2 M potassium phosphate buffer (pH 7.5) containing 1.0 mM EDTA. It was centrifuged at 10,000 rpm at 4°C for 10 min in a Beckman centrifuge J2–21 (rotor JA 20.1). The supernatant was used as the enzyme source. A 2.0 ml reaction mixture consisting of the following ingredients was taken in a clear quartz cuvette

(i) Potassium phosphate buffer 0.2 M containing 1.0 mM EDTA : 1.0 mL
(ii) 5–5' Di-thiobis (2 nitro benzoic acid) (DTNB) 3.0 mM pH 7.5 : 0.5 mL
(iii) β-NADPH 10 mM : 0.1 mL

(iv) Distilled water	:	0.460 mL
(v) Crude extract	:	0.005 mL
(vi) Glutathione oxidized (GSSG) 20 mM	:	0.1 mL

All the ingredients listed above except GSSG were added and used as the blank to calibrate the Spectrophotometer (Beckman DU 64). A kinetics Soft Pac module was used to assay the enzyme at every 30 seconds interval for a total period of 5 min at 412 nm. The reaction was initiated by the addition of 0.1 mL of GSSG. The increase in absorbance was calculated from the linear portion of the enzyme reaction curve and the enzyme activity was expressed as increase in absorbance min^{-1} mg^{-1} protein.

RESULTS

Peroxidase (POD)

CK 60A had significantly higher ($P<0.05$) peroxidase activity than IS 84 in control and heat shocked conditions. The increase in enzyme activity after heat shock was greater in CK 60A than in IS 84. The activity showed a rapid decline (from 53.85 to 24.35%) in IS 84 during recovery period as compared to CK 60A (Table 1; Fig. 1). The interaction was significant at $P<0.05$.

Table 1. Effect of heat shock and recovery on peroxidase activity of sorghum varieties.

Varieties	Control	Heat shocked	Heat shocked and Recovered
CK 60A	3.460	5.390	5.167
IS 84	2.600	4.000	3.233
	Varieties	Treatments	V×T
CD (0.05)	0.126	0.155	0.218

(Unit g^{-1} fresh weight)

Ascorbic acid oxidase (AAO)

The variety CK 60A had a significantly greater ($P<0.05$) activity of ascorbic acid oxidase when compared to IS 84. Up to 45% of increase in enzyme activity was observed in the tolerant variety whereas in susceptible the increase was only 21%. The enzyme activity declined to unstressed level in CK 60A on recovery (Table 2; Fig. 1). The interaction effect was significant ($P<.0.05$) only in CK 60A under heat shock condition.

Table 2. Effect of heat shock and recovery on ascorbic acid oxidase activity of sorghum varieties.

Varieties	Control	Heat shocked	Heat shocked and Recovered
CK 60A	0.320	0.460	0.317
IS 84	0.280	0.337	0.297
	Varieties	Treatments	V×T
CD (0.05)	0.018	0.022	0.031

(Unit g^{-1} fresh weight)

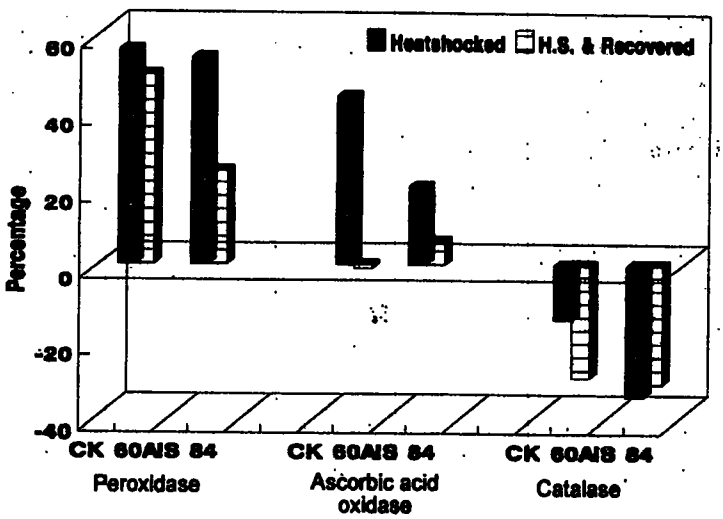

Fig. 1. Effect of heat shock on enzyme activities (per cent over control) of sorghum varieties.

Catalase (CAT)

The variety CK 60A had a significantly higher ($P<0.05$) catalase activity than IS 84 in all treatments. Both varieties had significant reduction in catalase activity after heat shock and the reduction was significant even after recovery in CK 60A, though IS 84 had a slight raise in catalase activity (only 3%) after recovery (Table 3; Fig. 1). The reduction due to heat shock was only 14% in CK 60A and around 35% in IS 84. At 5% level, all interaction effects were significant.

Superoxide dismutase (SOD)

The variety CK 60A had a significantly higher ($P<0.05$) superoxide dismutase activity than IS 84. The enzyme activity increased (27.50%) after heat shock and then decreased to 19% during recovery in CK 60A over its control. CK 60A had a significant reduction in SOD activity when the heat shock was recovered (Table 4; Fig. 2). V×T interaction was significant ($P<0.05$).

Table 3. Effect of heat shock and recovery on catalase activity of sorghum varieties.

Varieties	Control	Heat shocked	Heat shocked and Recovered
CK 60A	2.75	2.35	1.93
IS 84	1.81	1.18	1.24
	Varieties	Treatments	V×T
CD (0.05)	0.028	0.035	0.049

(Unit g^{-1} fresh weight)

Table 4. Effect of heat shock and recovery on superoxide dismutase activity of sorghum varieties.

Varieties	Control	Heat shocked	Heat shocked and Recovered
CK 60A	1.20	1.53	1.43
IS 84	1.03	1.13	1.13
	Varieties	Treatments	V×T
CD (0.05)	0.025	0.030	0.043

(Unit enzyme mg $protein^{-1}$ ml^{-1} min^{-1})

Glutathione reductase (GR)

Glutathione reductase activity was significantly higher ($P<0.05$) in CK 60A than in IS 84. The increase was more than two times in CK 60A while it was 1.8 times in IS 84. Though the level of decline in enzyme activity was similar in both varieties during the recovery period, CK 60A tended to maintain a high level of enzyme activity (Table 5; Fig. 2). All the interaction effects were significant at $P<0.05$.

Table 5. Effect of heat shock and recovery on glutathione reductase activity of sorghum varieties.

Varieties	Control	Heat shocked	Heat shocked and Recovered
CK 60A	0.703	1.657	1.010
IS 84	0.407	0.763	0.603
	Varieties	Treatments	V×T
CD (0.05)	0.010	0.012	0.017

(increase in absorbance min^{-1} mg^{-1} protein)

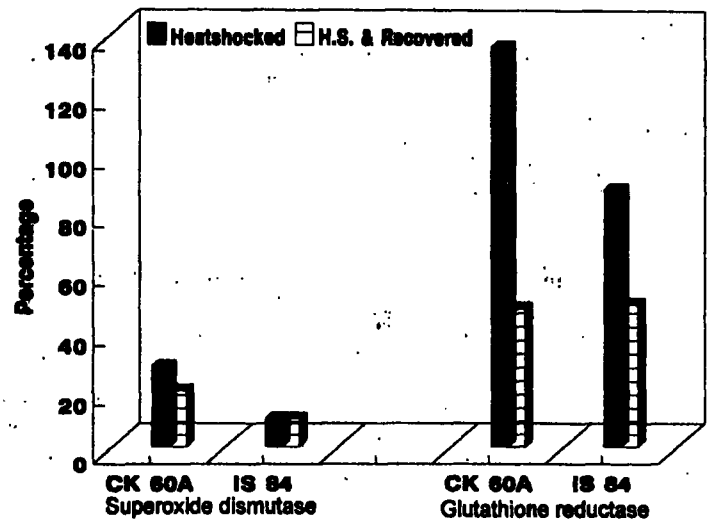

Fig. 2. Effect of heat shock on enzyme activities (per cent increase over control) of sorghum varieties.

DISCUSSION

Plant cells normally are protected from activated forms of oxygen and accumulation of free radicals associated with damage to membranes and build up of lipid peroxides due to various stresses by antioxidant systems in plants such as peroxidase, catalase, ascorbic acid oxidase and superoxide dismutase. An increase in oxidative enzymes upon water stress was reported in Vigna seedlings (Mukherjee and Choudhuri, 1981). Except catalase, the activities of all the other oxidative enzymes increased due to water stress. Badiani *et al.* (1990) considered the relation between drought stress and enzymatic antioxidant systems in different species.

Peroxidase (POD)

Peroxidase catalyses hydrogen peroxide dependent oxidation of substrates (RH2) according to the general equation.

$$RH_2 + H_2O_2 \rightarrow H_2O + R$$

POD activities of both the genotypes (CK 60A and IS 84) increased greatly upon heat stress and started decreasing after recovery. Elevated H_2O_2 concentrations could release (POD) from membrane structures with which it was normally associated. An increase in POD was observed in other stress conditions such as salt (Siegel, 1993). Increase in POD activities was reported in diploid and tetraploid wheat upon heat stress (Jingxian and Kirkham, 1994).

Activity of ascorbic acid oxidase which catabolises ascorbic acid, another antioxidant was reported to increase on heat stress (Upadhyaya *et al.*, 1991). Ascorbic acid activity increased in both varieties on heat shock and started to decline when the shock was removed. In CK 60A, the recovery was close to the unstressed levels. The results also suggested that a high degree of oxidative metabolism was involved in heat stressed leaves. Similar increase in AAO was reported upon heat stress in moth bean seedlings by Upadhayaya *et al.* (1991).

The breakdown of H_2O_2 is catalysed by catalase. CAT activity declined on heat shock and the decline was severe in the susceptible variety (IS 84). The decline in CAT activity favoured the accumulation of H_2O_2. This decrease in CAT activity was due to the inactivation by the accumulated H_2O_2 induced by heat stress and could be explained partly by the inactivation of the enzyme.

Dismutation of $^{\cdot}O_2$ results in formation of H_2O_2 and elevated levels of both (O_2 and H_2O_2) might create highly active hydroxyl radicals ($^{\cdot}OH^-$) and single oxygen (O_2) as a result of mutual interactions. These free radicals could react with numerous cell components, thereby causing inactivation of enzymes, pigment bleaching, lipid peroxidation and protein degradation with consequent membrane damage. Under normal conditions, damage to the photosynthetic apparatus was minimised by means of scavenger systems which catalysed the rapid decomposition of H_2O_2 and superoxide. Enzymes of chloroplast protective mechanisms are SOD, Glutathione reductase, ascorbic peroxidase and dehydro ascorbate reductase. The $^{\cdot}O_2$ is finally metabolised to water within a cycle involving ascorbic acid and glutathione. The concentration of active oxygen was likely to be increased in plants suffering from heat stress. If the variety failed to adapt, the active mechanisms might soon be overburdened and final results would be photo oxidative damage. The SOD activity increased on heat stress in both varieties and there was a gradual decline in SOD activity on recovery only in the tolerant variety. In the susceptible variety, the increased activity was retained at the elevated level even after the recovery. The enzyme activity was a result of both synthesis and degradation. Increase in net SOD activity under heat stress might be ascribed to increase synthesis of SOD and/or decrease degradation.

SOD showed an increase or was stable in the early phase of water stress and then decreased after rewatering (Jingxian and Kirkham, 1994) in wheat species. Davidson *et al.* (1996) reported that over expression of catalase and dismutase genes caused an increase in thermotolerance in *Saccharomyces cerevisiae*. In the present work, CK 60A had a better tolerance mechanism than IS 84 by way of having higher SOD activity to scavenge the toxic radicals generated during heat stress than IS 84. The activity decreased on the event of recovery.

Glutathione reductase is an important flavo protein which catalyses the enzymatic conversion of oxidised dimer (GSSG) to the reduced monomer (GSH). Chloroplast might be a primary site of damage caused by high temperature stress in plant tissues and heat stress caused an increase in GR activity. GR played an important role in protection of plants from both high and low temperature stresses by preventing the oxidation of enzymes and membranes. This increase might be in part due to feed back mechanisms responding to increase demand for GSH. In the susceptible variety, the elevation was not much and therefore, less activity. Mahan (1994) reported an increased activity of GR that played a role in the detoxification of oxidants that often resulted from exposure of plants to high/low temperature. Criessen *et al.* (1996) also regarded GR as a key enzyme in defence against oxidative stress in peas.

CONCLUSIONS

Many environmental stresses cause damages to plants directly or indirectly through formation of activated oxygen species. Among the two sorghum genotypes, CK 60A showed better enzymatic defence mechanisms against harmful H_2O_2 and other free radicals by way of elevated levels of enzymatic activity (antioxidants) of POD, AAO, SOD and GR than IS 84. The increased tolerance was correlated with increased activities of some anti-oxidant systems. Such enhanced activity of anti oxidant systems plays a role in the ability of plants to withstand high temperature stresses.

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REFERENCES

Badhiani, M., De Biasi, M.G.M., Colognota, M. and Artemi, F. (1990). Catalase, peroxidase and superoxide dismutase activities in seedlings submitted to increasing water deficit. Agronomica. 34: 90-102.

Beauchamp, C.O. and Fridovich, I. (1971). Superoxide dismutase improves assay and an assay applicable to acrylamide gels. Annal. Biochem. 44: 276-287.

Biswas, A.K. and Choudhuri, M.A. (1976). Control of senescence of rice leaf by foliar treatment with essential elements. Sci. Cult. 42: 236-240.

Chinoy, J.J., Singh, Y.D. and K. Gurumurthi (1976). Calorimetric determination of ascorbic acid turn over in plants. Indian J. Pl. Physiol. 19: 122-130.

Creissen, G., Broadbent, P., Stevens, R., Wellburn, A.R. and Mullineaux, P. (1996). Manipulation of glutathione metabolism in transgenic plants. Biochemical Society Transactions. 24(2): 465-469.

Davidson, J.F., Whyte, B., Bissinger, P.H. and Schiestl, R.H. (1996). Oxidative stress involved in heat induced cell death in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. 93(10): 5116-5121.

Jingxian, Z. and Kirkham, M.B. (1994). Drought stress induced changes in activities of superoxide dismutase, catalase and peroxidase in wheat species. Plant Cell Physiol. 35(5): 785-791.

Kar, M. and Mishra, D. (1976). Catalase, peroxidase and polyphenol oxidase activities during rice leaf senescence. Plant. Physiol. 57: 315-319.

Larson, R.A. (1988). The antioxidants of higher plants. Phytochemistry. 27: 969-978.

Levitt, J. (1972). Responses of plants to environmental stress. Academic Press Inc., London, UK. ISBN 0-12-445560-3.

Mahan, J.R., Burke, J.J. and Orzech, K.A. (1990). Thermal dependence of the apparent K_m of glutathione reductases from three plant species. Plant Physiol. 93: 822-824.

Mahan, J.R. (1994). Thermal dependence of GR. Thermal limitation on antioxidant protection in plants. Crop Sci. 34(6): 1550-1556.

Mukherjee, S.P. and Choudhuri, A. (1981). Effect of water stress on some oxidative enzymes and senescence in vigna seedlings. Physiol. Plant. 52: 37-42.

Oliver, M. (1967). Ascorbic Acid: Estimation. pp. 338-359. *In*: Sebrell, W.H. Jr. and Harris, R.S. (Eds). The Vitamins, Academic Press Inc., London, UK.

Peacock, J.M. (1982). Response and tolerance of sorghum to temperature stress. pp. 143-159. *In*: Sorghum in Eighties. Proc. Intl. Sump. Sorghum, Nov. 2-7, 1981. OCHRIEST, Patencheru, A.P., India.

Siegel, B.Z. (1993). Plant-peroxidases- an organisimic perspective. Plant Growth Regulators. 12: 303-312.

Smimoff, N. (1993). The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol. 125: 27-58.

Smith, I.K., Vietheller, T.L. and Thorne, C.A. (1988). Assay of glutathione reductase in crude tissue homogenates using 5-5' Di-thiobis (2-nitro-benzoic acid). Annal. Biochem. 175: 408-413.

Upadhyaya, A., Davis, T.D. and Sankla, N. (1991). Heat shock tolerance and anti oxidant activity in moth bean seedlings treated with tetcyclasis. Plant Growth Regulation. 10 (3): 215-222.

Yan Bin, Dai Qiujie, Liu Xianozhong, Huang Shaobai and Wang Xixia. (1996). Flooding induced membrane damage, lipid oxidation and activated O_2 generation in corn leaves. Plant and Soil. 179(2): 261-268.