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Implications of reactive oxygen species in heat shock induced germination and early growth impairment in *Amaranthus lividus* L.

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Abstract

An effort has been made to assess the role of reactive oxygen species in germination and subsequent growth of *Amaranthus lividus* under elevated temperature. Transfer of *A. lividus* seeds from 25 to 45 °C for 4, 8 and 12 h, during early imbibitional period reduced percentage of germination, relative germination performance, relative growth index and seedling length. Heat shock during early germination decreased also the activities of free radical scavenging enzymes like catalase, peroxidase and superoxide dismutase, increased the accumulation of superoxide, hydrogen peroxide and induced lipoxygenase mediated membrane lipid peroxidation. Membrane injury index and relative leakage ratio revealed a rise with concomitant reduction in membrane protein thiol content in heat shock raised seedlings. The results indicate that heat shock in *A. lividus* seeds induced an excessive generation of ROS and led to an oxidative membrane damage, causing early growth impairment.

Additional key words: free radicals, free radical scavengers, malondialdehyde, oxidative membrane damage, superoxide.

Introduction

High temperature influences germination and subsequent seedling growth (Alka and Khanna-Chopra 1995, Bewley and Black 1982, Bhattacharjee and Mukherjee 1998). In fact, imbibition and early germination event continue even at supra optimum temperature but embryo growth in most of the cases is inhibited. Such damage can be ascribed directly to metabolic disfunctioning pertaining to loss of membrane integrity in juvenile tissues (Alka and Khanna-Chopra 1995, Bhattacharjee and Mukherjee 1998). But, the studies related to mechanism that leads to such damages are really scanty.

It is currently assumed that adverse effects of various environmental stresses are at least partially due to generation of ROS or imposed oxidative stress (Shalata and Tal 1998, Bartoli *et al.* 1999, Krishnamurthy *et al.* 2000, Jiang and Zhang 2001). The over-production of ROS (superoxide, hydrogen peroxide, hydroxyl radical, peroxy radical, alkoxy radical, *etc.*) results from the exposure of various experimental stimuli like heat, chilling, salinity, *etc.* (Zhao and Blumwald 1998) that result in the development of various injury symptoms (Fadzillah *et al.* 1996, O'Kane *et al.* 1996).

Various authors have proposed that free radical mediated membrane lipid peroxidation during accelerated ageing (under elevated temperature) has a detrimental effect on germinability of seeds (Stewart and Bewley 1980). This has led to continuing interest in attempts to maintain germinability of seeds by using free radical scavengers (Robert *et al.* 1980, Woodstock *et al.* 1983). The involvement of ROS is indirectly implicated by

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Abbreviations: CAT - catalase; d.m. - dry mass; DTNB - 5,5'-dithiobis(2-nitrobenzoic acid) or Ellman's reagent; EC - electrical conductivity; LOX - lipoxygenase; MPT - membrane protein thiol; MDA - malondialdehyde; MII - membrane injury index; MLP - membrane lipid peroxidation; PUFA - polyunsaturated fatty acid; POD - peroxidase; ROS - reactive oxygen species; RGP - relative germination performance; RGI - relative growth index; RH - relative humidity; RLR - relative leakage ratio; SOD - superoxide dismutase; TBA - thiobarbituric acid; MLP - membrane lipid peroxidation;
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reduction in activities of free radical scavenging enzymes (Gasper and Dhindsa 1981, Bhattacharjee and Mukherjee 1998).

The present work was undertaken to evaluate the role of ROS in heat shock induced germination and early growth impairment. To examine the mechanistic aspect of imbibitional heat shock induced germination and early growth impairment in *A. lividus* several parameters were considered which include assessment of membrane injury, lipoxygenase mediated membrane lipid peroxidation, activities of free radical scavenging enzymes, accumulation of reactive oxygen species and membrane protein thiol content.

Materials and methods

Seeds of an important tropical leaf crop (*Amaranthus lividus* L.) were supplied by *Sutton Seed Company*, Calcutta, India. Seeds were washed in sterile water, immersed in two successive solutions of 0.1 g dm^{-3} $HgCl_2$ for 5 min each and finally washed in sterile distilled water for 15 min. Seeds were allowed to imbibe water in a beaker for 6 h and thereafter they were sown on moist filter paper in Petri dishes. Seeds were divided into different batches (30 seeds per dish) and kept at 45 °C in dark for 4, 8 and 12 h. The end of heat shock treatment for different durations was considered as start (0 h) of germination. Seeds were allowed to grow at 25 ± 2 °C for 12-h photoperiod (irradiance of 270 μmol m^{-2} s^{-1}) and relative humidity 78 ± 2 %.

Percentage of germination of seeds was calculated after 24, 48 and 72 h, respectively. Relative germination performance (RGP) was calculated as (number of seeds germinated after 72 h under treatment per dish / number of control seeds germinated after 72 h per dish) × 100.

Growth performance was estimated in terms of relative growth index (RGI) and calculated as (average dry mass of seedlings under treatment / average dry mass of control seedlings) × 100.

For all biochemical estimations, *e.g.* assessment of oxidative stress and membrane damage, root and shoot tissues of 72-h-old seedlings were used. Membrane lipid peroxidation was estimated in terms of malondialdehyde (MDA) accumulation. To estimate MDA accumulation, thiobarbituric acid (TBA) test was performed using the procedure of Heath and Packer (1968). For the extraction and estimation of lipoxygenase activity the method of Peterman and Siedow (1985) was followed. Hydrogen peroxide was extracted and estimated following the procedure of MacNevin and Uron (1953) using $Ti_2(SO_4)_3$. For the determination of superoxide, the method of Chaitanya and Naithani (1994) was followed with some necessary modifications. 500 mg of tissue (root and shoot) was homogenized in cold with 5 cm^3 of 0.2 M sodium phosphate buffer, pH 7.2, with addition of diethyldithiocarbamate (10^{-3} M) to inhibit SOD activity. The homogenate was immediately centrifuged at 2 000 *g* and 4 °C for 1 min. In the supernatant, superoxide anion was measured by its capacity to reduce nitroblue-tetrazolium (10^{-4} M). The absorbance of the end product was measured at 540 nm. Formation of superoxide was expressed as ΔA_{540} g^{-1}(d.m.) min^{-1}.

For the extractions and estimations of catalase and peroxidase, the method of Snell and Snell (1971) and Kar and Mishra (1976) were followed, respectively. For SOD, the method of Gianopolitis and Ries (1977) was followed. The enzyme activity was expressed according to Fick and Qualset (1975) as enzyme unit g^{-1}(d.m.) min^{-1}.

For the study of membrane injury index, the process of Bhattacharjee and Mukherjee (1998) was followed. Root and shoot tissues of seedlings (200 mg of each) from each treatment were placed in vials containing 15 cm^3 of deionised water and incubated at 25 °C for 24 h. Electical conductivity of bathing medium was measured at 25 °C with conductivity meter. The tissue with leachate was then autoclaved (at 6.6 × 10^6 P) and brought to 25 °C and EC was measured again. MII was calculated using the formula of Sullivan (1972). MII = [1- (T_1/T_2) / 1- (C_1/C_2)] × 100, where C_1 and C_2 are the ECs of the untreated control sample before and after autoclaving, and T_1 and T_2 are the ECs of the heat shock raised sample before and after autoclaving .

For relative leakage ratio the methods of Bhattarcharjee and Mukherjee (1996) was followed and UV-absorbing tissue leachate was measured. RLR was calculated as A_{280} (before autoclaving) / A_{280} (after autoclaving).

For determination of membrane protein thiol content, the membrane was prepared according to Singh (1997) with some necessary modifications. 1 gram of plant tissue was homogenised in 10 cm^3 ice cold buffer (0.05 M Tris-HCl, pH 7.0). The homogenate was centrifuged at 10 000 *g* at 4 °C for 30 min and pellet was discarded. The membranes were sedimented at 100 000 *g* at 4 °C for 3 h and the pellet containing membrane fractions was suspended in ice cold buffer (0.05M Tris-HCl, pH 7.0). The membrane fractions were stored under ice. The membrane associated protein bound thiol groups were assayed after protein precipitation with TCA (10 % m/v) and quantified with DTNB following the procedures of Ellman (1959) and Dekok and Kuiper (1986).

Results

With increasing duration of imbibitional heat shock, the germination performance of *A. lividus* (measured in terms of germination percentage and RGP) declined significantly (Table 1). The germination continued at supra-optimum temperature but embryo growth was inhibited gradually with increase of treatment time. This was reflected by the data of poor and delayed germination. The seedling growth and vigour (measured in terms of RGI and 72 h-old seedling length) decreased with increasing duration of imbibitional heat shock (Table 1). The heat shock during early imbibitional phase was detrimental to germination.

Table 1. Effect of high temperature (45 °C) treatment of different duration (4, 8 and 12 h) during early imbibitional period on germination percentage, relative germination performance (RGP), relative growth index (RGI), and seedling length of *A. lividus* seedlings (72 h after stress). Means ± SE, $n = 6$.

Treatment	Germination [%]	RGP [%]	Seedling length [mm]		RGI [%]
			root	shoot	
25 °C (control)	95 ± 0.66	100	17.2 ± 0.07	15.2 ± 0.03	100
45 °C for 4 h	75 ± 0.44	78.9 ± 0.61	13.8 ± 0.04	11.2 ± 0.07	80.1 ± 0.68
45 °C for 8 h	68 ± 0.37	71.5 ± 0.48	13.1 ± 0.09	10.2 ± 0.01	63.3 ± 0.81
45 °C for 12 h	52 ± 0.91	54.7 ± 0.42	6.7 ± 0.04	5.3 ± 0.02	56.1 ± 0.71

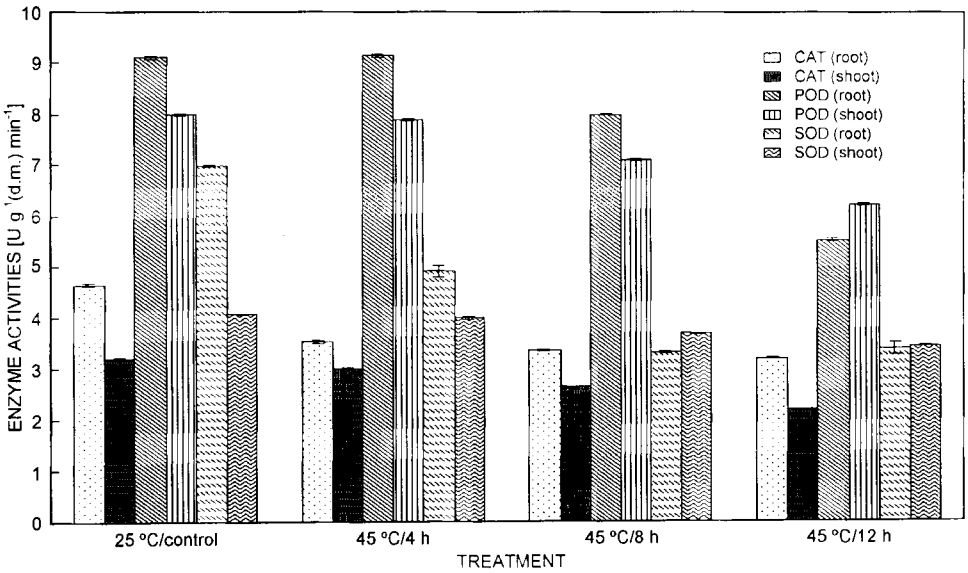

Fig. 1. Changes in the activities of ROS-scavenging enzymes (CAT, POD, SOD) in *A. lividus* seedlings (72-h-old) raised after heat shock. *Error bars* represent SE, $n = 6$.

Activities of CAT, POD and SOD (Fig. 1) showed a gradual decline with increasing duration of heat shock, maximum decrease was recorded for seedlings raised at the 45 °C 12 h treatment. Accumulation of $O_2^{\cdot-}$ and H_2O_2, on the other hand, increased significantly in the heat shock raised seedlings (in both root and shoot tissues) in a duration-dependent manner (Fig. 2A,*B*).

Assessment of one of the product of membrane lipid peroxidation, MDA, revealed its augmentation (in both root and shoot tissues) proportional to the duration of heat shock (Fig. 3*A*). This was associated with significant enhancement of lipoxygenase activities (Fig. 3*B*), maximum activity being recorded in seedlings raised from the 45 °C for 12 h treatment. The higher activities of LOX, accumulation of ROS, MDA and significant reduction in activities of free radical scavenging enzymes clearly suggest a possible role of ROS in germination impairment.

Estimation of membrane permeability, in terms of MII (% membrane injury) and RLR (of UV absorbing substances) clearly revealed the detrimental effect of heat shock mediated by ROS on newly assembled membrane systems of germinating *A. lividus* seedlings (Table 2). Both MII and RLR increased significantly in heat shock

raised seedlings, and this was also dependent on treatment duration. Estimation of MPT (Table 2) further corroborated the significance of oxidative damage of newly assembled membrane system of *A. lividus* seedlings raised from imbibitional heat shock. MPT content was found to drop significantly in heat shocked seedlings guided by duration of treatment.

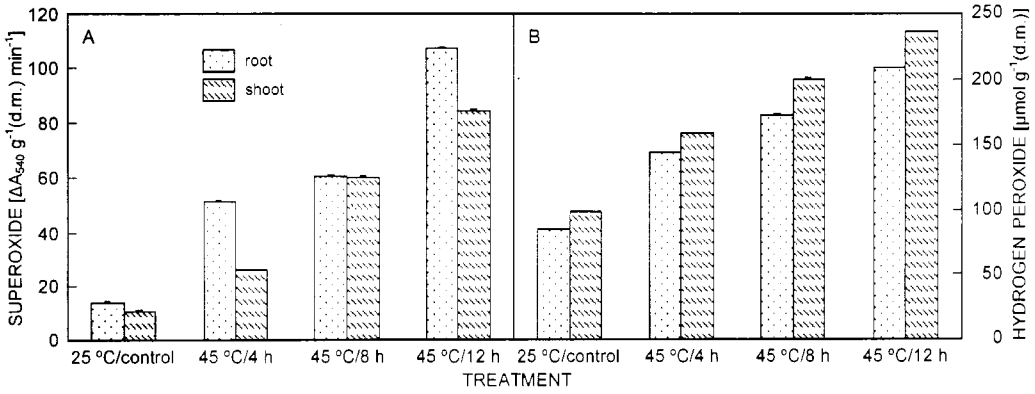

Fig. 2. Changes in the amount of superoxide (*A*) and hydrogen peroxide (*B*) in *A. lividus* seedlings (72-h-old) raised after heat shock. *Error bars* represent SE, $n = 6$.

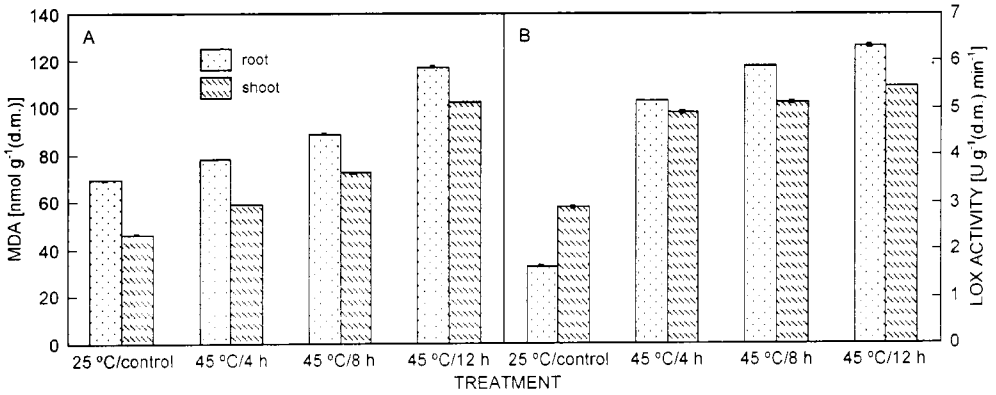

Fig. 3. Membrane lipid peroxidation, in terms of MDA accumulation (*A*) and lipoxygenase (LOX) activities (*B*) in *A. lividus* seedlings (72-h-old) raised after heat shock. *Error bars* represent SE, $n = 6$.

Table 2. Influence of imbibitional heat shock on membrane injury index (MII), relative leakage ratio (RLR), and membrane protein thiol content (MPT) in 72 h-old *A. lividus* seedlings. Means ± SE, $n = 6$.

Treatment	MII [%]		RLR [A_{280}/A'_{280}]		MPT [nmol g^{-1}(d.m.)]	
	root	shoot	root	shoot	root	shoot
25 °C (control)	0	0	0.16 ± 0.01	0.15 ± 0.01	116.4 ± 0.18	158.8 ± 0.30
45 °C for 4 h	16.4 ± 0.11	11.1 ± 0.12	0.23 ± 0.01	0.20 ± 0.01	89.9 ± 0.21	140.0 ± 0.12
45 °C for 8 h	23.0 ± 0.12	16.4 ± 0.12	0.29 ± 0.01	0.24 ± 0.01	57.0 ± 0.22	84.6 ± 0.10
45 °C for 12 h	31.2 ± 0.14	20.7 ± 0.17	0.42 ± 0.01	0.33 ± 0.01	27.0 ± 0.21	31.0 ± 0.08

Discussion

ROS like $O_2^{\cdot-}$ and H_2O_2 are controlled largely by free radical scavenging enzymes like CAT, POD and SOD (Fridovich 1976). In heat shocked germinating *A. lividus* seedlings the balance between generation of ROS and scavenging reactions might be disturbed in favour of the former, inducing an oxidative stress. The results here clearly suggest that the activities of free radical scavenging enzymes declined proportionately with duration of heat shock during early germination period, thus the accumulation of $O_2^{\cdot-}$ and H_2O_2 induced a

secondary oxidative stress that increased degradation of membrane lipid via peroxidation (Fadzillah *et al.* 1996, O'Kane *et al.* 1996). However, transient increase in H_2O_2 content under heat shock might induce a signal activation mechanism of acclimation, so the significance of accumulation of H_2O_2 may not be simply an injury symptom (Smirnoff 1998, Neill *et al.* 1999).

MDA which is one of the products of membrane lipid peroxidation of polyunsaturated fatty acids (PUFA) of biomembranes showed greater accumulation in heat shock raised *A. lividus* seedlings. Membrane lipid peroxidation (MLP) triggered by imbibitional heat shock in germinating *A. lividus* seedlings was significant not only from the point of view of disorganisation of newly assembled membrane system but was also of great significance from the point of view of generation of ROS (Winston 1990), which ultimately imposed a secondary oxidative stress in germinating tissues. Involvement of ROS in membrane lipid peroxidation in *A. lividus* seedlings subjected to heat shock might be further substantiated from the data of enhanced LOX activities, which is known to accelerate membrane lipid peroxidation (Hildebrand 1989). This is in agreement with the results of Robert *et al.* (1980) and Cakmak and Horst (1991). Data of MII and RLR of *Amaranthus* seedlings raised from imbibitional heat shock further substantiate temperature and duration dependent deterioration of membrane structure of germinating tissues. Givelberg *et al.* (1984) and Bhattacharjee and Mukherjee (1998) reported a large increase of electrolytes, macromolecules and UV-absorbing tissue leachates under the influence of elevated temperature. Various lines of evidence (Simon and Rajaharun 1972, Short and Lacy 1976, Bewley and Black 1982, Bhattacharjee and Mukherjee 1998) supported the view that heat shock induced leakage is related to membrane structure and function and ultimately this is the function of temperature and duration of exposure. Wismer *et al.* (1998) considered inhibition of reorganization of biomembrane structure and function, *i.e.* modification of biophysical properties of membrane, as an important cause of temperature stress induced injury.

The involvement of ROS in membrane damage of heat shocked seedlings could be further supported from the data of MPT content, which decreased steadily in a duration guided manner in *A. lividus*. ROS accumulation due to reduced efficiency of free radical scavenging enzymes and enhanced membrane lipid peroxidation might oxidize membrane protein thiol groups, leading to membrane injury and inactivation of certain enzymes. An estimation of MPT content has been used as indicator of oxidative stress injury (Bhaskar and Balasubramanian 1995).

There is little evidence which describes the effect of transient change in temperature on membranes of juvenile germinating tissues. The present work clearly supports the idea that the membranes are the primary target of oxidative injury initiated by elevated temperature (Levitt 1980, Steponkus 1984, Smirnoff 1998) and also the sensor of transient temperature changes in germinating seeds, corroborating well with the findings of King and Ludford (1983), Chan *et al.* (1985) and Marbach and Mayer (1985).

So, heat shock during imbibition may result in the induction of secondary oxidative stress in germinating tissues which might lead to early growth impairment.

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