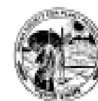
Indian J. Plant Physiol., Vol. 12, No. 4, (N.S.) pp. 337-343 (Oct.-Dec., 2007)



INVOLVEMENT OF CALCIUM AND CALMODULIN IN THE ACQUISITION OF THERMOTOLERANCE IN *AMARANTHUS LIVIDUS* L.

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Received on 30 April, 2007, Revised on 7 Nov., 2007

SUMMARY

Heat caused reduction in membrane protein thiol level and increased accumulation of thiobarbituric acid reactive substances in 72 hour old germinating tissues thereby reducing germination and early growth performances. Calcium chelator (EGTA), calcium channel blocker ($LaCl_3$) and calmodulin inhibitor (trifluroperazine) aggravated the effects of heating and added calcium reversed them, implying that protection against heat induced oxidative damage and improvement of germination in *Amaranthus* requires calcium and calmodulin. Imposition of heat stress during early germination also causes accumulation of reactive oxygen species (ROS) like $O_2^{\cdot-}$ and H_2O_2. Calcium treatment significantly reduced the accumulation of both the toxic ROS, while EGTA, $LaCl_3$ and TFP treatment enhanced the accumulation. Activities of antioxidative enzymes catalase (CAT), ascorbate peroxidase (AP) and glutathione reductase (GR) and total thiol content decreased under heat stress in germinating *Amaranthus* seedlings. Seedlings raised with Ca^{2+} treatment under heat stress exhibit higher activities of CAT, GR and AP and total thiol level than the untreated plants. EGTA, $LaCl_3$ & TFP treatment, on the other hand significantly reduce the activities of all anti-oxidative enzymes and total thiol level. The work supports the view that Ca^{2+}-signalling pathway plays significant role in limiting heat induced oxidative injury during recovery phase of post germination event in *Amaranthus lividus*.

Key words : **Calcium, calmodulin, germination, heat stress, oxidative stress**

INTRODUCTION

Plants are often subjected to temperature extremes limiting growth and development involving many physiological and biochemical changes including oxidative stress (Jiang and Huang 2001, Bewly and Black 1982, Bhattacharjee and Mukherjee 2006). High temperature influences germination and subsequent seedlings growth (Bewly and Black 1982, Alka and Khanna-Chopra 1995, Bhattacharjee and Mukherjee 2003/2004). In fact, imbibitions 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 to metabolic dysfunctioning pertaining to loss of membrane integrity in juvenile tissues, which are largely due to secondary oxidative stress (Alka and Khanna-Chopra 1995, Bhattacharjee and Mukherjee 2003, 2004).

Several studies showed that Ca^{2+} is involved in the regulation of plant responses to various environmental stresses including heat (Knight and Knight 1993, Gong *et al.* 1998, Jiang and Huang 2001, Nayyar 2003). Increased cytosolic Ca^{2+} content under heat stress alleviates heat injury and enable plant cells to survive better (Knight and Knight 1993, Wang and Li 1999).

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However, excessive Ca^{2+} released into cytosol and high Ca^{2+} concentration causes cytotoxicity (Wang and Li 1999, Jiang and Huang 2001).

The treatment of *Zea mays* and *Amaranthus* seeds with 15 and 20 mM Ca^{2+} solution enhanced intrinsic heat tolerance of seedlings (Gong *et al.* 1997, Bhattacharjee 2001). In contrast, it has been reported that heat induced growth retardation could not be alleviated by external Ca^{2+} treatment in excised coleoptile of wheat (Onwueme and Laude 1972). Therefore, the role of Ca^{2+} in regulation of heat tolerance is still unclear. Trofimova *et al.* (1999) suggests that Ca^{2+} may be involved in signal transduction involving new gene expression under oxidative and heat stress. Other workers reported that Ca^{2+} control guard cell turgor and stomatal aperture (Mansfield *et al.* 1990, Webb *et al.* 1996) and helps in turgor maintenance under stress (Hare *et al.* 1998).

The present investigation was undertaken to investigate the role of calcium in heat tolerance and survival during early germination in a tropical leaf crop *Amaranthus lividus* L. and to examine its effect in mitigation of oxidative stress.

MATERIALS AND METHODS

Seeds of the tropical leaf crop *Amaranthus lividus* L., selected as experimental material was supplied by local harvest. Effect of Ca^{2+} on plant survival under heat shock and its relationship with ROS metabolism and associated oxidative damages were studied. For this surface sterilized seeds of *Amaranthus lividus* were imbibed in the following solutions separately for 20 hours in darkness: (i) 20 mM $CaCl_2$, (ii) 1 mM Lanthanum chloride ($LaCl_3$), (iii) 2 mM EGTA, (iv) 200 µM Trifluroperazine, (v) Glass distilled water

Seeds were air dried in room temperature and finally sown in Petri plates on moist filter paper (30 seeds per plate) and subsequently kept either at $40^0 \pm 2^0$C or at room temperature for 24 hours. Finally they were allowed to grow at $25^0 \pm 2^0$C with 12 hour photoperiod (270 E m^{-2} s^{-1}) and 78 ± 2% RH.

Survival assay, growth performance, extent of oxidative membrane damage (in terms of thiobarbituric acid reactive substances and membrane protein thiol level), efficiency of antioxidative defense systems (in terms of the activities of ascorbate peroxidase, catalase, glutathione reductase, superoxide dismutase and total thiol content) and accumulation of reactive oxygen species ($O_2^{\cdot-}$ and H_2O_2) were also performed after 72 hours of treatment. For studying survival assay and growth performance, relative germination performance (RGP) and relative growth index (RGI) were calculated (Bhattacharjee and Mukherjee 2003/2004). Relative germination performance (RGP) was calculated as

$$RGP = \frac{\text{Number of seeds germinated after 72 hours of treatment}}{\text{Number of control seeds germinated after 72 hours of treatment}} \times 100$$

Relative growth index (RGI) was calculated as

$$RGI = \frac{\text{Average dry mass of ten seedlings under treatment}}{\text{Average dry mass of ten control seedlings}} \times 100$$

To estimate membrane lipid peroxidation test for thiobarbituric acid reactive substances (TBARS) was performed using the procedure of Heath and Packer (1968).

For the determination of membrane protein thiol content, the membrane was prepared according to Singh (1997) with some necessary modifications. 1 gram of plant tissue was homogenized in 10 cm^3 ice cold buffer (0.05 M Tris-HCl, pH 7.0). The homogenate was centrifuged at 10,000 g at 4^0C for 30 minutes and the pellet was discarded. The membranes were then sedimented at 1,00,000 g at 4^0C for 3 hours and the pellet containing the membrane fractions was suspended in ice cold buffer (0.05 M Tris-HCl, pH 7.0). The membrane fractions were stored under ice. The membrane associated protein bound thiol group were assayed after protein precipitation with TCA (10% mass/volume) and quantified with DTNB following the procedures of Ellman (1959) and Dekok and Kuiper (1986). Membrane permeability status was estimated in terms of membrane injury index (MII) and relative leakage ratio (RLR) of UV absorbing tissue leachates using the procedure of Bhattacharjee and Mukherjee (2003/2004).

H_2O_2 was extracted and estimated following the procedure of MacNevin and Uron (1953) using titanic sulfate. For the determination of superoxide, the method of Chaitanya and Naithani (1994) was followed with some necessary modifications. 500 mg of tissues was homogenized in cold with 5 cm^3 of 0.2 M sodium phosphate buffer, pH 7.2, with addition of diethyldithiolcarbomate (10^{-3} M) to inhibit SOD activity. The homogenate was immediately centrifuged at 2000 g at 4^0C for 1 min. In the supernatant, superoxide anion was measured by its capacity to reduce nitrobluetetrazolium (2.5 X 10^{-4} M). The absorbance of the end product was measured at 540 nm. Formation of superoxide was expressed as ΔA_{540} g^{-1} (dm) min^{-1}. For the extraction and estimation of lipoxygenase the process of Peterman and Siedow (1985) was followed. For the extraction and estimation of catalase and superoxide dismutase the methods of Snell and Snell (1971) and Gianopolitis and Ries (1977) were followed.

Ascorbate peroxidase (APX) activity was determined according to Nakano and Asada (1981) using homogenates previously supplemented with 0.5 mM ascorbic acid and 0.1 mM EDTA. Parallel experiments in presence of p-chloromercuribenzoate (50mM) were performed to rule out any interference from guaiacol peroxidases. Glutathione reductase (GR) activity was measured according to Schaedle and Bassham (1977). The reaction mixture contained 50 mM Tris-HCl (pH 7.6), 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), 3 mM $MgCl_2$ and 100 ml homogenate (7 mg protein ml^{-1}). NADPH oxidation was followed at 340 nm. The enzyme activity was expressed according to Fick and Qualset (1975) as enzyme unit g^{-1} dm min^{-1}.

For the estimation of total thiol content the method of Tietze (1969) was followed. Total –SH content was assayed in acid soluble extract (in 3% w/v TCA solution) followed by a brief centrifugation. The supernatant was then diluted 10-fold in 100 mM phosphate buffer (pH 7.5). Thiol contents was determined measuring absorbance at 412 nm in presence of 0.5 mM 5, 5′-dithiobis 2-nitrobenzoic acid (DTNB), 0.5 U ml^{-1} glutathione reductase and 0.2 mM NADPH.

RESULTS AND DISCUSSION

Germinating *Amaranthus lividus* seeds kept at 40^0C for 24 hours, subsequently transferred to normal growth conditions, exhibited low survival (measured in terms of germination after 72 hours of treatment) as well as reduced early growth (measured in terms of relative growth index). The relative germination and relative growth index for heat stress treated *Amaranthus*

Table 1. Effect of calcium (20 mM $CaCl_2$), calcium channel blocker (1 mM $LaCl_3$), calcium chelator (2 mM EGTA) and calmodulin inhibitor (250 mM Trifluroperazine) on oxidative stress index (in terms of thiobarbituric acid reactive substances and membrane protein thiol level), survival (relative germination performances) and early growth performances (relative growth index) in response to heat stress (40^0C for 24 hours) in *Amaranthus lividus* L. (values are mean of three replicates ± SE).

Treatment	Oxidative Stress index				Survival RGP (%)	Growth performances RGI (%)
	TBARS (mmol g^{-1} dm)		MPTL (nmol g^{-1} dm)			
	Root	Shoot	Root	Shoot		
Untreated	68.0±0.11	44.6±0.09	114.0±0.28	158.0±0.30	100.0	100.0
Untreated & heat stress	88.1±0.14	71.6±0.12	58.5±0.11	85.8±0.21	54.7±0.11	56.1±0.19
Ca^{2+} & heat stress	72.6±0.12	53.5±0.10	83.1±0.20	110.5±0.26	71.5±0.17	72.0±0.10
$LaCl_3$ & heat stress	136.5±0.11	122.5±0.13	48.8±0.09	72.6±0.17	44.4±0.08	44.0±0.05
EGTA & heat stress	101.7±0.19	92.1±0.21	52.7±0.12	83.2±0.17	46.4±0.04	48.2±0.04
TFP & heat stress	98.4±0.08	79.9±0.10	48.6±0.08	78.3±0.11	48.0±0.03	46.2±0.04

reduced to 54% and 56%, respectively, as compared to the cent percent values of untreated control (Table 1). Hyperthermia also caused significant increase in TBARS in germinating *Amaranthus* seedlings (Table 1). As TBARS assay measures oxidative damage to membrane, so to corroborate it further membrane protein thiol level (MPTL) was estimated for high temperature treated and control *Amaranthus* seedlings. Significant decline in MPTL in the heat shock treated *Amaranthus* seedlings was observed as compared to untreated control (Table 1). Ca^{2+} pretreated *Amaranthus* seedlings exhibited reversal in oxidative damage as well as improved survival and early growth. There was significant reduction in the accumulation of TBARS and restoration of MPTL for the Ca^{2+} pretreated seedlings, thereby, implying the involvement of Ca^{2+} in mitigating oxidative stress. Treatment with inhibitors ($LaCl_3$, EGTA, and TFP) increased heat shock induced accumulation of TBARS with a concomitant reduction in MPTL, accompanied by low survival rate and poor early growth. $LaCl_3$ treated seedlings had maximum oxidative membrane damage with a corresponding reduction in the survival and early growth.

Ca^{2+} pretreatment caused significant decline in oxidative membrane damage under heat stress as indicated from the data of membrane injury index (MII) and relative leakage ratio (RLR) of UV absorbing tissue leachate (Table 2). Heat induced oxidative membrane damage was found to be associated with significant enhancement of lipoxygenase activity (Table 2).

Imbibitional heat shock at 40^{0}C causes significant increase in the level of $O_2^{.-}$ and H_2O_2 as compared to untreated control (Fig 1). Ca^{2+} pretreatment prior to heat shock reduced the accumulation of both the ROS ($O_2^{.-}$ and H_2O_2), whereas, the treatment with inhibitors ($LaCl_3$, EGTA, TFP) enhanced the accumulation of ROS (Fig. 1). Heat stress during early phase of germination induced oxidative injury and significantly reduced the activities of antioxidative enzymes (during early phase of recovery) including CAT, APX, SOD, GR as well as total thiol level (Table 3). Ca^{2+} treatment prior to heat shock helped maintain higher APX, CAT and GR activities and total thiol level. The Ca^{2+} treatment, however, did not affect SOD activity. Activities of the antioxidant enzymes and total thiol level decreased significantly when treated with Ca^{2+} channel blocker, chelator or calmodulin inhibitor, hinting the role of Ca^{2+} and calmodulin in the maintenance of the antioxidative defense during recovery phase of oxidative stress.

Results indicate that the secondary oxidative stress and related oxidative membrane damage to the juvenile tissue occurs in *Amaranthus lividus* during early germination after the exposure of heat stress and the levels of the damage continues 3 days post exposure. The results are similar to the work described by Gong

Table 2. Effect of Calcium (20 mM $CaCl_2$), calcium channel blocker (1mM $LaCl_3$), calcium chelator (2 mM EGTA) and calmodulin inhibitor (250 mM Trifluroperazine) on membrane permeability status (membrane injury index and relative leakage ratio) and lipoxygenase activities in response to heat stress (40^{0}C for 24 h) in *Amaranthus lividus* seedlings. (values are mean of three replicates ± SE).

Parameters		Treatment					
		Untreated	Untreated and heat stress	Ca^{2+} & heat stress	$LaCl_3$ & heat stress	EGTA & heat stress	TFP & heat stress
Membrane Injury Index (%)	Root	0	23±.21	17.4±.11	28±.17	26.5±.13	24±.21
	Shoot	0	16±.11	12.8±.09	18.9±.11	19±.09	18.2±.08
Relative leakage ratio	Root	0.16±0.01	0.29±.01	0.20±.04	0.32±.05	0.31±.009	0.27±.02
	Shoot	0.15±0.02	0.22±.02	0.18±.007	0.28±05	0.29±.03	0.27±05
Lipoxygenase activities ($U\ g^{-1}\ dm\ min^{-1}$)	Root	1.5±0.02	5.6±.01	4.97±.04	6.98±.02	6.68±.01	6.14±.005
	Shoot	2.8±0.01	5.1±.02	4.3 4±.01	5.89±.02	6.34±.02	6.0±.01

Table 3. Effect of calcium (20 mM $CaCl_2$), calcium channel blocker (1 mM $LaCl_3$), calcium chelator (2 mM EGTA) and calmodulin inhibitor (250 mM Trifluroperazine) pretreatment on heat (40^{0}C for 24 hours) induced changes in activities of antioxidative enzymes (ascorbate peroxidase, catalase, superoxide dismutase and glutathione reductase) and total thiol content in *Amaranthus lividus* L. seedlings. (values are mean of three replicates ± SE).

Treatment	Activities of antioxidative enzymes (Ug^{-1} dm min^{-1})								Total thiols (μmol g^{-1} dm)	
	AsPOD		CAT		SOD		GR			
	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot
Untreated	9.80±0.04	8.30±0.03	4.14±0.01	3.28±0.010	4.80±0.02	4.00±0.02	0.17±0.010	0.11±0.007	1.07±0.007	0.85±0.002
Untreated & heat stress	8.06±0.30	7.00±0.01	3.30±0.02	2.60±0.050	3.70±0.02	3.64±0.02	0.12±0.010	0.07±0.002	0.75±0.002	0.48±0.001
Ca^{2+} & heat stress	8.5±0.07	7.90±0.04	3.79±0.01	2.98±0.020	3.71±0.01	3.60±0.03	0.16±0.006	0.09±0.001	0.91±0.003	0.60±0.08
$LaCl_3$ & heat stress	8.0±0.03	6.75±0.02	3.20±0.01	2.41±0.007	3.50±0.01	3.19±0.02	0.10±0.002	0.05±0.001	0.63±0.001	0.35±0.004
EGTA & heat stress	8.0±0.02	6.80±0.01	3.10±0.01	2.50±0.002	3.40±0.03	3.10±0.02	0.10±0.002	0.06±0.002	0.70±0.001	0.40±0.003
TFP & heat stress	7.90±0.02	6.77±0.01	3.15	2.54±0.003	3.50±0.03	3.20±0.03	0.09±0.002	0.06±0.002	0.72±0.004	0.38±0.001

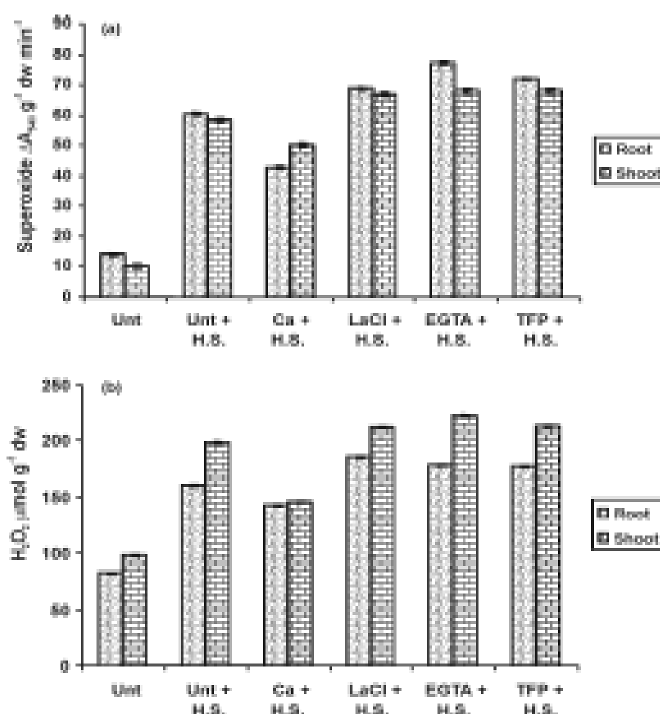

Fig. 1. Effect of Calcium (20 mM $CaCl_2$), calcium channel blocker (1mM $LaCl_3$), calcium chelator (2 mM EGTA) and calmodulin inhibitor (250 mM Trifluroperazine) pretreatment on the accumulation of reactive oxygen species (a) superoxide and (b) hydrogen peroxide, in response to heat stress (40^{0}C for 24 h) in *Amaranthus lividus* seedlings. (values are mean of three replicates ± error bar).

et al. 1998, Larkindale and Knight 2002, Bhattacharjee 2005, Bhattacharjee and Mukherjee 2003/2004, 2006.

The levels of oxidative damage measured correlated with ultimate survival (germination) and early growth performances of *Amaranthus*. Although the two variables, i.e., survival and oxidative damage are not completely linked, but in all experiments survival declined with higher levels of TBARS and reduced MPTL. This suggests that survival after heat shock requires ability to tolerate or repair oxidative damage; it also requires ability to tolerate or minimize other kinds of heat induced damage (Woodstock *et al.* 1983, Gong *et al.* 1998, Bhattacharjee 2001, Larkindale and Knight 2002, Bhattacharjee 2005, Bhattacharjee and Mukherjee 2006).

The development of thermo-tolerance (in terms of increased survival i.e., germination and improved early growth performances) in Ca^{2+} pretreated and subsequently heat stressed *Amaranthus* seedlings reinforced the idea that Ca^{2+} might be required in some signaling pathway in *Amaranthus* leading to improved survival. This suggests that a flux of Ca^{2+} ion is required to switch on some mechanism by which plants prevent or repair oxidative damage caused by heating and thus a calcium flux is required for the plant to germinate under

elevated temperature. The pretreatment with Ca^{2+} channel blocker and Ca^{2+} chelator substantiate the requirement of calcium flux.

The Ca^{2+}-dependent pathway is presumed to act through calmodulin, as calmodulin inhibitor (trifluroperazine) significantly increased the oxidative stress in heat stressed seedlings. A role for calmodulin is supported by the fact that higher levels of calmodulin have been obtained in thermo-tolerant maize cells than in those that are sensitive to heat (Gong *et al.* 1997). Higher calmodulin levels have also been linked to lower levels of heat induced membrane damage in maize (Gong *et al*. 1997a) and in Arabidopsis (Larkindale and Knight 2002). Price *et al.* (1996) have reported links between Ca^{2+} signaling and oxidative stress in tobacco plant where oxidative stress induces Ca^{2+} levels.

ACKNOWLEDGEMENT

The author gratefully acknowledges University Grants Commission, New Delhi, for providing assistance in the form of Research Project (Project Sanction No.FPS/ 007/04-05W,ERO).

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