

Full Length Research Paper

Induction of thermotolerance through heat acclimation and salicylic acid in *Brassica* species

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High temperature stress is the second most important stress, which can strike crop plants at any time and impose severe limitations on crop growth and development. Developing crop plants with improved thermotolerance can mitigate the adverse effects of heat stress. However, a thorough understanding of physiological responses of plants to high temperature and mechanisms involved for thermotolerance is imperative. *Brassica* is an important oilseed crop and its early sowing implies many important advantages. However, the crop sown early encounters high temperature stress, which causes a great yield lost. Many putative signaling molecules like SA, ABA, H₂O₂ and CaCl₂ and heat acclimation have been found to be involved in inducing thermotolerance as well as in initiating the underlying signal transduction pathway for upregulating various genes involved in thermotolerance. In the present investigation, we observed the effects of heat shock, heat acclimation and SA in four genotypes of *Brassica*; TL15, PBT37, RL1359 and PBR210. Heat acclimation for 3 h at sublethal temperature and SA pretreatments at 10 and 20 µM for 2 h prior to heat shock were found to be effective in imparting thermoprotection at seedling stage, which is the crucial stage of plant establishment. These pretreatments helped seedlings to recover from heat stress by increasing seedling length, reduced electrolyte leakage and conferring membrane protection. Increased level of total soluble sugars, fresh/dry weight, and also increase in enzymatic activities of invertase, CAT, POX conferred thermotolerance. Further, enhanced expression of some new proteins including heat shock proteins (HSPs) was observed by both of the pretreatments through SDS-PAGE. We assume that heat acclimation and SA pretreatments induced thermotolerance and definitely play a role in initiating various mechanisms involved in overcoming high temperature limitations.

Key words: *Brassica*, heat acclimation, heat shock proteins, high temperature stress, salicylic acid, thermotolerance.

INTRODUCTION

Plant productivity is severely affected by abiotic stress factors which include salinity, drought, high and low temperature, and heavy metals. In general, physiological and biochemical responses in plants vary and cellular aqueous and ionic equilibria are disrupted. Also, hundreds of genes and their products respond to these stresses at transcriptional and translational level (Cushman and

Bohnert, 2000; Umezawa et al., 2006; Yamaguchi - Shinozaki and Shinozaki, 2007).

Out of the various abiotic stresses, high temperature is the second most important stress, which can strike crop at any time and impose many limitations on growth and development. Thermotolerance using various genetic approaches can mitigate the adverse effects of heat stress. For this purpose, however, a thorough understanding of physiological responses of plants to high temperature, mechanisms of heat formation of some new proteins in TL15, PBT37, RL1359 and PBR210 genotypes of *Brassica* tolerance and possible strategies for improving crop thermotolerance is imperative (Wahid et al., 2007).

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Abbreviations: ABA, abscisic acid; CAT, catalase; HSPs, heat shock proteins; POX, peroxidase; ROS, reactive oxygen species; SA, salicylic acid.

Brassica is an important oilseed crop of winter season and its early sowing implies many important advantages. First, early harvest of *Brassica* is desirable to avoid disease infestation and aphid attack that normally coincides with the flowering stage. Secondly, shattering of fruits can be avoided during the time of harvest when crop encounters high temperature. However, *Brassica* encounters high temperatures which impose severe limitations on their germination pattern and subsequent seedling establishment and thus yield. Moreover high temperature causes a profound modification of all aspects of cell and whole metabolism (Stone, 2000).

There are many *Brassica* varieties which are resistant to high temperature; so there is a need to identify those thermotolerant varieties. Many other varieties are there which have high yielding abilities but are otherwise thermosusceptible. Identification of genes is required to be done in these varieties, so that they can be used for getting better yield by working upon expression of those genes, which are responsible for their low resistance to high temperature. Literature is available on the heat acclimation treatments and also on the exogenous application of certain biomolecules, which have enabled seeds of different crop species to overcome thermosusceptibility or lead to induction of thermotolerance (Zhou and Leul, 1999; Dhaubhadel et al., 2002).

Acquired thermotolerance is the ability of a plant to survive normally lethal temperature after an exposure to mild or sublethal temperature or by exogenous application of biomolecules (Howarth and Ougham, 1993). It is a complex phenomenon, which relies on the induction of specific pathways during acclimation period and a subsequent development (i.e. acquisition) of thermotolerance. For instance, the exogenous applications of cytokinins (Caers et al., 1985), GA₃ (Chen et al., 1986) and ABA (Bonham-Smith et al., 1988) have been shown to reverse the effect of heat shock and impart thermotolerance.

The enhanced expression to the elevated temperature is due to the few conservative genes found in plants, which respond to high temperature. Secondly, by the synthesis of polypeptides encoded by these genes, some of which are known as the 'heat shock proteins' (HSPs). HSPs facilitate growth and survival of plants not only over the course of transient extremes of temperature but also under conditions of severe heat stress whereby lethal temperature can be tolerated for short periods. Heat shock proteins accumulation is accompanied by the increase in the cell thermotolerance (Trofimova et al., 1997). Therefore, the present study was planned to study associated with thermoprotection of seedlings, changes in the activity patterns of antioxidant enzymes and

MATERIALS AND METHODS

Plant material and growth conditions

In this study, an attempt was made to induce thermotolerance in 4-

day old *Brassica* seedlings of different genotypes; TL15, PBT37, PBR210 and RL1359. TL15 and PBT37 were designated as thermosensitive while PBR210 and RL1359 as thermotolerant, based on our previous studies (data not included).

Uniform sized seeds of each variety were surface sterilized with 0.1% HgCl₂ for 1 min, rinsed thoroughly with distilled water and germinated in dark for 4 days at 25±1°C in Petri dishes on filter paper moistened with 5 ml of distilled water in 3 replicates of each cultivar. A seed was considered to have germinated when its radicle emerged at least 4 cm.

Treatments

Heat shock at 40-55°C was applied to 4-day-old seedlings of *Brassica* for 1 to 3 h followed by recovery period of 3 days at 25±1°C in dark. In all subsequent experiments, 45°C for 3 h was selected as the standard heat shock treatment for thermosensitive genotypes and 55°C for 3 h was selected as the standard heat shock treatment for thermotolerant genotypes.

Heat acclimation at 30-45°C were given to 4-day-old seedlings for 1 to 3 h followed by heat shock at 45°C for 3 h and the recovery period at 25±1°C for 3 days in dark. Pre-incubation at 35°C 3 h, followed by challenge with a lethal temperature at 45°C for 3 h proved to be effective in imparting thermotolerance to the seedlings of PBR210 and RL1359 genotypes.

While in case of TL15 and PBT37 genotypes heat acclimation at 45°C for 3 h before exposure of seedlings to 55°C (heat shock temperature), helped in induction of thermotolerance in the seedlings. Salicylic acid pretreatments (10 and 20 µM) at normal temperature for 2 h were given to 4 day old seedlings followed by heat shock at 45°C for 3 h to thermosensitive genotypes and 55°C for 3 h to thermotolerant genotypes followed by recovery growth at normal temperature for 3 days in dark. The effect of these treatments on membrane integrity, carbohydrate metabolism and antioxidant enzymes was studied. The effect of high temperature and pretreatments was also recorded in terms of changes in proteins through SDS-PAGE.

Biochemical analysis

Membrane integrity was determined by measuring electrolyte conductivity of the seedlings with different treatments. Estimation was undertaken in three replication of each treatment, which was repeated twice. Values as percent leakage are presented as mean ± S.E and it was calculated as:

$$\text{Leakage (\%)} = \frac{\text{Conductivity before boiling}}{\text{Conductivity after boiling}} \times 100$$

Total soluble sugars were extracted and estimated by the method of Dubois et al. (1956). The activity of soluble Invertase enzyme was detected by the method of Nelson (1944). The activity levels of membrane thermostability and sugar metabolism antioxidant enzymes; catalase and peroxidase were measured by the methods given by Aebi et al. (1983) and Shannon et al. (1966), respectively.

Extractions of proteins and its profiling

Proteins were separated on the 12.5% SDS-PAGE using the method of Walker (1996). For this 2.5 mg of fresh tissue was 4°C. of supernatant was loaded on mini gel. The gel was run homogenized with .5 M Tris-H buffer of pH 6.8 and centrifuged at 20 µl at 1.5 mA current/cm gel for 90 min. The gel was stained with 0.1% coomassie brilliant blue R-250 in methanol : acetic acid : water (50: 10: 40) for 6 h than destained with solution containing acetic acid : water : methanol in ratio 70: 830:100 ml.

Table 1. Effect of heat acclimation, SA and heat shock on the seedling length (cm) of different genotypes of *Brassica* spp.

Treatment	Seedling length (cm)			
	TL 15	PBT 37	RL 1359	PBR 210
Control	13.10±0.473	12.85±0.519	13.65±0.317	14.10±0.379
Heat Shock	5.10±0.298	5.25±0.226	4.05±0.193	4.25±0.177
Heat Acclimation	8.90±0.237	9.02±0.275	8.40±0.290	9.15±0.255
Salicylic acid (10 µM)	5.92±0.177	6.34±0.193	6.75±0.237	7.30±0.275
Salicylic acid (20 µM)	6.20±0.226	6.52±0.298	6.85±0.309	8.00±0.224

CD1 (5%) = 0.852 (for treatments); CD2 (5%) = 0.852 (for genotypes). Data represents mean±SE of three replicate.

Table 2. Effect of heat acclimation, SA and heat shock treatment on fresh / dry weight (mg) of different genotypes of *Brassica* spp.

Varieties	Fresh / dry weight (mg)							
	TL 15		PBT 37		RL 1359		PBR 210	
Treatments	FW	DW	FW	DW	FW	DW	FW	DW
Control	397.9±2.500	32.50±0.381	385.0±6.061	30.9±0.191	545.0±7.620	38.8±1.300	535.3±6.420	29.1±0.803
Heat Shock	177.5±7.201	13.0±0.981	200.0±4.082	16.0±0.943	227.6±3.540	13.2±1.300	206.6±7.201	10.1±0.950
Heat Acclimation	307.9±6.420	25.8±0.720	283.0±2.490	26.7±0.471	358.0±5.250	27.6±1.190	351.3±5.171	28.4±1.211
Salicylic acid (10 µM)	340.8±5.250	24.8±0.803	310.0±3.540	24.7±0.950	438.3±4.910	25.3±0.720	361.0±4.900	25.6±1.361
Salicylic acid (20 µM)	365.0±5.171	26.0±0.950	335.2±4.082	26.0±0.943	465.0±6.061	26.9±0.471	487.0±5.464	27.6±1.190

Weight of ten seedlings is given in the table. Data represents mean ±SE of three replicates.

RESULTS

The efficacy of various pre-treatments like heat acclimation and use of salicylic acid was studied in inducing thermotolerance in *Brassica* spp. The inhibition of seedling growth was observed after heat shock at 45 to 55°C (Table 1). However, heat acclimation and salicylic acid pre-treatment showed a significant effect on seedling length and helped in imparting thermotolerance by slightly increasing the seedling length (Table 1). Both the pre-treatments improved the fresh/dry weight of seedlings thereby overcoming lethal effects of heat shock (Table 2).

The electrical conductivity of the leachate from heat-shocked seedlings was significantly higher than control seedlings. Heat acclimation and SA pretreatments reduced the electrolyte leakage from the seedlings compared with heat shocked ones in all the genotypes (Table 3) suggesting the initiation of repair processes. However, genotype PBT37 performed significantly better than the rest of genotypes.

Further, the effect of heat shock on carbohydrate metabolism showed significant increase in the total soluble sugars in *Brassica* seedlings (Table 4). Heat acclimation and SA pretreatments also showed significant effect on total soluble sugars content; an increase in total soluble sugars was recorded among all the geno-

types. The activity of soluble neutral invertase also increased with heat acclimation and SA pretreatments (Figure 1). Increased invertase activity in heat shocked and pre-treated seedlings corroborates the higher level of total soluble sugars in these seedlings.

The activity of oxidative enzymes catalase (CAT) and peroxidase (POX) increased significantly in both the genotypes tested with the above said treatments over control, following heat stress (Figures 2 and 3). However, this response of POX was recorded maximum with SA pretreatments, in both thermosusceptible (RL1359 and PBR210) and thermotolerant genotypes (TL 15 and PBT37). The response of living tissue subjected to a sudden temperature rise is almost characterized by a cessation or reduction in synthesis of normal proteins and rapid production of large amounts of a group of proteins collectively known as heat shock proteins (HSPs). Pre-incubation of germinating seedlings at 35 and 45°C for 3 h each in thermosusceptible and thermotolerant varieties prior to heat shock resulted in synthesis of many new proteins in the low molecular weight range as evidenced by lanes 6 and 10 in heat acclimatized PBT 37 and TL15 (Figure 4), respectively and lanes 1 and 6 in heat acclimatized RL1359 and PBR 210 in Figure 5, respectively. Pretreatment with SA at 10 and 20 µM gave similar results. Both the concentrations resulted in the synthesis

Table 3. Effect of heat acclimation, SA and heat shock on electrolyte leakage (%) in different genotypes of *Brassica* spp.

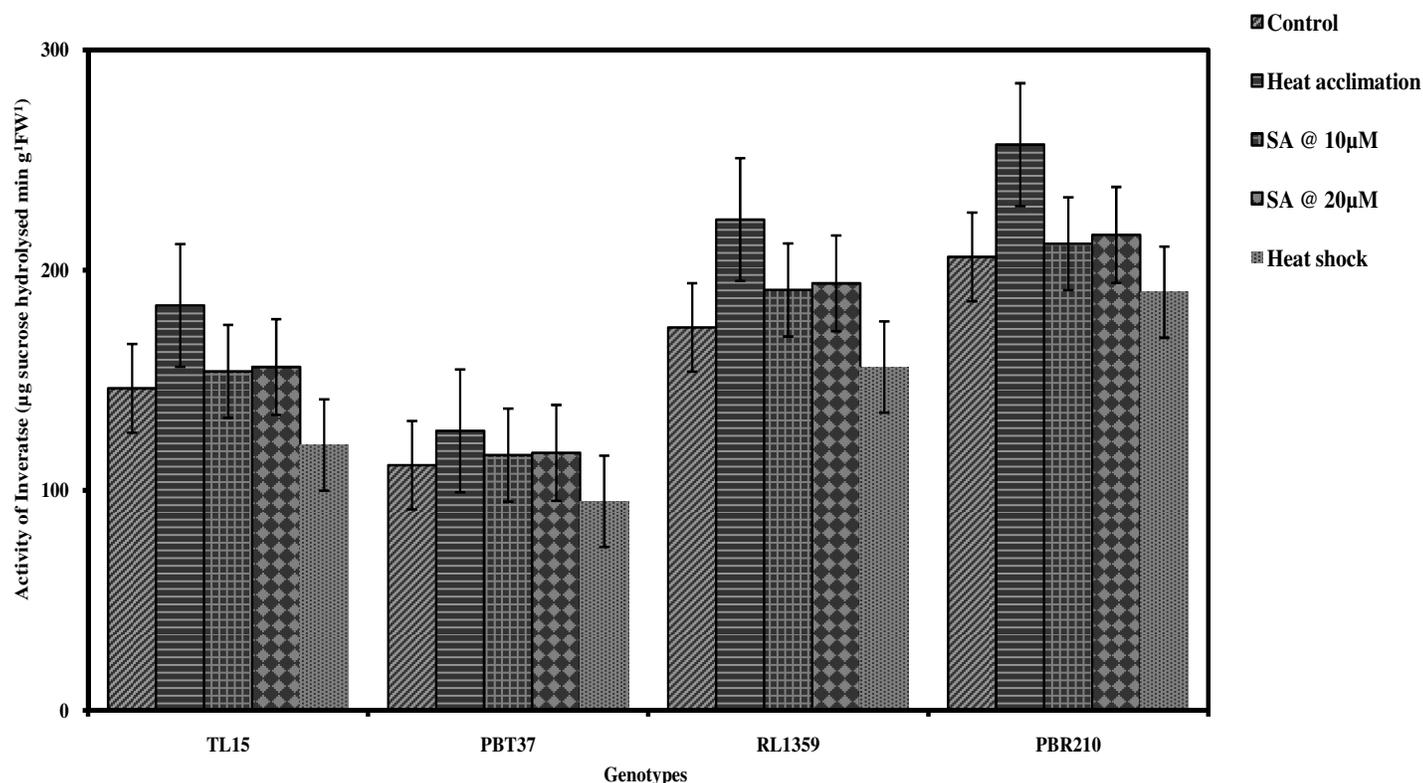
Treatment	Electrolyte leakage (%)			
	TL 15	PBT 37	RL 1359	PBR 210
Control	58.69±1.064	78.05±0.710	59.84±0.131	52.25±1.840
Heat Shock	91.33±0.544	95.70±0.272	88.44±0.362	95.75±0.103
Heat Acclimation	65.63±0.230	80.32±0.510	67.67±0.414	65.83±0.678
Salicylic acid (10 µM)	72.80±0.707	88.62±0.720	76.91±0.291	80.66±0.544
Salicylic acid (20 µM)	70.32±0.510	84.40±0.820	71.22±0.318	77.50±0.231

CD1 (5%) = 5.922 (for treatments); CD2 (5%) = 6.621 (for genotypes).
Data represents mean±SE of three replicates.

Table 4. Effect of heat acclimation, SA and heat shock on total soluble sugars (mg /g FW) in different genotypes of *Brassica* spp.

Treatment	Total soluble sugars (mg /g FW)			
	TL 15	PBT 37	RL 1359	PBR 210
Control	9.33±1.420	8.08±1.100	6.94±0.661	8.40±0.200
Heat Shock	9.54±0.075	8.28±0.035	7.22±0.009	8.69±0.061
Heat Acclimation	16.01±0.086	15.60±0.061	14.85±0.040	15.71±0.100
Salicylic acid (10 µM)	11.40±0.431	9.22±0.776	7.86±0.172	8.96±0.086
Salicylic acid (20 µM)	12.10±0.689	11.12±0.517	8.99±0.017	9.79±0.033

CD1(5%) = 0.718 (for treatments); CD2(5%) = 0.802 (for genotypes).
Data represents mean±SE of three replicates.

**Figure 1.** Effect of heat acclimation, SA and heat shock treatments on the activity of invertase in the different genotypes of *Brassica* spp.

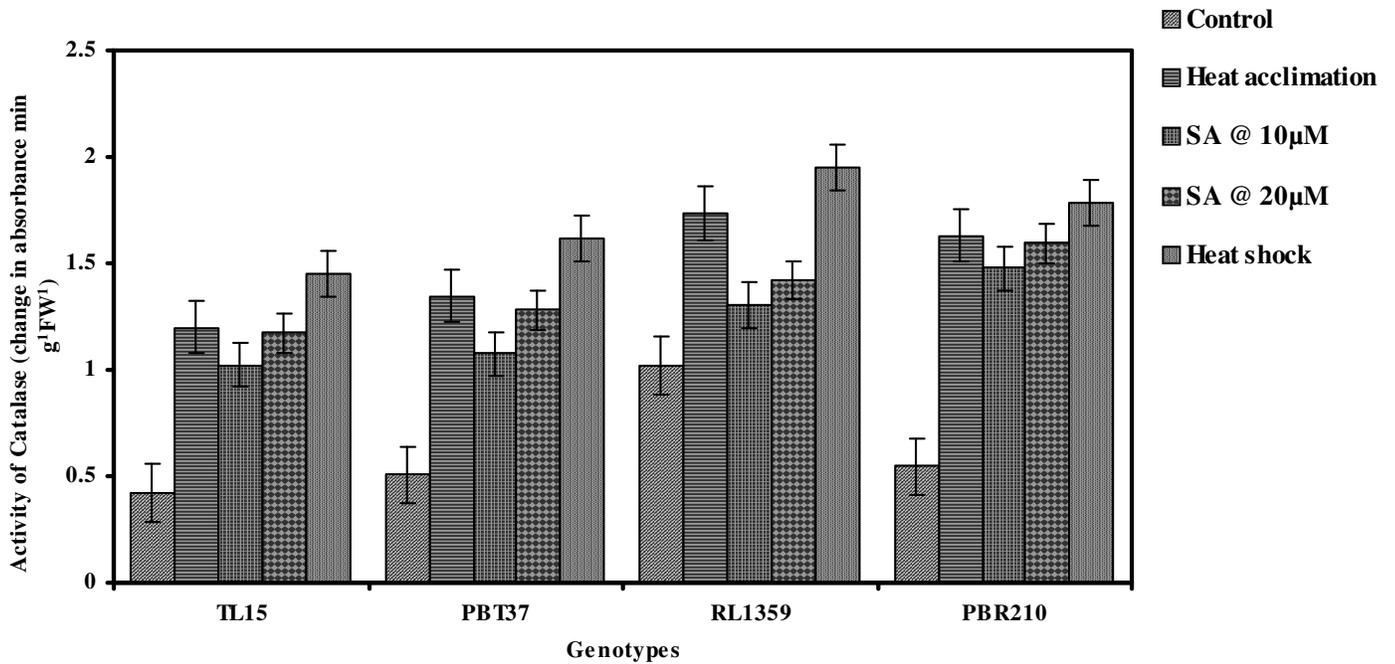


Figure 2. Effect of heat acclimation, SA and heat shock treatments on the activity of catalase in the different genotypes of *Brassica* spp.

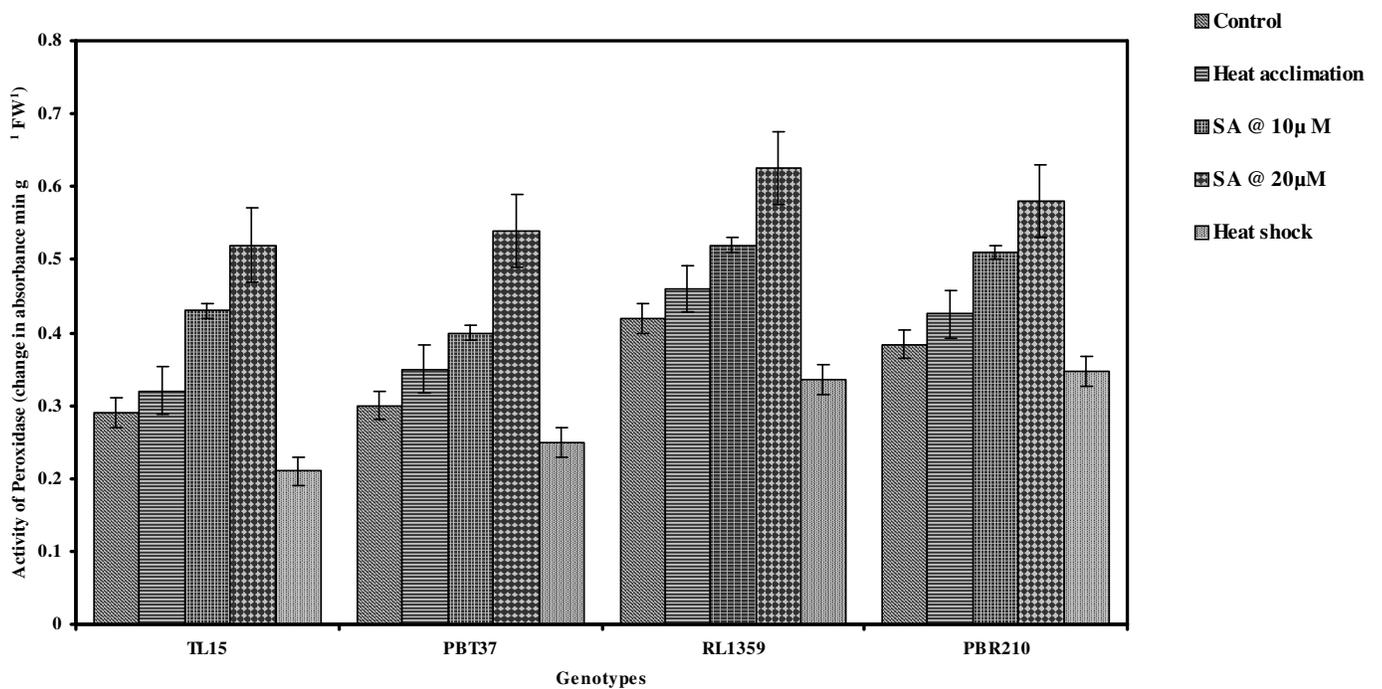


Figure 3. Effect of heat acclimation, SA and heat shock treatments on the activity of peroxidase in the different genotypes of *Brassica* spp.

of low molecular weight proteins in PBT 37, TL15 and RL1359, PBR 210 genotypes as observed in lanes 4 and

8 as well as in lanes 2, 3, 7 and 8 of the SDS-PAGE (Figures 4 and 5, respectively).

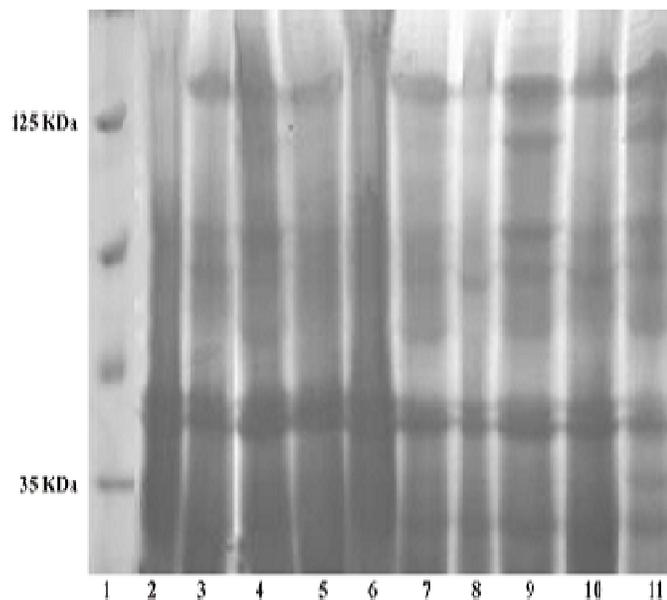


Figure 4. Electrophoretic banding pattern of TL15 and PBT37. Lane 1, standard; lane 2, PBT 37 heat acclimatized; lane 3, TL 15 heat acclimatized; lane 4, PBT 37 salicylic acid treated; lane 5, PBT 37 heat shocked; lane 6, PBT 37 heat acclimatized; lane 7, PBT 37 control; lane 8, TL 15 salicylic acid treated; lane 9, TL 15 heat shocked; lane 10, TL 15 heat acclimatized; lane 11, TL 15 control.

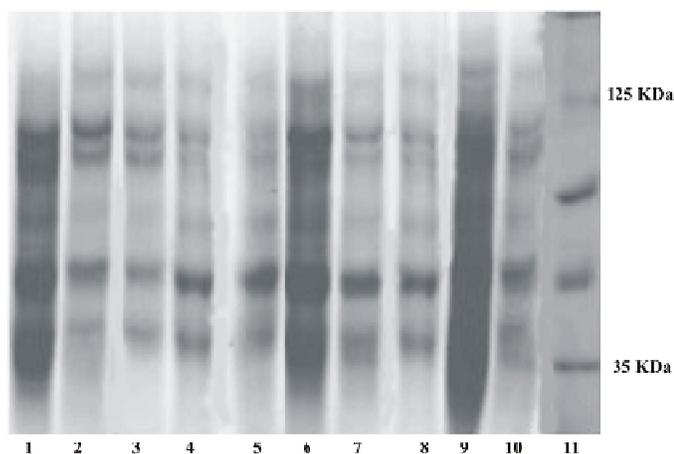


Figure 5. Electrophoretic protein banding pattern of RL 1359 and PBR 21. Lane 1, RL 1359 heat acclimatized; lane 2 RL 1359, salicylic acid at 10 μ M; lane 3 RL 1359 salicylic acid at 20 μ M; lane 4, RL 1359 control; lane 5, RL 1359 heat shocked; lane 6, PBR 210 heat acclimatized; lane 7, PBR 210 salicylic acid at 10 μ M; lane 8 PBR 210 salicylic acid at 20 μ M; lane 9, PBR 210 control; lane 10 PBR 210 heat shocked; lane 11, standard.

DISCUSSION

In the present investigation, exposure of *Brassica* seedlings to high temperature caused inhibition of seedling

growth significantly. However, pretreatments with heat acclimation and SA helped the seedlings to recover from heat-shock induced injury. Kaur (2000) has also reported an improvement in growth of heat stressed mungbean seedlings, pretreated with heat acclimation and SA. Improvement in growth could also be related to increased accumulation of fresh/dry weight in pretreated heat-stressed seedlings.

Increase in level of total soluble sugars was recorded with heat shock, heat acclimation and salicylic acid treatments in comparison to their respective controls. Accumulation of sugars in heat stressed mungbean seedlings has been reported by Thind et al. (1997). The significant increased level of total soluble sugar may be linked to increased invertase activity in all the four genotypes. In addition, the increase in soluble sugars may be acting as an adaptive mechanism for exerting protective effects under heat stress, as sugar acts as signalling molecule during abiotic stress.

It has been reported that accumulation of compatible solutes may protect cell against different stress factors (Rhizsky et al., 2004; Uemura et al., 2003). Moreover, invertase plays an important function in cell elongation and plant growth (Gibeaut et al., 1990). Secondly, it also helps in sucrose metabolism, which in turn has a crucial role in germination, seedling growth of different crop and greatly increases the osmotic potential of the stressed cell.

Plants protect their cells and subcellular systems from the cytotoxic effects of the reactive oxygen species (ROS) by activating antioxidative enzyme system. In the present investigation CAT and POX was found to increase in all the four genotypes under high temperature stress, which confirms that seedlings of the four genotypes encountered heat stress, due to which these enzymes got activated and measured by their increased activity. Other investigators have also reported increase in POX activity in grasses and cucumber under UV-stress and under high temperature stress in wheat (Almeselmani et al., 2006). CAT activity is also associated with the scavenging of toxic H_2O_2 and decreases the H_2O_2 levels in plant cells conferring stress tolerance. However, activity of CAT is not always found to be increasing; in some of the crops it decreases suggesting that its activity varies from species to species. Therefore, in the present investigation pretreated seedlings better adapt themselves to confront heat stress, by activation of its antioxidative system.

Plants show the heat tolerance by the virtue of synthesis of heat-shocked proteins (HSPs). The expression of HSPs has been investigated in a number of different plants and positive correlation was found between high temperature and this protein (Shridevi et al., 1999). Chakraborty and Tongden (2005) observed a low molecular weight protein of 14 Kda and another protein of molecular weight 36 Kda in SA pretreated plants challenged with lethal temperature. In the present

investigation, heat acclimatized seedlings showed *de novo* synthesis of some of the low molecular proteins and simultaneously disappearance of some of existing proteins, which also confirm that *Brassica* seedlings protect themselves against heat stress by expressing different HSPs.

The results of present investigation also suggest that although both the pretreatments i.e. heat acclimation and SA is successful in induction of thermotolerance, heat acclimation confers better tolerance than SA.

Thus physiological traits and biochemical changes may be linked with enhanced tolerance to heat stress damage at the seedlings stage and could be used as a marker for screening against heat stress.

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