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Heat stress tolerance in relation to oxidative stress and antioxidants in *Brassica juncea*

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Abstract

In the present study fifty genotypes of *Brassica juncea* were evaluated for heat stress tolerance in terms of biochemical components, in four day old seedlings. Heat shock was given at 45°C for 4.5 hr and thereafter survival percentage, electrolyte leakage and chlorophyll content were estimated. Tolerant genotypes (10) registered survival greater than 65%, moderately tolerant (20) between 35-65% and susceptible (20) less than 35%. Electrolyte leakage was significantly ($p<0.001$) higher in susceptible genotypes than in tolerant ones with respect to control seedlings. Chlorophyll content showed no significant variation among the tolerant, moderately tolerant and susceptible genotypes, although it registered a decline in response to heat stress. Lipid peroxidation, assessed by malondialdehyde (MDA) in stressed conditions was 4.66 (MDA g^{-1} f. wt. of tissue) in tolerant genotypes, 7.44 (MDA g^{-1} f. wt. of tissue) in susceptible genotypes and correlated significantly ($r=0.563$) with electrolyte leakage. Increase in POD activity under heat stress was maximum in tolerant class with respect to control. CAT activity showed decrease after heat shock treatment in all the three classes but the decrease was 1.3 fold in tolerant genotypes as compared to 1.6 fold in susceptible genotypes. The non-enzymatic antioxidants glutathione and proline registered a significantly (<0.01) high value in tolerant genotypes on heat shock treatment in comparison to susceptible genotypes corroborating the role of antioxidants in mitigating the effect of heat stress in *B juncea*. The antioxidants and proline seemed to play role in mitigating the effect of heat stress.

Key words

Antioxidant relationship, *Brassica juncea*, Heat stress, Oxidative stress

Introduction

The rise in temperature due to global warming has implications on the world wide crop production systems (Porter 2005 and Wahid *et al.*, 2007). Indian mustard (*Brassica juncea*), a cool season crop, is the major oilseed *rabi* crop of north-western India and is grown on six million hectares during winter season. It is endowed with high productivity, vigorous seedling growth, quicker ground covering ability, greater tolerance to heat and drought with enhanced resistance to insects and disease (Wright *et al.*, 1995, Norton *et al.*, 2004). High temperature (42.5ºC) prevailing at sowing time reduces germination, emergence and survival of seedlings (Dat *et al.*, 1998) resulting in loss of productivity. The optimum temperature required for germination and seed development in *B. juncea* is 25-33ºC. High temperature (33-35ºC) during seed filling period also causes yield loss due to floral sterility and impaired seed filling (Young *et al.*, 2004, Bjorkman and Pearson, 1998).

Heat stress manifestations are mediated via. oxidative damage through generation of reactive oxygen species (ROS) (Knight and Knight, 2001). The ROS damage various biomolecules like DNA, lipid, proteins etc. and thus fatally affect plant metabolism and limit growth and yield (Sairam and Tyagi, 2004). Plants have developed both enzymatic and non-enzymatic detoxification systems to counteract ROS, thereby protecting cells from oxidative damage (Sairam and Tyagi, 2004). These enzymes include peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) etc.; whereas non-enzymatic metabolites include glutathione (GSH), proline, glycinebetaine etc. (Wahid *et al.*, 2007). Improved thermo tolerance in plants has been observed due to the synthesis of isoprene (Singaas *et al.*, 1997) or glycinebetaine (Wahid *et al.*, 2007), production of antioxidant enzymes (Kubo *et al.*, 1999) and reduction in α-linoleinic acid concentration (Wahid *et al.*, 2007). Protection against oxidative stress is an important component in determining the survival of a plant under heat stress and antioxidant defence strength is correlated with acquisition of thermotolerance.

Screening of germplasm for high temperature tolerant/ resistant genotypes and understanding their biochemical basis for heat stress tolerance would help in designing strategies for sustainable crop yield under high temperature stress. Since *B. juncea* is an important oilseed crop of India, the present study was carried out to investigate the heat stress tolerance in *B. juncea* at seedling stage; the stage highly vulnerable to heat stress, in terms of oxidative stress and antioxidative status.

Materials and Methods

The seeds of *B.juncea* (50 genotypes) were procured from Oilseeds Section, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. To study heat stress tolerance, 20 seeds of each genotype were placed in a jar on four layers of rough filter paper soaked in double distilled water. The jars were run in duplicates. The mouth of each jar was covered with aluminium foil. Seeds were germinated and grown for four days at 25 ± 2 ºC under light/dark period in an incubator. Four days after germination, one set of the jar containing seedlings was given heat shock treatment at LT_{50} i.e. 45 ºC for 4.5 hours (LT_{50}: lethal temperature at which 50% seedlings of released variety of *B. juncea*, PBR-210 were killed). Second set of jars was considered as control. Resistance/tolerance to stress was evaluated on the basis of seedlings survived 24 hr after heat shock treatment (Dat *et al.*1998). Seedling death was assessed by stem collapse 1-2 cm below apex. Percentage survival was calculated as the number of seedlings which survived 24 hr after heat shock treatment upon total number of seedlings germinated, multiplied by 100. On the basis of percent survival, genotypes were categorized into tolerant, having survival greater than 65%, moderately tolerant, having survival within the range of 35-65% and susceptible, having survival less than 35%. Cell membrane stability was estimated by electrolyte leakage (Deshmukh *et al.*, 2001). Chlorophyll content was estimated following the method of Johnson *et al.* (1984).

Lipid peroxidation was measured in terms of malondialdehyde content (MDA) (Dhindsa *et al.*, 1981). Peroxidase activity was measured following the method of Claiborne and Fridovich (1979). Four day old seedlings (shoot portion) were homogenized in chilled 50mM phosphate buffer (pH 6.5) and centrifuged at 13000 rpm for 30 min at 4ºC. Supernatant was used as enzyme extract. The reaction mixture consisted of 50mM phosphate buffer (pH 6.5), enzyme extract and 0.1% O-Dianisidine solution. The mixture was brought to 30ºC in water bath and 6% H_2O_2 was added to it. The change in absorbance was recorded at 430nm for 3 min at 30 sec interval against reagent blank. The enzyme activity was expressed as change in absorbance (ΔE) $minute^{-1}$ g^{-1} f.wt. Catalase activity was measured following the method of Aebi (1983). Four day old seedlings (shoot portion) were homogenized in chilled 50mM phosphate buffer (pH 7.0) and centrifuged at 13000 rpm for 30 min at 4ºC. Supernatant was used as enzyme extract. The reaction mixture consisted of 50mM phosphate buffer (pH 7.0), enzyme extract and 30mM H_2O_2 solution. Decrease in absorbance after every 30 sec interval for 3 min was recorded at 240nm against reagent blank. Enzyme activity was calculated using extinction coefficient of H_2O_2 (0.036$mM^{-1}cm^{-1}$) and was expressed as μmole of H_2O_2 decomposed min^{-1} g^{-1} f.wt. Glutathione and proline contents were measured by the methods of Beutler *et al.* (1963) and Chinard (1952), respectively. The experiments were performed in a completely randomised design (CRD). One way analysis of variance (ANOVA) was done with all the data to confirm the variability of data and validity of results, and least square difference (LSD) was performed to determine the significant difference between treatments using AgRes (3.01) statistical software ©, 1994. Pascal International software solution, USA.

Results and Discussion

Heat stress tolerance evaluation of *B.juncea* genotypes in terms of survival percentage after heat shock treatment registered ten genotypes to be tolerant, twenty moderately tolerant and rest of the twenty genotypes to be susceptible (Fig.1). Tolerant and moderately tolerant genotype seedlings registered survival percentage of 85.03% and 46.06% respectively, which was four fold and three fold greater than mean percent survival (17.86%) of susceptible genotypes respectively. The three classes of genotypes were then compared in terms of electrolyte leakage, chlorophyll content, lipid peroxidation and antioxidant status. Electrolyte leakage increased significantly ($p<0.001$) after heat stress in seedlings of all the three classes of genotypes as compared to their respective controls (Fig.1). The susceptible genotypes registered highest value (78.96%) of electrolyte leakage in response to heat stress as compared to tolerant (52.48%) and moderately tolerant genotypes (60.09%). The electrolyte leakage data is an indicator of cell membrane stability (Du *et al.*, 2009) and the present study, depicted cell membrane damage in seedlings during heat stress. A significant negative correlation ($r= -0.799$) between survival and electrolyte leakage was observed. Chlorophyll content (Fig.1), which determines the photosynthetic capacity of plants, decreased significantly ($p<0.001$) in heat shock treated seedlings in comparison to controls in the three classes of genotypes. The

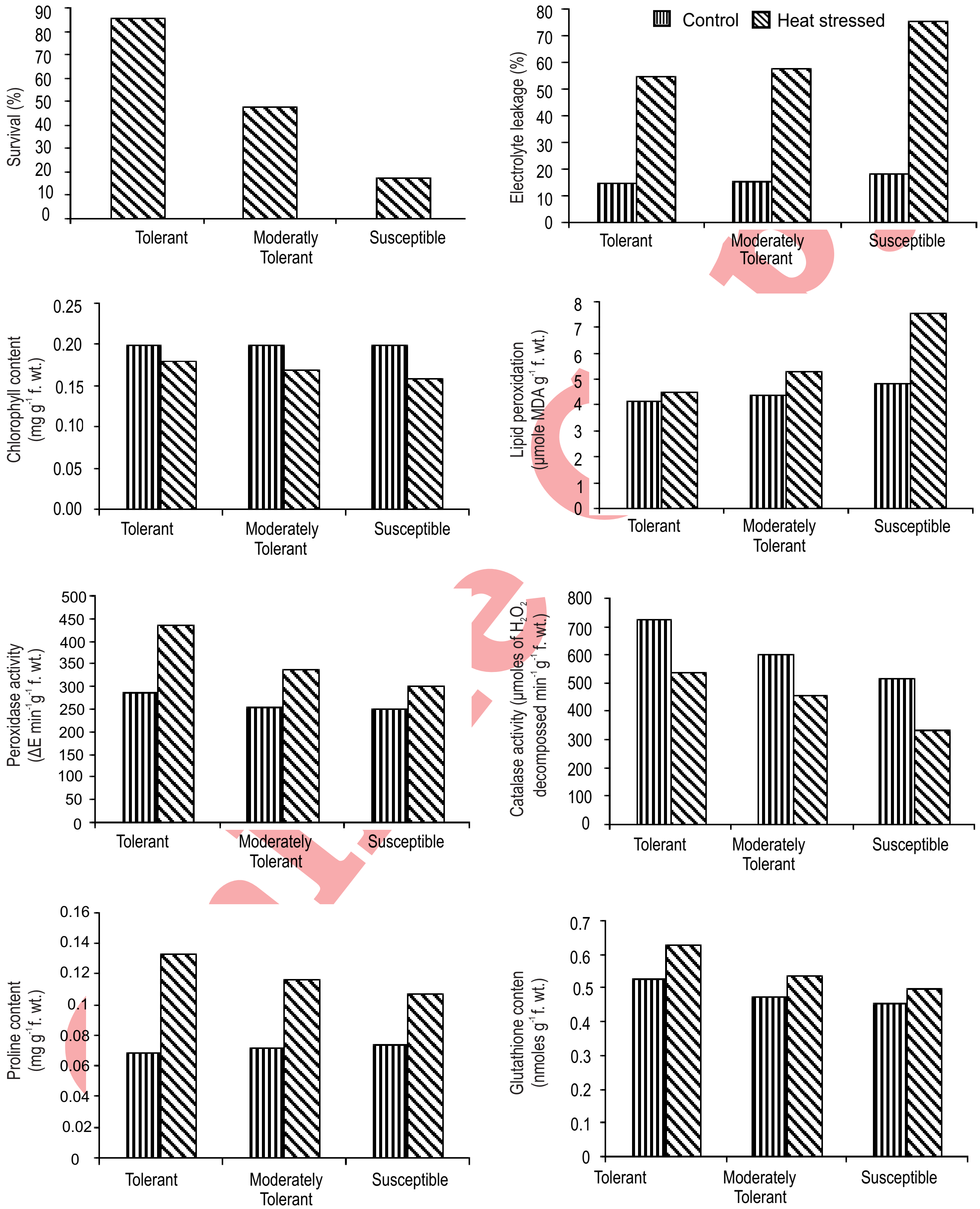

Fig. 1 : Effect of heat stress on survival (percent), electrolyte leakage, chlorophyll, lipid peroxidation, peroxidase activity, catalase activity, glutathione and proline content of four day old seedings of *Brassica juncea.*

decline was at par with all the three classes which suggested damage to photosynthetic apparatus mediated by oxy radicals (Karim *et al.*, 1997) due to high temperature. Similar decline in chlorophyll content was observed in Turfgrass upon exposure to drought, heat or combined stresses (Jiang and Huang, 2001). Decreased or unchanged chlorophyll level has also been observed in other species during draught stress ((Arjenaki *et al.*, 2012 and Mensah *et al.*, 2006).

Lipid peroxidation increased significantly ($p<0.001$) after heat stress. The increase was maximum in susceptible genotypes (7.44±1.3 µm MDA g^{-1} f.wt.) followed by moderately tolerant genotypes (6.12±1.3 µm MDA g^{-1} f.wt.) and tolerant genotypes (4.66±1.1 µm MDA g^{-1} f.wt.) (Fig.1). The respective controls were however at par with each other. Highest lipid peroxidation registered in susceptible genotypes indicated lower membrane stability. Low cell membrane integrity is a repercussion of lipid peroxidation of membrane lipids caused by ROS generated during stress (Liu and Huang, 2000). Liu and Huang (2000) stated that ROS generated during heat stress react with unsaturated lipids in membranes and cause lipid peroxidation leading to MDA accumulation. MDA is a product of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage (DaCosta and Huang, 2007). Similar observation has been reported by Ismail and Hall (1999) in cowpea, Jiang and Huang (2001) in turfgrass and Liu and Huang (2000) in bentgrass. A significant correlation between lipid peroxidation and electrolyte leakage ($r=0.563$) suggests that the oxidative damage to lipids disrupts the membrane structural integrity, which in turn causes electrolyte leakage (Liu and Huang, 2000).

The POD activity increased significantly ($p<0.001$) after heat shock treatment. Following heat stress the mean POD activity increased by 52.26% in tolerant genotypes, 32.42% in moderately tolerant and 21.11% in susceptible genotypes, respectively. Higher activity in tolerant genotypes could be responsible for heat stress tolerance as POD is known to decompose H_2O_2 and thus prevent lipid peroxidation (Chakraborty and Tongden, 2005). Negative correlation between lipid peroxidation and POD ($r=-0.471$) supported the data. The effect of heat stress on CAT activity registered an opposite trend. Heat stress significantly ($p<0.001$) decreased the CAT activity, which is a H_2O_2 scavenging peroxisomal enzyme, in all the three classes. The activity declined by 59.62% in seedlings of susceptible genotypes, 40.84% in moderately tolerant genotypes and 27.29% in tolerant genotypes, respectively. The decrease in activity could be due to its non-robust nature and sensitivity to heat stress (Fadzillah *et al.*, 1996). Reports on the effect of stresses on CAT activity vary. Increased CAT activity was reported in chickpea under draught stress (Mafakheri *et al.*, 2011) and decreased CAT activity was observed in heat stressed wheat leaves (Hameed *et al.*, 2012) and Kentucky bluegrass (He *et al.*, 2011). CAT showed a highly significant correlation with survival($r=0.909$) in tolerant genotypes which indicated its role in conferring resistance against heat stress. A significant correlation between CAT and electrolyte leakage ($r=-0.789$) as well as between CAT and lipid peroxidation ($r=0.631$) was observed. This further supported the fact that CAT acted as an important antioxidant scavenging ROS, minimising lipid peroxidation and thus electrolyte leakage.

Among the non-enzymatic antioxidants, the contents of GSH and proline were monitored. A highly significant increase ($p<0.001$) in GSH content (Fig.1) after heat stress was observed in seedlings of all the three classes of genotypes. The value of GSH recorded in tolerant, moderately tolerant and susceptible genotypes was 0.623, 0.521 and 0.509 n moles g^{-1} f. wt., respectively. The basal level of GSH in all the classes was at par. High GSH content could be protective against heat shock generated oxidative stress as GSH has been reported to participate in removal of H_2O_2 (Nocter and Foyer, 1998), which may get accumulated during high temperature stress. GSH is considered as the first line of defence. The increased GSH levels would not only protect against free radicals but also switch the whole panoply of stress resistance response (Noctor and Foyer, 1998). The proline content did not vary significantly among control seedlings of tolerant, moderately tolerant and susceptible genotypes. Heat shock treatment resulted in significant ($p<0.001$) increase in proline content. The increase was almost two fold in seedlings of tolerant genotypes, 1.6 fold in moderately tolerant and 1.4 fold in susceptible genotypes as compared to control. Increased proline content during heat stress might have resulted from stimulation of proline synthesis from glutamate by loss of feed back inhibition, decline in proline oxidation or due to decreased incorporation into proteins (Handa *et al.*, 1986). Proline has been reported to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses like heat (Wahid *et al.*, 2007) draught and salinity stress (Ashraf and Foolad, 2007). Significant positive correlation between survival (%) and proline content ($r= 0.621$) indicated its role in protection during heat stress and further supported the previous reports. A significant negative correlation ($r=-0.668$) between electrolyte leakage and proline suggested its role as an antioxidant.

In summary, out of fifty genotypes ten genotypes were found to be tolerant to heat stress. Heat stress induced oxidative injury, assessed in terms of electrolyte leakage and lipid peroxidation, was higher in susceptible genotypes but lower in tolerant genotypes. The tolerant genotypes also showed increased levels of antioxidant enzymes and metabolites and thus register lesser oxidative damage. The information so generated can pave way for improving the quality of the existing high yielding cultivars.

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