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Biochemical and Physiological Parameters: Swift Tools for Screening High Temperature Tolerance in Barley

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ABSTRACT

High temperature (HT) stress is a wide-spread problem seriously affecting barley production and quality. Development of heat-tolerant cultivars is of prime importance; however, the progress is hampered by a lack of swift and efficient tools for selection of tolerant germplasm that can be used as a source of candidate genes. This study was conducted to explore the thermotolerance behaviour of barley with respect to germination, dry mass accumulation, membrane thermal stability, TTC (2,3,5-triphenyl tetrazolium chloride) cell viability, lipid peroxidation and chlorophyll content. Twenty genotypes of barley were subjected to a brief heat shock (HS) episode of 45°C for 2 h followed by transfer to a normal temperature (25°C) for five days. HS reduced the germination, dry mass accumulation and damaged the integrity of cellular membranes as indicated by increased electrolyte leakage after exposure to HS. Genotypic testing, using TTC reduction as a measure of tissue viability, following HS treatment, confirmed the thermal responsiveness of seedlings. The inhibitory effect of HS was reflected by increased lipid peroxidation and a decline in chlorophyll content. The tolerant genotypes registered less reduction in germination, dry mass accumulation, TTC cell viability and chlorophyll content under HS conditions compared to susceptible ones. Oxidative damage, in terms of generation of malondialdehyde, was markedly higher in susceptible genotype. These results suggest that the biochemical and the physiological parameters can be used as tools for screening HT tolerance in barley to facilitate the development of HT-tolerant germplasm.

Keywords: electrolyte leakage, chlorophyll, heat shock, lipid peroxidation, membrane thermal stability, thermotolerance, TTC

INTRODUCTION

High temperature (HT) is a major agricultural problem in many areas of the world responsible for reduced yield and quality of cereals (Wardlaw *et al*. 2002), including barley. According to a report of the Intergovernmental Panel on Climatic Change (IPCC), global mean temperature will rise 0.3°C per decade (Jones *et al*. 1999) reaching approximately 1 and 3°C above the present value by 2025 and 2100, respectively, and leading to global warming. Transitory or constantly high temperatures cause an array of morpho-anatomical, physiological and biochemical changes in plants affecting their growth and development and may lead to a drastic reduction in economic yield (Wahid *et al*. 2007). At very HT, severe cellular injury and even cell death may occur within minutes, which could be attributed to a catastrophic collapse of cellular organization (Schoffl *et al*. 1999) eventually leading to starvation, inhibition of growth, enhanced ion leakage, production of toxic compounds and reactive oxygen species (ROS) (Schoffl *et al*. 1999; Howarth 2005) and hence affect crop establishment and final yield. Consequently, the development of heat-tolerant cultivars is of prime importance to alleviate future threats to food availability in a rapidly expanding human population. The progress using either traditional breeding or genetic engineering to improve heat tolerance has been restricted by the lack of heat-tolerant germplasm or the genes that control heat tolerance. HT tolerance can be characterized by measuring whole-plant productivity traits (e.g., yield components) or by utilizing bioassays (Porter *et al*. 1995). Evaluating grain yield under HT stress has long been practiced by breeders to identify cultivars better adapted to hot conditions. This approach has the advantage of combining the effects of many different factors without having to know the relative or physiological relevance of each factor. Field screening has the advantage of using the relative stress level, however, the variable climate, limited seasonal duration for screening, large plot sizes, time-consuming breeding protocols, inconveniently situated target environments and interaction of various stresses and constraints on plant responses complicate the assessment (Howarth 2005). So there is a call for swift and efficient screening tools which are not affected by these problems and can be conducted under controlled conditions. Biochemical and physiological screening techniques, as a complement to empirical methods could increase selection efficiency, securing heat tolerance genes that may be lost during empirical selection (Reynolds *et al*. 1994).

Although resistance to HT involves several complex tolerance and avoidance mechanisms, one of the sites of primary physiological injury by heat is thought to be the membrane. Cell membrane stability under stress condition has been suggested for estimating cellular thermotolerance in plants (Huang *et al*. 2001). Elevated temperature results in rapid loss of water from the plant surface and causes a state of dehydration. This leads to the disruption of cellular membrane, making them more permeable to ions (Jiang and Huang 2001). An association between heat tolerance measured by electrolyte leakage (EL) at the seedling stage and tolerance to post-anthesis HT stress was reported by Saadalla *et al.* (1990b). The reduction of photosynthesis by HT stress is related to a disruption of chloroplasts and inactivation of many enzymes, mainly induced by oxidative stress (Dekov *et al*. 2000). Ristic *et al.* (2007) reported that under HT conditions loss of chlorophyll (Chl) and damage to thylakoid membranes are closely associated in wheat. They suggested that loss of Chl under HT could be used as a reliable and high throughput approach for screening HT tolerance.

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tive damage to cell membranes by ROS, resulting in lipid peroxidation (LPO) and consequently membrane injury (Liu and Huang 2000). The abilities to maintain cell membrane integrity and diminish oxidative stress have been proposed as good indicators of thermotolerance in plants (Huang *et al*. 2001). The TTC cell viability assay is based on the principles of tetrazolium salt reduction to formazan by dehydrogenase respiratory enzymes. Therefore, the TTC test evaluates the mitochondrial electron transport chain, and thus, it represents respiratory activity. Cell viability measurement based on the reduction of the TTC has been used to characterize HT tolerance by Porter *et al.* (1995). They concluded that the TTC assay is an efficient and reliable technique for measuring differences in acquired HT tolerance. Fokar *et al.* (1998) evaluated genetic variation in thermotolerance among wheat cultivars at the seedling stage of growth by TTC assays. They suggested that the results by TTC did not change with plant age and the association between thermotolerance at the seedling growth stages and at the flowering growth stages across cultivars was linear and highly significant for the TTC assay. That result is extremely important for selection work, since seedling tests require less resources than assays with fully grown plants.

In view of these findings, the present work was an effort to screen HT tolerant barley germplasm at the germination stage using physiological and biochemical parameters in the controlled laboratory conditions.

MATERIALS AND METHODS

Plant material and stress treatment

Grains of genetically diverse two- and six-rowed barley (*Hordeum vulgare* L.) genotypes *viz*, VJM 201, VJM 318, VJM 514, VJM 531, VJM 540, BH 393, RD 2668, DWRUB 52, BK 201, BK 203, PL 796, BL 16, BL 48, BL 53, BL 61, BL 71, BL 73, DWR 28, K 551 and RD 2503 were procured from the Department of Plant Breeding and Genetics, Punjab Agricultural University, Punjab, India. The genotypes chosen were comprised of advanced commercial released cultivars, however, no information about their thermal responsiveness was available. Among two-rowed genotypes VJM 201 for Punjab, DWR 28, DWRUB 52 and RD 2668 for North Western plain zone (NWPZ) and among six-rowed genotypes, BH 393 for Haryana, K 551 and RD 2503 for NWPZ were the released commercial cultivars.

Uniform sized grains of each variety were hand-picked and surface sterilized with 0.1% $HgCl_2$ for 1 min followed by thorough rinsing with distilled water. Twenty grains were imbibed in distilled water (20 ml) for 24 h in the dark and germinated in Petri dishes (9.0 cm) (Borosil, India) on double layer Whatman No. 1 filter paper moistened with 5.0 ml of distilled water under controlled conditions (light/dark cycle of 16/8 h at 25 ± 2°C and photosynthetic photon flux density of 400 μmol m^{-2} s^{-1}) in growth chamber for 5 days. Moisture level was visually checked daily and topped-up as necessary. HS treatment was applied as described earlier in Singh and Asthir (2009). Briefly, HS at 45°C for 2 h was applied after 24 h of germination and thereafter Petri dishes were maintained at normal temperature (25°C) for five days. In the control experiment, seedlings were maintained at 25°C for five days. All the chemicals used in this study were of analytical grade.

Physiological and biochemical parameters

Germination percentages were estimated after five days of germination using root/shoot protrusion as a criterion (appearance of root/shoot 1.5 cm in length). Fresh weights of individual seedlings of control and treated plants were measured after five days of germination. Dry weights of same seedlings were recorded after drying in an oven at 110°C for 10 min followed by at 60°C till constant weight. Dry mass percentage = [(dry weight/fresh weight) ×100].

Chl content of shoots was estimated according to Arnon (1949). 0.5 g of fresh tissue samples were extracted with 5 ml of ammonical acetone and centrifuged at 3000 × g for 5 min. The absorbance of supernatants was determined at 645, 663 and 710 nm; 710 nm was used as an isobetric point, deducted from all other absorbance readings. Chl content was calculated as per a standard method (Arnon 1949).

EL was estimated as per Basra and Basra (2001). Ten roots and shoots were excised from residual grain and washed with deionized water to remove adhering electrolytes. Seedling samples were taken in 20 ml of deionized water in two sets. One set was subjected to continuous stirring at 28°C for 5 h and its conductivity (C1) was recorded using a conductivity meter (Systronics, TDS 307). A second set was kept in a boiling water bath (100°C) for 30 min and its conductivity (C2) was also recorded. EL percentage was = [(C1/C2) × 100].

LPO products were determined from the thiobarbituric acid reactive substances (TBARS) contents resulting from the thiobarbituric acid (TBA) reaction as described by Larkindale and Knight (2002). Briefly, 0.5 g of fresh tissue was homogenized in 2.0 ml of 5% trichloroacetic acid (TCA) solution and centrifuged at 13,500 × *g* for 15 min at room temperature. The supernatant of tissue extract was mixed with an equal volume of 20% (w/v) TCA containing 0.5% (w/v) TBA and incubated at 95°C for 30 min. The reaction was stopped by placing the reaction tubes in an ice bath and centrifuged at 9500 × g for 10 min. Absorbance of supernatant was read at 532 nm using the 0.5% TBA in 20% TCA solution as the blank. The value for non-specific absorption at 600 nm was subtracted from the 532 nm value. The LPO contents, as malondialdehyde (MDA) were determined by its extinction coefficient of 155 mM^{-1} cm^{-1}.

TTC cell viability was measured in terms of the reduction of TTC to a red colour formazan as per Boamah-Asare and Fletcher (1983). Twenty root tips (each 2 cm) were floated in 0.1% (w/v) aqueous TTC solution in dark for 3 h. The root tips were homogenized with ethyl acetate using a glass homogenizer and centrifuged at 2500 × g for 10 min at 0°C. Absorbance of the sample supernatants was measured at 486 nm.

Statistical analysis

The data were analysed in a factorial completely randomized design (CRD) by using CPCS1 software package. The means were compared for significant differences at $P \leq 0.05$ by the Student's *t*-test using GSTAT04 software package. Correlation analyses were carried out using MS Excel 2003.

RESULTS

The mean data clearly show that there are differences between the control values of all the genotypes. Therefore it would not be legitimate to interpret data on a mean basis. Thus the results have been presented as a percentage of the control value.

No genotype showed significant differences in percent germination under control, however, HS treatment resulted in -3 to 29% reduction in percent germination (**Table 1**). Germination of shoots was inhibited relatively more than roots in all genotypes, with maximum reduction was observed in genotype BL 71>VJM 540>PL 796>BK 203, while VJM 514, BK 201, RD 2503<VJM 201 and K 551 were able to minimize the reduction.

The inhibition of seedling growth was observed after HS in all genotypes. Shoots had relatively higher dry masses compared to roots (**Table 1**). Under control conditions genotypic differences were not so evident for root and shoot dry masses, however, a significant ($P \leq 0.05$) decrease in percent dry mass was seen in all genotypes under HS. Maximum reductions was observed for roots of genotypes VJM 540>DWR 28>DWRUB 52 and shoots of VJM 540>RD 2503>DWR 28. However, genotypes VJM 318<BL 53<BH 393<BL 61 for roots and VJM 318<BL 61<BL 16 for shoots registered minimum reductions in dry masses. Genotype VJM 318 was least affected by HT while VJM 540 was maximum affected. Reduction in dry mass accumulation in genotype VJM 540 was 2.3 and 2.6 fold higher for roots and shoots respectively, than genotype VJM 318.

Cell membrane stability was estimated by measuring

Table 1 Effect of heat shock on percent germination and dry mass accumulation of barley seedling. Values in parenthesis indicate percent reduction as compared to control.

Genotypes	Germination (%)						Dry mass (%)					
	Root			Shoot			Root			Shoot		
	Control	Heat Shock		Control	Heat Shock		Control	Heat Shock		Control	Heat Shock	
VJM 318	100	95 ± 1.4	(5)	100	90 ± 2.4	(10)	62.7 ± 1.7	52.9 ± 1.5	(16)	71.4 ± 1.9	59.4 ± 1.5	(17)
BL 61	98 ± 1.5	93 ± 4.3	(5)	95 ± 3.0	85 ± 2.9	(11)	61.4 ± 1.2	50.7 ± 1.2	(17)	68.2 ± 1.8	54.6 ± 1.4	(20)
VJM 540	95 ± 1.7	90 ± 2.9	(5)	98 ± 1.4	72 ± 1.4	(27)	55.4 ± 1.2	35.4 ± 1.3	(36)	61.2 ± 1.3	34.6 ± 1.9	(44)
DWR 28	100	98 ± 1.4	(2)	95 ± 2.3	80 ± 2.9	(16)	52.1 ± 2.6	37.2 ± 1.6	(29)	64.8 ± 1.7	39.7 ± 2.8	(39)
VJM 201	98 ± 1.1	95 ± 1.9	(3)	98 ± 1.7	90 ± 5.8	(8)	54.4 ± 1.4	42.1 ± 2.1	(23)	65.2 ± 1.8	43.0 ± 2.0	(34)
VJM 514	100	100	(0)	95 ± 1.6	92 ± 4.3	(3)	59.2 ± 1.7	47.1 ± 1.7	(20)	66.6 ± 1.4	42.3 ± 2.5	(37)
VJM 531	93 ± 1.4	95 ± 2.7	(-2)	93 ± 1.4	82 ± 4.6	(12)	60.6 ± 2.0	48.9 ± 1.8	(19)	71.3 ± 2.1	55.7 ± 2.0	(22)
BH 393	95 ± 2.4	98 ± 1.8	(-3)	93 ± 2.6	82 ± 3.3	(12)	60.5 ± 1.8	50.1 ± 2.4	(17)	70.2 ± 1.0	50.5 ± 1.2	(28)
RD 2668	98 ± 1.2	95 ± 2.2	(3)	98 ± 1.2	82 ± 1.9	(16)	57.4 ± 3.3	46.3 ± 1.9	(19)	71.8 ± 1.5	49.6 ± 1.5	(31)
DWRUB 52	98 ± 2.5	95 ± 1.5	(3)	98 ± 1.5	85 ± 5.7	(13)	54.9 ± 2.1	40.8 ± 1.7	(26)	71.4 ± 3.7	55.9 ± 2.6	(22)
BK 201	98 ± 1.4	98 ± 1.7	(0)	95 ± 1.7	92 ± 4.3	(3)	60.3 ± 3.1	49.8 ± 2.6	(17)	70.8 ± 2.0	50.8 ± 1.5	(28)
BK 203	98 ± 1.6	93 ± 4.3	(5)	100	75 ± 5.8	(25)	62.1 ± 1.7	51.0 ± 1.3	(18)	71.1 ± 2.4	51.8 ± 2.0	(27)
PL 796	100	95 ± 2.8	(5)	93 ± 1.4	67 ± 4.3	(26)	54.7 ± 1.5	44.1 ± 1.8	(19)	68.7 ± 2.7	46.8 ± 1.0	(32)
BL 16	100	93 ± 1.4	(7)	98 ± 2.0	75 ± 2.9	(24)	55.6 ± 2.4	42.4 ± 1.3	(24)	67.5 ± 1.9	52.9 ± 2.5	(22)
BL 48	93 ± 2.0	95 ± 4.3	(-2)	90 ± 1.1	72 ± 1.4	(20)	57.1 ± 1.6	45.7 ± 2.9	(20)	67.1 ± 1.4	45.2 ± 1.4	(33)
BL 53	95 ± 1.6	95 ± 2.5	(0)	100	87 ± 4.3	(13)	59.4 ± 1.4	49.4 ± 2.5	(17)	54.8 ± 1.1	35.7 ± 1.1	(35)
BL 71	98 ± 1.5	98 ± 1.2	(0)	98 ± 1.9	70 ± 5.8	(29)	55.2 ± 1.7	44.6 ± 1.7	(19)	71.2 ± 2.6	48.7 ± 1.5	(32)
BL 73	95 ± 2.9	98 ± 2.9	(-3)	98 ± 1.2	80 ± 2.9	(18)	60.7 ± 1.9	48.9 ± 2.3	(19)	67.5 ± 1.8	41.6 ± 1.3	(38)
K 551	98 ± 2.1	98 ± 1.5	(0)	98 ± 2.0	90 ± 5.8	(8)	57.5 ± 3.7	45.6 ± 1.8	(21)	70.5 ± 2.4	44.7 ± 2.6	(37)
RD 2503	98 ± 1.7	95 ± 2.0	(5)	93 ± 1.6	90 ± 2.9	(10)	61.1 ± 2.1	49.7 ± 2.5	(16)	65.6 ± 1.0	39.7 ± 2.6	(17)
CD (P≤0.05)	Treatment:		NS			1.95			1.29			1.23
	Genotypes:		1.31			6.17			4.07			3.89
	T × G:		NS			8.73			NS			5.50

Table 2 Effect of heat shock on electrolyte leakage and TTC cell viability of barley seedling. Values in parenthesis indicate percent increase and percent reduction for electrolyte leakage and TTC cell viability, respectively, as compared to control.

Genotypes	Electrolyte leakage (%)						TTC cell viability (Absorbance at 486 nm × 10)		
	Root			Shoot			Root		
	Control	Heat Shock		Control	Heat Shock		Control	Heat Shock	
VJM 318	41.2 ± 1.8	42.3 ± 1.7	(3)	33.3 ± 2.4	35.0 ± 2.4	(5)	3.2 ± 0.1	2.4 ± 0.2	(25)
BL 61	40.0 ± 2.3	41.8 ± 1.5	(5)	35.0 ± 3.3	37.9 ± 1.7	(8)	3.1 ± 0.2	2.4 ± 0.2	(23)
VJM 540	46.7 ± 1.7	54.1 ± 2.4	(16)	35.0 ± 1.8	44.6 ± 1.5	(27)	2.9 ± 0.1	1.3 ± 0.1	(55)
DWR 28	45.7 ± 2.1	53.0 ± 2.1	(16)	31.4 ± 5.2	41.8 ± 1.3	(33)	2.3 ± 0.1	1.6 ± 0.2	(30)
VJM 201	43.3 ± 1.6	48.9 ± 1.7	(13)	26.7 ± 3.7	29.2 ± 1.4	(9)	3.1 ± 0.3	2.1 ± 0.1	(32)
VJM 514	41.8 ± 2.4	48.2 ± 1.8	(15)	31.4 ± 2.3	40.0 ± 3.0	(27)	2.7 ± 0.3	1.6 ± 0.2	(41)
VJM 531	41.2 ± 3.0	43.3 ± 2.1	(5)	35.0 ± 2.4	40.0 ± 2.4	(33)	3.0 ± 0.1	2.1 ± 0.1	(30)
BH 393	42.3 ± 1.8	48.2 ± 2.2	(14)	26.7 ± 2.1	36.1 ± 1.6	(9)	3.1 ± 0.2	2.2 ± 0.5	(29)
RD 2668	45.7 ± 2.4	47.5 ± 2.4	(4)	26.7 ± 2.6	32.2 ± 2.3	(27)	2.8 ± 0.2	1.7 ± 0.1	(39)
DWRUB 52	44.6 ± 2.3	51.7 ± 2.9	(16)	35.0 ± 1.9	40.0 ± 2.1	(14)	2.7 ± 0.1	1.6 ± 0.5	(41)
BK 201	40.0 ± 2.1	43.3 ± 2.1	(8)	26.7 ± 1.6	32.8 ± 1.5	(23)	3.0 ± 0.4	2.1 ± 0.3	(30)
BK 203	43.3 ± 1.6	45.7 ± 1.3	(6)	27.4 ± 2.2	32.2 ± 1.8	(18)	2.4 ± 0.3	1.8 ± 0.1	(25)
PL 796	42.7 ± 1.3	49.0 ± 2.4	(15)	31.4 ± 3.1	37.8 ± 2.0	(20)	3.0 ± 0.1	2.2 ± 0.2	(27)
BL 16	42.3 ± 1.8	44.2 ± 2.3	(5)	26.1 ± 2.2	34.3 ± 2.2	(31)	3.0 ± 0.1	2.1 ± 0.1	(30)
BL 48	43.3 ± 1.5	45.7 ± 2.9	(6)	31.4 ± 2.8	37.8 ± 1.3	(20)	2.6 ± 0.4	2.1 ± 0.2	(19)
BL 53	41.2 ± 2.9	44.3 ± 2.1	(8)	35.0 ± 2.3	40.0 ± 2.4	(14)	3.1 ± 0.3	2.2 ± 0.1	(29)
BL 71	43.3 ± 2.0	46.7 ± 2.9	(8)	26.7 ± 4.3	35.0 ± 3.0	(31)	2.7 ± 0.1	1.8 ± 0.2	(33)
BL 73	45.7 ± 2.4	47.5 ± 3.0	(4)	31.4 ± 2.8	37.8 ± 1.8	(20)	3.1 ± 0.2	2.1 ± 0.2	(32)
K 551	41.2 ± 3.3	48.2 ± 1.5	(17)	35.0 ± 1.8	41.8 ± 2.9	(19)	2.8 ± 0.2	2.2 ± 0.4	(21)
RD 2503	44.6 ± 1.7	51.7 ± 1.8	(16)	35.0 ± 2.4	40.0 ± 2.4	(14)	2.3 ± 0.1	1.8 ± 0.1	(22)
CD (P≤0.05)	Treatment:		1.38			1.56			0.14
	Genotypes:		4.35			4.93			0.43
	T × G:		NS			NS			NS

EL. The electrical conductivity of the leachate from heat shocked seedlings was significantly higher than control seedlings (**Table 2**). Shoots had relatively higher EL than roots in all genotypes except VJM 201, BH 393, DWRUB 52 and RD 2503. In response to HS, maximum percent increase in EL of root was observed for genotypes VJM 540, DWR 28, DWRUB 52, K 551 and RD 2503, while VJM 318<RD 2668, BL 73<BL 61 had minimum percent increase in EL among all the genotypes. Maximum increases in shoot EL was in case of DWR 28>BL 71>VJM 540 while VJM 318<BL 61<BH 313 and VJM 201 showed minimum increase in EL.

HT treatment resulted in reduction of TTC cell viability in all the genotypes (**Table 2**). Although genotypes VJM 540>VJM 514, DWRUB 52>RD 2668 were relatively more affected by HS, as indicated by greater percent reduction in TTC assay, VJM 318, BL 61, BL 48, K 551 and RD 2503 were able to minimize the adverse effect of HT by minimizing the reduction in TTC cell viability (**Table 2**).

Genotypic differences were evident with respect to the above parameters under the influence of HT treatment. Accordingly, genotypes VJM 540, DWRUB 52, DWR 28 and genotypes VJM 318 and BL 61 were classified as susceptible and tolerant to HT, respectively. Among this group, only genotypes VJM 540 and VJM 318 behaved contrastingly for all the studied parameters. To authenticate the aforesaid parameters, these two contrasting genotypes were selected to further analyze their thermal responsiveness.

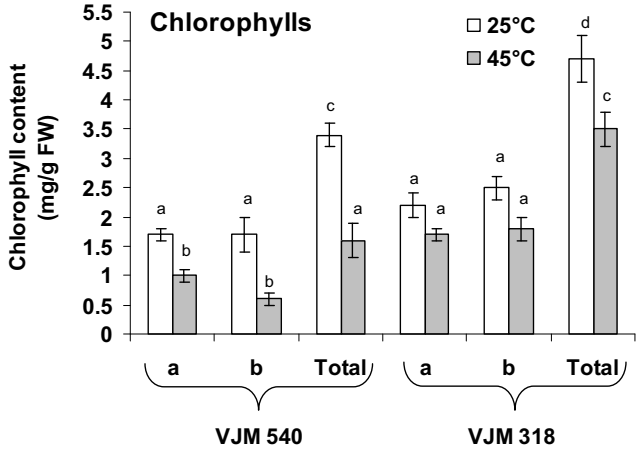

Fig. 1 Effect of heat shock on chlorophyll contents of barley seedling. Values with same letter do not differ significantly (P≤0.05, Student's *t*-test).

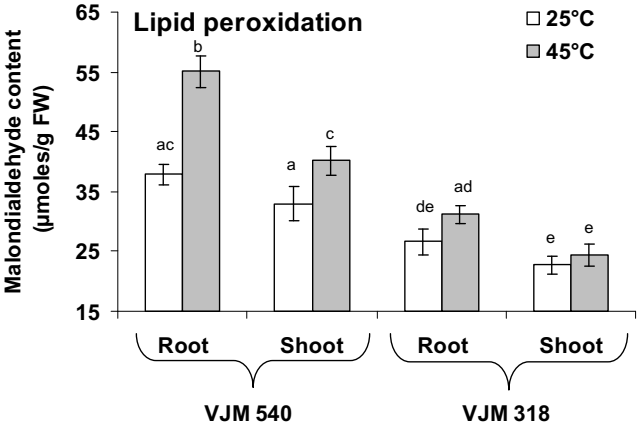

Fig. 2 Effect of heat shock on lipid peroxidation of barley seedling. Values with same letter do not differ significantly (P≤0.05, Student's t-test).

HT resulted in reduction of Chl contents of shoots in both genotypes (**Fig. 1**). Genotype VJM 318 showed 25% reduction while 53% reduction in total Chl contents was observed for VJM 540 under HT. Chl *a* and Chl *b* had similar trend as that of total Chl.

MDA is a final product of peroxidation of unsaturated fatty acids in phospholipids that is often used as a measure of level of LPO (Halliwell and Gutteridge 1989). The MDA contents of roots and shoots were significantly higher in heat-shocked seedling than in control seedling for both genotypes (**Fig. 2**). The increases in MDA content under HS were more pronounced in roots compared to shoots. During HS, significantly higher increase in MDA content was observed in genotype VJM 540 for both root (45%) and shoot (22%) as compared to root (17%) and shoot (8%) of genotype VJM 318.

DISCUSSION

Drastic effects of HT on plant productivity are due to its interference with normal plant development and the restraint of plant growth. The results for different parameters of twenty genotypes presented here clearly show the variable response of these genotypes to HT.

Germination starts by the passive process of water imbibition, swelling of existing tissue, and rupture of the seed coat, followed by cell division and tissue elongation in the embryo. The decline in dry mass of root and shoot in response to HS may be attributed to altered metabolism of germinating seeds which affected the supply of nutritional reserves from the cotyledons to the developing root/shoot. The maximum reduction in dry mass accumulation in response to HS treatments in genotypes VJM 540, DWR 28, DWRUB 52 and RD 2503 indicates their susceptibility to HT compared to other genotypes. However, genotypes VJM 318, BL 53, BH 393, BL 61 and BL 16 with least reductions, hint at their relatively thermotoleranat nature. Genotypes with high biomass have been reported to sustain better growth and development after release of stress (Srivastava *et al*. 2007). In our study a significant positive correlation (r=0.46; P≤0.05) was observed between percent reduction in root dry mass and percent increase in EL (**Table 3**). The same correlation was less strong, though still significant (r=0.37; P≤0.12) for shoot dry mass and EL. The positive correlation indicates that the genotypes with lesser HS induced reduction in dry mass were also registered lesser increase in their EL and maintained their membrane stability. Marcum (1998) noted a positive relationship between membrane thermal stability (MTS) and shoot dry weight for Kentucky bluegrass cultivars, and regarded it as a reliable screening tool for HT response.

Membranes are highly sensitive to alterations caused by temperature. Exposure of germinating seedling to HT leads to leakage of cell solutes. EL indicates cell MTS and is used widely as an indicator of stress injury (Fokar *et al.* 1998). The genotypes VJM 318, RD 2668, BL 73, BL 61, BH 313 and VJM 201 tolerated the adverse effect of HS by maintaining their membrane integrity as reflected by lesser increase in their HS-induced EL. However, the relatively higher increase of EL in VJM 540, DWR 28, DWRUB 52, K 551, RD 2503 and BL 71 indicated that membrane system of these genotypes was damaged to a greater extent by HS. The regulation of membrane injury has earlier been reported to be a good index of HT tolerance (Ibrahim and Quick 2001; Madhava Rao *et al*. 2002). Reynolds *et al.* (1994) compared MTS for seedlings versus field-grown flag leaves and found a significant positive correlation. They suggested that the MTS determined at the two developmental stages was well associated in wheat. Saadalla *et al.* (1990a) concluded that conducting a MTS assay with 4-cm long germinated seedlings was appropriate for assaying heat tolerance of wheat and that assays in germinating seedlings predicted the thermotolerance of the fully developed plant. This indicated that the screening procedure for MTS using seedlings raised under artificial conditions may be a viable alternative to the use of field-grown tissue. The use of seedlings is favorable logistically since the conditions of plant acclimation can be controlled, while this is not possible in the field. Another advantage of using seedlings over more mature tissue is that MTS is unlikely to be affected by phenology at such an early stage of development (Reynolds *et al.* 1994).

Metabolic activity measured as TTC cell viability indicates respiratory activity which gets reduced during HT as reported by Porter *et al.* (1995) in wheat. Greater percent reduction in TTC cell viability of genotypes VJM 540, VJM 514, DWRUB 52 and RD 2668 suggests relatively higher thermal susceptibility of mitochondrial system of these genotypes. TTC assay was earlier found to be an efficient technique for quantification of HT tolerance in wheat (Porter *et al.* 1995; Dhanda and Munjal 2006). Reduction in respiratory activity indicates less formation of ATP and carbon skelton for various biosynthetic activities and other processes are expected to be controlled leading to inhibition of seedling growth (Kittock and Law 1968). This is evident since genotype VJM 540 which exhibited highest percent reduction in TTC assay, also accumulated minimum dry mass of root and shoot under HT (**Table 1**). Correlation analysis also indicated a highly significant (P≤0.01) and positive correlation (r=0.67) between percent reductions in dry mass and TTC (**Table 3**).

Thylakoid membranes and photosystem II (PS II) are considered the most heat-labile cell structures. In cereals, for example, thylakoids are more affected than the chloroplast envelope, stromal enzymes, or the integrity of cell compartments. Thylakoids harbor Chl, a portion of which is associated with the proteins of PS II (Vacha *et al.* 2007).

Table 3 Correlation coefficients between heat shock induced percent changes in various characters in twenty barley genotypes

Character	Root				Shoot		
	Germination	Dry mass	Electrolyte leakage	TTC	Germination	Dry mass	Electrolyte leakage
Germination	1.00	0.24	0.05	0.09	1.00	0.20	0.36*
Dry Mass		1.00	0.46**	0.67***		1.00	0.37*
Electrolyte leakage			1.00	0.26			1.00
TTC				1.00			

*, **, ***Significant at P≤0.12, P≤0.05and P≤0.01 respectively.

Damage to thylakoids caused by HT could be, therefore, expected to lead to Chl loss. Indeed, heat-induced damage to thylakoid membranes and Chl loss have been observed in many crop plants (Ristic *et al*. 2007). Genotype VJM 540, identified as susceptible to HT, exhibited maximum reduction in Chl contents under HS conditions, as compared to tolerant genotype VJM 318. Genotype VJM 540, when compared to genotype VJM 318, also exhibited 2.6-fold higher reductions in shoot dry mass (**Table 1**). It is therefore evident that reduced photosynthetic activity in terms of decreased Chl contents leads to reduced dry mass accumulation under HT conditions, and these parameters are affected most in susceptible genotype. Similar observation has been reported in maize by Srivastava *et al*. (2007). Chl loss naturally occurs in plants undergoing senescence and it can also prematurely occur in plants experiencing HT (Al-Khatib and Paulsen 1984). In our case, control seedlings did not show any significant changes in Chl content or senescence. Therefore, it is likely that the Chl loss in heat-shocked seedling was primarily due to the effects of HT rather than natural senescence. The mechanism by which HT may have caused Chl loss is, however, unclear. Al-Khatib and Paulsen (1984) and Ristic *et al.* (2007) have suggested that a major effect of HT is acceleration of senescence, which is manifested by an increase in the activity of proteolytic enzymes leading to protein degradation and Chl loss. We speculate that this may be the case in our study. Alternatively, Chl loss may be a consequence of HS-induced damage to thylakoid membranes and PS II. Further studies are needed to elucidate the mechanisms of chlorophyll loss in barley under HT conditions.

Exposure of plants to HT often results in production of ROS (Almeselmani *et al*. 2006). Although plants have developed enzymatic and non enzymatic scavenging systems to quench ROS, however under HT, the scavenging system may lose its function and results in accumulation of hydroxyl-free radicals via Herbert-Weiss reaction (Bowler 1992). The hydroxyl-free radicals can directly attack unsaturated fatty acids of lipid to induce TBARS in the cell. The level of LPO, measured as MDA, has been used as an indicator of free radical damage to cell membranes under stress conditions (Huang *et al.* 2001). An increase in MDA contents in response to HS suggests that LPO has occurred leading to degradation of phospholipids. The higher MDA content of VJM 540 as compared to VJM 318, under HS indicates that VJM 318 was better able to curtail LPO. Huang *et al.* (2001) reported that HT caused severe oxidative damage by limiting antioxidant activities and inducing LPO in creeping bent grass. They suggested that low level of LPO was related to the better tolerance to HT. Enhanced LPO under HT may cause greater destabilization of membranes leading to greater solute leakage and hence, elevation of HT susceptibility. In other independent experiments, performance of genotypes VJM 540 and VJM 318 in terms of thermal responsiveness of their carbohydrate metabolism at germination (Singh S and Asthir B, unpublished data) and at reproductive stages (Singh and Asthir 2008) was clearly contrasting, which further strengthened the utilization of aforesaid tools for screening HT tolerant germplasm.

CONCLUSION

The present investigation indicates that HT-tolerant genotypes exhibit lesser reductions in, germination, dry mass accumulation, TTC cell viability and chlorophyll contents under HS conditions. Susceptible genotypes are more prone to HT induced oxidative damage as indicated by markedly higher MDA levels. These biochemical and physiological tools may be taken to screen HT tolerant genotypes of barley which, in turn, can be used as a source of candidate genes to facilitate the breeding programs aimed at development of thermotolerant germplasm.

REFERENCES

Al-Khatib K, Paulsen GM (1984) Mode of high temperature injury to wheat during grain development. *Physiologia Plantarum* **6**, 363-368

Almeselmani M, Deshmukh PS, Sairam RK, Kushwaha SR, Singh TP (2006) Protective role of antioxidant enzymes under high temperature stress. *Plant Science* **171**, 382-388

Arnon DI (1949) Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. *Plant Physiology* **24**, 1-15

Basra RK, Basra AK (2001) Phenolics biosynthesis inhibitors accentuate the effects of heat shock injury in mung bean seedlings: alleviation by salicylic acid. *Journal of New Seeds* **3**, 41-51

Boamah-Asare NK, Fletcher AR (1983) Physiological and cytological effects of BAS 9052 OH on corn (*Zea mays*) seedlings. *Weed Science* **31**, 49-55

Bowler C, Van Montagu M, Inzé D (1992) Superoxide dismutase and stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**, 83-116

Dekov I, Tsonev T, Yordanov I (2000) Effects of water stress and high temperature stress on the structure and activity of photosynthetic apparatus of *Zea mays* and *Helianthus annuus*. *Photosynthetica* **38**, 361-366

Dhanda SS, Munjal R (2006) Inheritance of cellular thermotolerance in bread wheat. *Plant Breeding* **125**, 557-564

Fokar M, Nguyen HT, Blum A (1998) Heat tolerance in spring wheat. I. Estimating cellular thermotolerance and its heritability. *Euphytica* **104**, 1-8

Howarth CJ (2005) Genetic improvements of tolerance to high temperature. In: Ashraf M, Harris PJC (Eds) *Abiotic Stresses: Plant Resistance through Breeding and Molecular Approaches*, Haworth Press Inc., New York, pp 277-300

Huang B, Liu X, Xu Q (2001) Supraoptimal soil temperatures induced oxidative stress in leaves of creeping bentgrass cultivars differing in heat tolerance. *Crop Science* **41**, 430-435

Ibrahim AMH, Quick JS (2001) Genetic control of high temperature tolerance in wheat as measured by membrane thermal stability. *Crop Science* **41**, 1405-1407

Jiang Y, Haung B (2001) Effects of calcium on antioxidant activities and water relations associated with heat tolerance in two cool-season grasses. *Journal of Experimental Botany* **52**, 341-349

Jones PD, New M, Parker DE, Mortin S, Rigor IG (1999) Surface area temperature and its change over the past 150 years. *Reviews of Geophysics* **37**, 173-199

Kittock DL, Law AG (1968) Relationship of seedling vigor, respiration and tetrazolium chloride reduction by germinating wheat seedlings. *Agronomy Journal* **60**, 286-288

Larkindale J, Knight MR (2002) Protection against heat stress-induced oxidative damage in Arabidopsis involves calcium, abscisic acid, ethylene, and salicylic acid. *Plant Physiology* **128**, 682-695

Liu X, Huang B (2000) Heat stress injury in relation to membrane lipid peroxidation in creeping bent grass. *Crop Science* **40**, 503-510

Madhava Rao KV, Sridevi V, Satyanarayana NV (2002) Heat shock induced lipid changes and solute leakage in germinating seeds of pigeonpea. *Biologia Plantarum* **45**, 71-76

Marcum KB (1998) Cell membrane thermostability and whole plant heat tolerance of Kentucky bluegrass. *Crop Science* **38**, 1214-1218

Porter DR, Nguyen HT, Burke JJ (1995) Genetic control of acquired high temperature tolerance in winter wheat. *Euphytica* **81**, 153-157

Reynolds MP, Balota M, Delgado MIB, Amani MIB, Fisher RA (1994) Physiological and morphological traits associated with spring wheat yield under hot irrigated conditions. *Australian Journal of Plant Physiology* **21**, 717-730

Ristic Z, Bukovnik U, Vara Prasad PV (2007) Correlation between heat stability of thylakoid membranes and loss of chlorophyll in winter wheat under

heat stress. *Crop Science* **47**, 2067-2073

Sadalla MM, Shanahan JF, Quick JS (1990a) Heat tolerance in winter wheat: I. Hardening and genetic effects on membrane thermostability. *Crop Science* **30**, 1243-1247

Saadalla MM, Shanahan JE, Quick JS (1990b) Heat tolerance in winter wheat: II. Membrane thermostability and field performance. *Crop Science* **30**, 1248-1251

Schoffl F, Prandl R, Reindl A (1999) Molecular responses to heat stress. In: Shinozaki K, Yamaguchi-Shinozaki K (Eds) *Molecular Responses to Cold, Drought, Heat and Salt Stress in Higher Plants*, RG Landes Co, Austin, Texas, pp 81-98

Singh S, Asthir B (2009) Interactive effects of high temperature and phyothormones on carbohydrate metabolism in barley seedlings. *Seed Science and Biotechnology* **3**, 44-47

Singh S, Asthir B, Bains NS (2008) High temperature tolerance in relation to carbohydrate metabolism in barley. *Ecology, Environment and Conservation* **14**, 561-565

Srivastava JP, Gangey SK, Shahi JP (2007) Waterlogging resistance in maize in relation to growth, mineral compositions and some biochemical parameters. *Indian Journal of Plant Physiology* **12**, 28-33

Vacha F, Adamec F, Valenta J, Vacha M (2007) Spatial location of photosystem pigment-protein complexes in thylakoid membranes of chloroplasts of *Pisum sativum* studied by chlorophyll fluorescence. *Journal of Luminescence* **122-123**, 301-303

Wadlaw IF, Blumenthal C, Larroque O, Wrigley C (2002) Contrasting effects of heat stress and heat shock on kernel weight and flour quality in wheat. *Functional Plant Biology* **29**, 25-34

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: An overview. *Environmental and Experimental Botany* **61**, 199-223