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Wheat cultivars differing in heat tolerance show a differential response to monocarpic senescence under high-temperature stress and the involvement of serine proteases

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Abstract

High temperature is a common constraint during anthesis and grain-filling stages of wheat leading to huge losses in yield. In order to understand the mechanism of heat tolerance during monocarpic senescence, the present study was carried out under field conditions by allowing two well characterized *Triticum aestivum* L. cultivars differing in heat tolerance, Hindi62 (heat-tolerant) and PBW343 (heat-susceptible), to suffer maximum heat stress under late sown conditions. Senescence was characterized by measuring photosynthesis related processes and endoproteolytic activity during non-stress environment (NSE) as well as heat-stress environment (HSE). There was a faster rate of senescence under HSE in both the genotypes. Hindi62, having pale yellow flag leaf with larger area, maintained cooler canopy under high temperatures than PBW343. The tolerance for high temperature in Hindi62 was clearly evident in terms of slower green-leaf area degradation, higher stomatal conductance, higher stability in maximum PSII efficiency, Rubisco activity and Rubisco content than PBW343. Both the genotypes exhibited lower endopeptidase activity under HSE as compared to NSE and this difference was more apparent in Hindi62. Serine proteases are the predominant proteases responsible for protein degradation under NSE as well as HSE. Flag leaf of both the genotypes exhibited high-molecular-mass endoproteases (78 kDa and 67 kDa) isoforms up to full grain maturity which were inhibited by specific serine protease inhibitor in both the environments. In conclusion, the heat-tolerant Hindi62 exhibited a slower rate of senescence than the heat-susceptible PBW343 during HSE, which may contribute towards heat stability.

Additional key words: heat stress; photosynthesis; Rubisco; senescence; serine protease; wheat.

Introduction

High temperature is a common constraint during anthesis and grain-filling stages of wheat across different parts of the world including India (Reynolds *et al.* 1994, Sinha *et al.* 1998). Wheat is particularly susceptible to yield losses as a result of high-temperature-induced heat stress affecting grain mass and grain number (Ferris *et al.* 1998; Mullarkey and Jones 2000) leading to almost 75% loss in the yield (Zhong-Hu and Rajaram 1993). In the moderately high-temperature range, wheat yield declined approximately by 3 to 4% for each 1°C rise in the average temperature above 15°C under controlled as well as field conditions (Wardlaw and Wrigley 1994). Reduction in grain yield under heat stress is due to reduction in both the source availability and the sink realization (Viswanathan and Khanna-Chopra 1996).

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Abbreviations: DAA – days after anthesis; DTT – dithiothreitol; g_s – stomatal conductance; F_0 – initial fluorescence; F_v/F_m – maximum efficiency of PSII; F_m – maximum chlorophyll fluorescence of the dark adapted state; F_m' – maximum chlorophyll fluorescence of the light adapted state; FM – fresh mass; HSE – heat-stress environment; LSU – large subunit; PMSF – phenylmethylsulfonyl fluoride; NSE – non-stress environment; PMSF – phenylmethylsulphonylfluoride; PPFD – photosynthetic photon flux density; P_N – net photosynthetic rate; PSII – photosystem II; PVP – polyvinyl pyrrolidone; PVPP – polyvinyl polypyrrolidone; q_N – non-photochemical quenching; RuBP – ribulose-1,5-bisphosphate; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; SDS-PAGE – sodium dodecyl sulfate - polyacrylamide gel electrophoresis; T_{max} – maximum temperature; T_{mean} – mean temperature; T_{min} – minimum temperature; TCA – trichloroacetic acid.
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from major wheat-growing regions in the developing world identified heat stress as one of their top research priorities (CIMMYT 1995). Late sowing of wheat is necessitated in some regions because of multiple cropping systems. For example, rice-wheat cropping system in North India and soybean-potato-wheat system in Central India has compelled wheat crop to be subjected to rapidly ascending temperatures coupled with hot dry winds during the post-anthesis stage, especially during grain development. Perhaps the greatest challenge to understanding the physiological problems associated with heat stress is to encompass the diversity of hot environments all over the world (Reynolds *et al.* 2000). Growing food demand and global warming would further push the wheat crop to heat-stress environments. Rising temperatures may also lead to altered growing season of agricultural crops by allowing the threshold temperature for the start of the season and crop maturity to reach earlier (Porter 2005).

Monocarpic senescence in crop plants is a deteriorative process at the cellular, tissue, organ or whole-plant level, especially in leaves, which occurs along with reproductive development leading ultimately to the death of the plant (Nooden 1988). Leaf senescence is an integrated response of leaf cells to age and other internal and environmental signals which provides the plants with optimal fitness (Lim *et al.* 2007). The generic nature of senescence across the plant kingdom has been validated *via* several transcriptomic (Gregersen and Holm 2007) and proteomic studies (Hebeler *et al.* 2008). The metabolic changes occurring during monocarpic senescence in wheat are further hastened by high temperature (Al-Khatib and Paulsen 1984) as plants tend to divert resources to cope with the heat stress and thus limited photosynthates would be available for reproductive development. Several components of the photosynthetic apparatus and associated metabolic processes are heat-labile such as PSII and overall, photosynthesis is the physiological process considered to be most sensitive to heat stress (Wahid *et al.* 2007).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) is an important and major source of nitrogen for developing grains in wheat leaf (Peoples and Dalling 1988). An understanding of the mechanism of breakdown of Rubisco during high-temperature-induced monocarpic senescence is required to establish an optimal mobilization of leaf nitrogen to developing grains through enhanced endoproteolytic activities (Benbella and Paulsen 1998). Several studies have been undertaken to find out the type of major endoproteases involved during senescence in wheat. Some studies have reported that cysteine proteases play a major role during post-anthesis and stress-induced senescence such as drought and darkness (Mae *et al.* 1989; Martínez *et al.* 2007), while others reported that serine proteases are the major enzymes responsible for Rubisco degradation during monocarpic senescence in wheat (Roberts *et al.* 2003).

Screening of suitable germplasm and breeding for heat tolerance is an important objective of wheat improvement programme. Senescence is usually studied in some model systems, in which leaf senescence is induced by either incubating detached leaves or whole plants in the dark. Most of the studies on heat stress have also been conducted in pots or under controlled environments (Zhao *et al.* 2007), so its direct relationship with field or natural stress conditions may not be easy to interpret (Smart 1994).

In order to understand the mechanism of heat tolerance, it is necessary to study the physiological responses of tolerant and susceptible cultivars to heat stress at various stages of development, especially during grain development. The present investigation was carried out under field conditions by allowing the wheat crop to suffer maximum heat stress under late-sown conditions. For this purpose, two well characterized bread wheat cultivars differing in heat tolerance were chosen for studying the impact of heat stress on the mechanism of senescence including various photosynthesis-related processes and the type of endopeptidases involved in protein degradation during monocarpy.

Materials and methods

Plant material and sampling: Two bread wheat (*Triticum aestivum* L.) genotypes Hindi62 (heat-tolerant) and PBW343 (heat-susceptible) were selected on the basis of their heat susceptibility index for yield and yield components under variable temperature environments and across normal and late-sown conditions, Patil *et al.* 2008. All experiments were conducted in the fields of Water Technology Centre, Indian Agricultural Research Institute, New Delhi, India (77°12'E; 28°40'N; 228.6 m a.s.l.). Wheat genotypes were sown in the field on two sowing dates, mid November for normal sowing and mid January for late sowing during the winter season. The November-sown crop environment was considered as non-stress environment (NSE) and January-sown crop environment was considered as heat-stress environment (HSE). The size of plot was 2 × 2 m and the plant-plant and row-row distance was 10 and 25 cm, respectively. Fertilizer was used at the rate of 100:50:50 kg ha^{-1} of N:P:K as a single dose. The plants were allowed to grow under natural field conditions with irrigation. Each genotype was replicated three times in a randomized complete block design in each sowing. The main shoots were tagged in each genotype at the time of anthesis. Flag leaf of the main shoot was taken for analysis. Sampling was done at 7-d and 5-d intervals in NSE plants and HSE plants, respectively, from anthesis to maturity. For the

biochemical assays, leaves were cut into small pieces after weighing. Three replicates were used for all measurements.

Weather data from sowing to harvesting of crop for all three years were collected from the meteorological station, Water Technology Centre, IARI, New Delhi. Weather data included maximum (T_{max}) and minimum (T_{min}) temperatures, relative humidity (RH), rainfall and evapotranspiration (ET).

Green flag-leaf area was determined by using non-destructive method of Aggarwal and Sinha (1987). For measuring the green leaf area, length and maximum breadth of the leaves were measured on sampling dates. Leaf area was calculated based on the formula leaf area = length × maximum breadth × 0.7.

Chlorophyll (Chl) and carotenoid (Car) contents were measured according to Lichtenthaler (1987).

Total soluble proteins were measured according to Lowry *et al*. (1951).

Net photosynthetic rate (P_N) and stomatal conductance (g_s) were measured in wheat flag leaves from anthesis to maturity under normal as well as high-temperature conditions in the morning between 10:00 to 11:00 using *LI-COR-6200* portable photosynthesis instrument (*LI-COR Inc*., Lincoln, NE, USA). The measurements were done on the adaxial surface of the flag leaf in the field under saturating photosynthetic photon flux density (PPFD) 1,200–1,500 μmol m^{-2} s^{-1}, CO_2 concentration ~ 380 ppm, air temperature 27–32°C (NSE), 33–35°C (HSE) and air humidity 50± 5% (NSE), 40± 5% (HSE) between anthesis and maturity. During the measurements relative humidity of air entering the *LI-COR* chamber was set to match ambient humidity with a ± 3% tolerance by varying the air flow rate.

Chl fluorescence parameters were measured in the flag leaves (adaxial surface) in the field using a portable fluorometer (*MINI-PAM, Heinz Walz GmbH*, Effeltrich, Germany). The fluorimeter employees 0.3-s pulses of light-emitting diode with the peak emission at 650 nm. Fluorescence is detected at wavelenghts above 710 nm. Heat-filtered "white light" from a halogen lamp serves for actinic irradiation and saturation pulses. Prior to measurement, the leaves were dark-adapted for 10 min, with the help of leaf clips (provided with the instrument). The ratio of F_v/F_m, termed as maximum efficiency of PSII photochemistry and nonphotochemical quenching (q_N) were assessed following Ferreira *et al*. (2007) with some modifications. The procedure was as follows: The initial fluorescence (F_0) obtained with modulated low irradiance (<0.1 μmol m^{-2} s^{-1}), maximum fluorescence (F_m) determined with a pulse of saturating irradiance (3000 μmol m^{-2} s^{-1}) with duration of 0.3 s, and maximum quantum yield of PSII; $F_v/F_m = (F_m - F_0)/F_m$. Hence, these parameters were used for obtaining the nonphotochemical quenching: $q_N = (F_m - F_m')/(F_m - F_0)$. These quenching coefficients were automatically calculated by *MINI-PAM* q_N was measured in response to "actinic light" 589 μmol m^{-2} s^{-1} of 20 s duration following a pulse of saturating radiation (3000 μmol m^{-2} s^{-1}) for 0.3 s. Before the measurements with actinic radiation, one measurement was performed in the dark (Schreiber *et al*. 1994).

Rubisco activity was estimated by RuBP-dependent incorporation of $^{14}CO_2$ into acid-soluble products using the procedure of Jiang *et al*. (1993) with some modifications. Frozen leaf samples [0.25 g(FM)] were ground in a mortar and pestle with liquid nitrogen and homogenized in 1.5 ml of 50 mM Bicine-KOH buffer, pH 7.8, containing 20 mM $MgCl_2$, 5 mM DTT, 0.1 mM Na-EDTA, and 0.1 mM PMSF. PVP [2 % (w/v)] was added at the time of grinding. The homogenates were centrifuged at 10 000 × *g*, 4°C for 2 min. Ten microlitres of the supernatant were incubated with 480 μl of 100 mM Bicine-KOH buffer, pH 8.2 containing 20 mM $MgCl_2$, 5 mM DTT and 250 mM $NaH^{14}CO_3$ (specific activity 1.839 GBq mol^{-1}), at 25°C for 9 min after which 10 μl of 20 mM RuBP was added. The reaction was stopped after 1 min by adding 200 μl of 3 N HCl and dried at 80°C. The acid stable ^{14}C was estimated by liquid scintillation counter (*Wallac 1409, Pharmacia*, Uppsala, Sweden).

Preparation of samples for Rubisco large subunit (LSU) quantification: Leaf samples frozen in liquid nitrogen were ground in a mortar and a pestle with liquid nitrogen and then extracted [3 ml per 0.25 g(FM)] in 30 mM Tris buffer, pH 7.8, containing 1 mM ascorbic acid, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF (Zivy *et al*. 1983). PVPP [4% (w/w)] was added at the time of grinding. The extracted samples were passed through four layers of cheesecloth and centrifuged at 10 000 × *g* for 20 min. To the supernatant, eight volumes of acetone were added and the protein precipitated overnight. The samples were again centrifuged at 10 000 × *g* for 20 min. To the pellet, electrophoresis sample solution [125 mM Tris-HCl buffer, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol (β-ME), and 2% SDS] was added and boiled for 4 min. Aliquots of the protein samples were subjected to electrophoresis on a 10% SDS-PAGE (Laemmli 1970). In all cases, 10 μg of total soluble protein was loaded.

Western blot analysis of Rubisco large subunit (LSU) was done according to Towbin *et al*. (1979). After electrophoresis, the separated polypeptides were electro-transferred at 4°C onto a nitrocellulose membrane (0.45 micron, *Bio-Rad*, Richmond, CA, USA) at 50 V for 1 h in transfer buffer using a transblot unit (*Bio-Rad,* Richmond, CA, USA). The membrane was probed with 1:2 000

dilution of polyclonal antibodies against Rubisco LSU. The antibodies were prepared as reported earlier (Srivalli *et al.* 2001). The immunoreactive protein bands were visualized using anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate antiserum in 1:20 000 dilution with 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT) as substrate. Rubisco LSU was quantified by scanning the blots using a gel documentation system (*GelDocMega system*, *Bio-systematica,* Wales, UK).

Endopeptidase activity was measured following the modified version of Peoples *et al*. (1983). Samples (0.5 g) frozen in liquid nitrogen were ground using a mortar and pestle in liquid nitrogen and after that suspended in 2 ml of 250 mM Tris-HCl buffer, pH 7.0, containing 10 mM ß-ME. PVP [2% (w/v)] was added during homogenization and the extract was centrifuged at 10 000 × *g*, 4°C for 20 min. The supernatant was collected and passed through three layers of cheesecloth. The supernatant was dialysed overnight against 25 mM Tris-HCl buffer, pH 7.0, containing 10 mM ß-ME. The reaction mixture contained 50 µl of crude extract, 125 µl of 250 mM sodium acetate buffer, pH 4.8, containing 10 mM ß-ME and 75 µl of Rubisco [0.45% (w/v)] (*Sigma-Aldrich*, Delhi, India) as a substrate. After incubation at 50ºC for 1h, the reaction was stopped by adding 1 ml of 10% TCA solution and incubated at 4ºC for 1 h. After centrifugation at 25,000 × *g* for 10 min, the absorbance by TCA-soluble peptides generated during the reaction were estimated of the supernatants at 340 nm using a spectrophotometer (*Lambda 2S UV/VIS spectrophotometer*, *Perkin Elmer*, Massachusetts, USA). Controls were kept for zero time, *i.e*. the reaction was stopped immediately without further incubation and without substrate. One unit of proteolytic activity in 1 cm cuvette was defined as an increment of 0.01 in A_{340} in 1 h. For inhibitor studies, the reaction mixture containing 50 µl of the crude extract was pre-incubated for 10 min with one of the following inhibitors - 3.8 µl of 2 mM PMSF, 18.8 µl of 10 mM EDTA, 9.4 µl of 25 µM DL-norleucine and 3.8 µl of 10 mM iodoacetamide for serine-, metallo-, aspartate-, and cysteine proteases, respectively. The remaining procedure was the same as for the endopeptidase activity. All the inhibitors were dissolved in water except PMSF which was dissolved in isopropanol.

Protease activity profiling based on the gel assay was done using the procedure of Ye and Varner (1996) with some modifications. Frozen leaf tissue (0.2 g) was homogenized in 1.5 ml of 50 mM Tris-HCl (pH 7.5) containing 10 mM of β-ME and centrifuged at 12 000 × *g*, 4°C for 15 min. The supernatant was collected and the total soluble proteins were estimated (Lowry *et al.* 1951). 10 µg of total soluble protein was loaded on a 5.0–12.5% polyacrylamide gradient gel containing 0.1% (w/v) gelatin. The extracts were mixed with an equal volume of electrophoresis sample solution (as mentioned for Rubisco LSU content) and incubated at room temperature for 10 min. After electrophoresis, the gel was incubated in renaturing buffer containing 2.5% Triton X-100 at room temperature for 1 h which helped to keep the proteins in their active state. The gel was then incubated in the developing buffer containing 50 mM sodium citrate, pH 5.0, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 10 mM β-ME and 0.05% Brij-35 (polyoxyethyleneglycol dodecylether), a non-ionic detergent, at 37°C overnight. The gel was stained with 0.5% Coomassie Brilliant Blue R-250 staining solution and destained with a methanol (40%) and glacial acetic acid (10%). Protease activity was detected on a dark-blue background as colourless bands. Molecular masses of the endoproteases were estimated by SDS-PAGE under the same conditions by using broad range molecular markers (29.0–205 kDa, *Sigma-Aldrich*, Delhi, India). Identification of the different classes of endopeptidases was done using class-specific proteinase inhibitors. The protease extracts were incubated on ice for 30 min in the presence of each of the following inhibitors, 2 mM PMSF, 10 mM iodoacetamide, 10 mM EDTA and 1 mM pepstatin A for serine-, cysteine-, metallo- and aspartate proteases before electrophoresis.

Results

Average T_{mean} for the genotypes was 2.9ºC and 5.8ºC higher in HSE than NSE during the pre- and post-anthesis phase, respectively (Table 1). The differences between sowings were higher in the mean maximum temperatures (average of daily maximum temperatures) than in the mean minimum temperatures (average of daily minimum temperatures). Due to regular irrigation, crop canopies always maintained the temperatures lower than the ambient ones, with Hindi62 having lower canopy temperatures than PBW343. The average canopy temperature depression during the post anthesis phase for Hindi62 was 6.6 ± 0.4ºC at NSE and 7.0 ± 0.6ºC at HSE, and for PBW343, it was 5.7 ± 0.3ºC at NSE and 6.2 ± 0.5ºC at HSE.

The two genotypes showed very little difference in their days to anthesis during both NSE which was 107 ± 0.6 for Hindi62 and 105 ± 0.3 for PBW343, and HSE which was 75 ± 0.6 for Hindi62 and 74 ± 0.3 for PBW343. Crop growth duration (CGD) or days to maturity were reduced drastically under HSE (100 ± 1.2 for Hindi62 and 97 ± 1.0 for PBW343) than NSE (144 ± 1.2 for Hindi62 and 137 ± 0.6 for PBW343). However, the magnitude of difference between the genotypes remained the same.

Table 1. Average maximum (T_{max}), minimum (T_{min}) and mean (T_{mean}) temperatures during crop growth duration of wheat genotypes under normal sown environment (NSE) and heat-stress environment (HSE). Means ± SD, $n = 3$.

Genotype	Air temperature [°C]					
	NSE			HSE		
	T_{max}	T_{min}	T_{mean}	T_{max}	T_{min}	T_{mean}
Pre-anthesis phase (sowing to anthesis)						
Hindi62	21.2 ± 0.8	8.6 ± 0.5	13.9 ± 0.5	23.6 ± 1.1	11.1 ± 0.9	16.8 ± 1.0
PBW343	21.1 ± 0.8	8.5 ± 0.6	13.8 ± 0.6	23.5 ± 1.1	11.0 ± 0.9	16.7 ± 0.9
Post-anthesis phase (grain development)						
Hindi62	30.5 ± 1.0	15.8 ± 0.9	23.2 ± 0.9	36.1 ± 1.0	20.3 ± 0.9	28.3 ± 0.8
PBW343	28.8 ± 1.2	14.7 ± 1.0	21.7 ± 1.0	35.9 ± 1.0	20.2 ± 0.9	28.1 ± 0.8

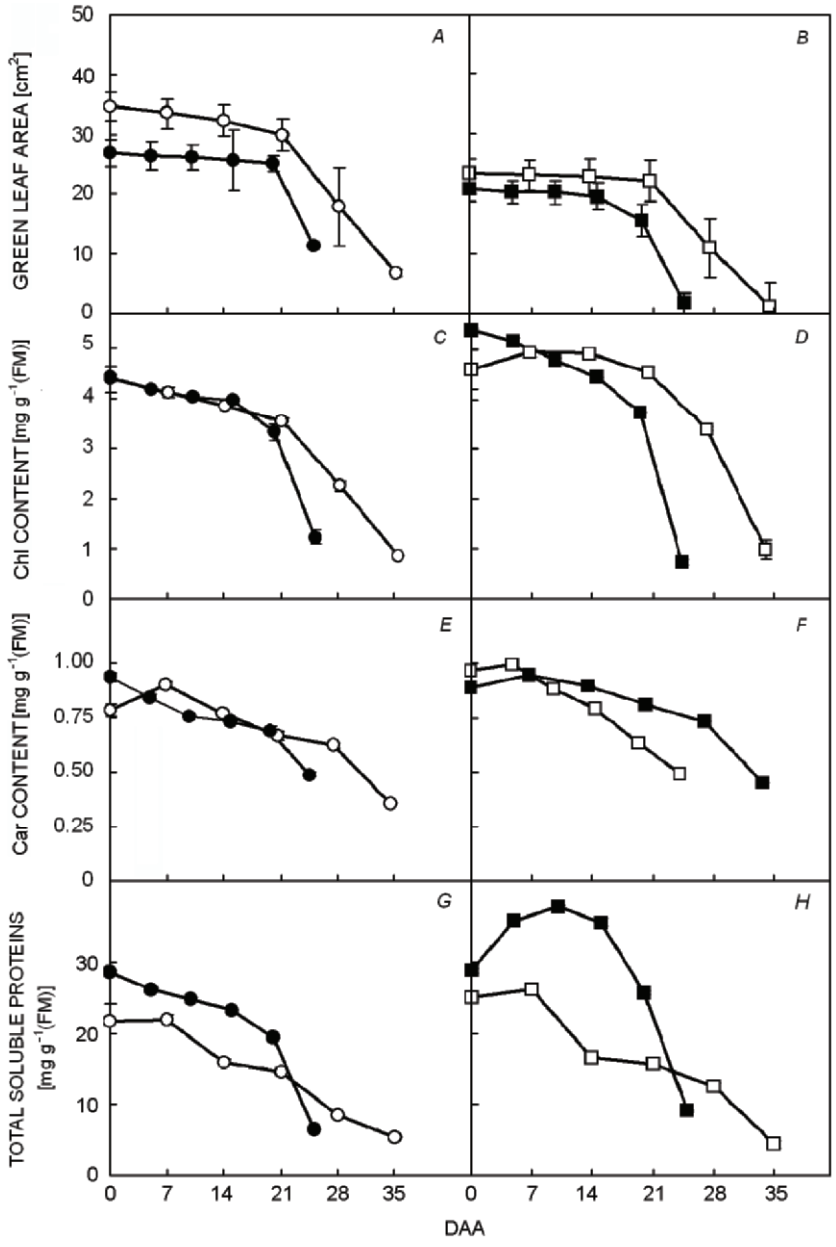

Fig. 1. Effect of high temperature on green flag-leaf area (*A,B*); chlorophyll (Chl) content (*C,D*); carotenoid content (*E,F*) and total soluble proteins (*G,H*) of wheat genotypes, Hindi62 and PBW343 during grain development. Vertical bars indicate SD ($n = 4$, *A,B*; $n = 3$, *C,D,E,F,G* and *H*). In some cases the bars are smaller than the symbols. Hindi62, ○ – NSE; ● - HSE (*A*, *C*, *E* and *G*); PBW343, □ – NSE; ■ – HSE (*B*, *D*, *F* and *H*), $n = 3$. Stages represent days after anthesis (DAA) on which sampling has been done and which varies for NSE and HSE as described in Materials and methods.

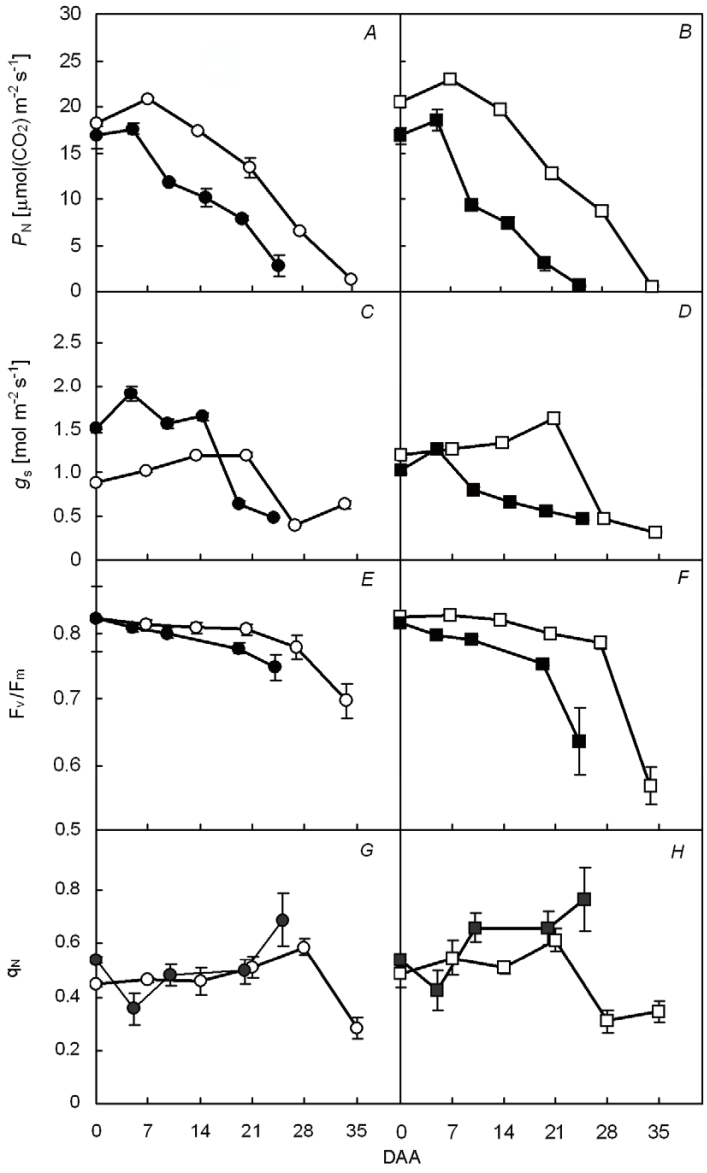

Fig. 2. Effect of high temperature on photosynthetic rate (P_N) (*A,B*); stomatal conductance (g_s) (*C,D*); maximum PSII efficiency expressed as F_v/F_m ratio (*E,F*) and nonphotochemical quenching (q_N) of chlorophyll fluorescence (*G,H*) of wheat genotypes, Hindi62 (*A,C,E*, and *G*) and PBW343 (*B,D,F*, and *H*) during grain development. Symbols as in Fig. 1. Vertical bars indicate SD. In some cases the bars are smaller than the symbols, $n = 3$.

Both the genotypes differed in size and colour of flag leaf. Hindi62 had pale yellow leaf with large area while PBW343 had dark green leaf with small leaf area. Under HSE, the rate of decline in leaf area and Chl content during grain development was not only slower in Hindi62 than PBW343 (Fig. 1 *A,B,C,D*), Hindi62 also had some green flag leaf area left on main shoot as well as on tillers at full grain maturity under NSE as well as HSE. In comparison to NSE, PBW343 showed a higher level of Chl and Car content up to 5 days after anthesis (DAA) during HSE, after which it was lower (Fig.1 *D,F*). In HSE, both the genotypes showed higher amount of total soluble proteins during grain development as compared to NSE (Fig. 1*G,H*). Moreover, the total soluble proteins were more in PBW343 than Hindi62 under both the environments. PBW343 showed an increase in total soluble protein up to 10 DAA under HSE, which then declined drastically during later stages of grain development.

P_N decreased in both the genotypes during grain development under both the environments. PBW343 had higher P_N than Hindi62 during most of the grain development period under NSE. Under HSE, PBW343 showed drastic reduction in P_N and g_s (Fig. 2*B,D*), whereas, Hindi62 performed very well under HSE by increasing its g_s which may be resulting in a higher canopy temperature depression and cooler canopy (Fig. 2*A,C*). The maximum PSII efficiency was better in PBW343 than Hindi62 for most of the grain development period under NSE with a severe reduction at the end (Fig. 2*E,F*). Under HSE, it was Hindi62 which had a higher maximum PSII efficiency than PBW343 especially at 25 DAA. Due to heat stress, gradual reduction in maximum PSII efficiency was observed during grain development, which was more severe in PBW343 than in Hindi62 (Fig. 2*E,F*). q_N increased under heat stress in both genotypes, but

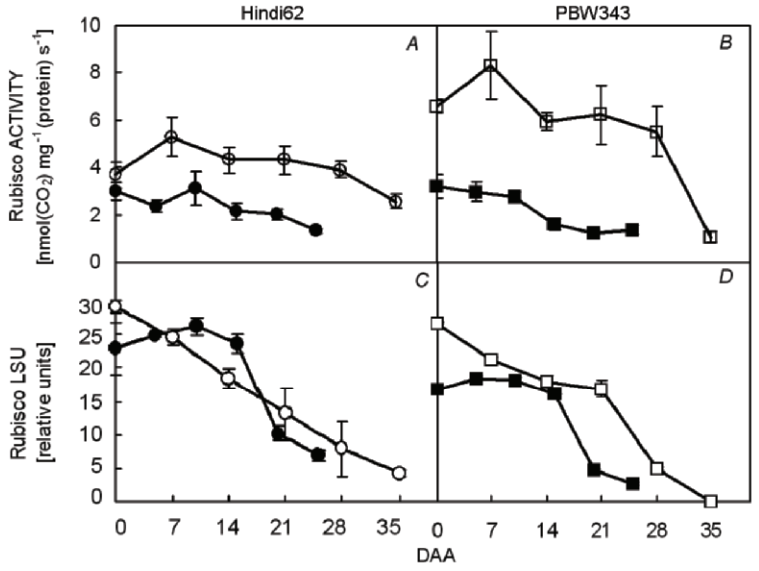

Fig. 3. Effect of high temperature on Rubisco activity (*A,B*); and Rubisco LSU content (*C,D*) of wheat genotypes, Hindi62 (*A,C*) and PBW343 (*B,D*) during grain development. Symbols as in Fig. 1. Vertical bars indicate SD. In some cases the bars are smaller than the symbols. Immunoblots showing effect of high temperature on Rubisco LSU degradation, using polyclonal antibodies against LSU in wheat flag leaf of Hindi62 (*E*) and PBW343 (*F*). In each case 10 μg of protein per lane was loaded. The lanes numbered as 7 to 35 and 5 to 25 represent the stages for days of sampling beginning with anthesis (An) under NSE and HSE respectively. M – molecular mass marker of Rubisco LSU, $n = 3$.

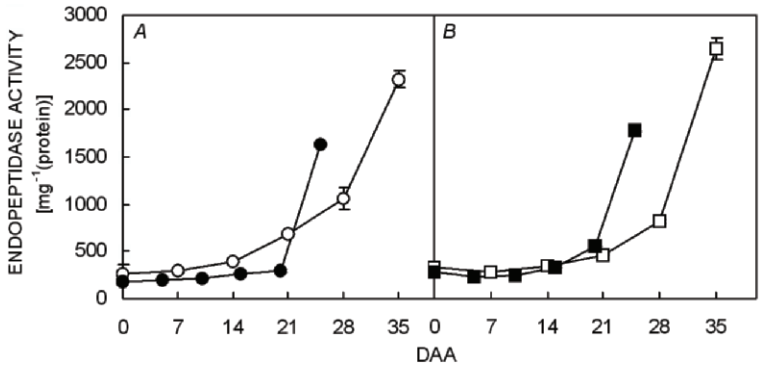

Fig. 4. Effect of grain development on endopeptidase activity of wheat genotypes, Hindi62 (*A*) and PBW343 (*B*) during grain development. Symbols as in Fig. 1. Vertical bars indicate SD. In some cases the bars are smaller than the symbols, $n = 3$.

Table 2. A summation of various proteases showing >10% inhibition during different stages of grain development in the flag leaf of wheat genotypes under NSE and HSE. DAA – days after anthesis.

Treatment	DAA	Nature of proteases	
		Hindi62	PBW343
NSE	0	serine	serine
	7	serine, metallo	serine
	14	serine, aspartate	serine, metallo
	21	serine, cysteine	cysteine, serine, metallo, aspartate
	28	aspartate	serine, aspartate
	35	metallo	serine, metallo, aspartate cysteine
HSE	0	serine	
	5	serine	metallo, serine, aspartate
	10	serine	serine
	15	serine	metallo, serine, aspartate
	20	serine, aspartate	serine, metallo
	25	serine, aspartate, metallo	aspartate, metallo

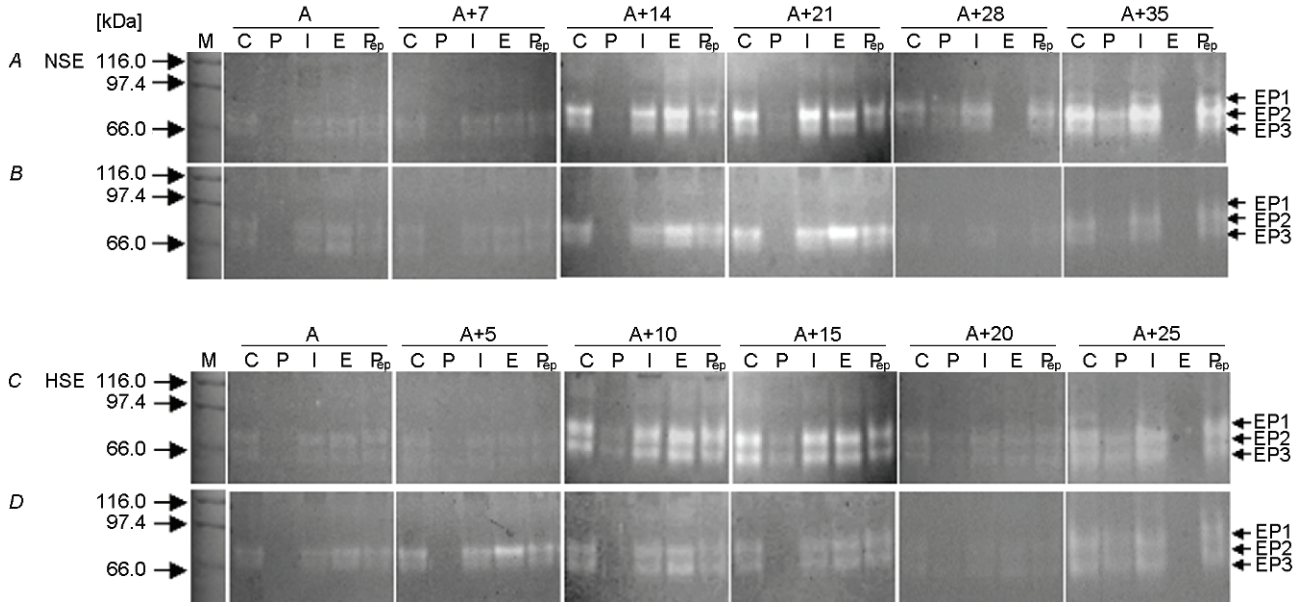

Fig. 5. Protease activity gels showing the inhibition of different proteases using specific inhibitors in the flag leaf of Hindi62 (*A,C*) and PBW343 (*B,D*) during grain development under NSE [non-stress environment] and HSE [heat-stress environment]. In each case 10 μg of protein per lane was loaded. M – molecular mass marker; C – control; P – PMSF [phenylmethylsulphonylfluoride]; I – iodoacetamide; E – EDTA, P_{ep} – pepstatin A.

slightly more in PBW343 than in Hindi62 (Fig. 2*G,H*).

Under NSE, PBW343 maintained a higher Rubisco activity during grain development as compared to Hindi62 (Fig. 3*A,B*). Both the genotypes showed reduction in Rubisco activity during grain development under HSE, which was observed to be more in PBW343 as compared to Hindi62. Both the genotypes exhibited decline in Rubisco LSU during grain development under both the environments (Fig. 3*C,D*). This was found to be higher under HSE especially for PBW343 than Hindi62. Immunoblot analysis using polyclonal antibodies against Rubisco LSU revealed degradation of Rubisco LSU into a lower molecular mass of 52 kDa by-product during later stages of senescence (Fig. 3*E,F*). In Hindi62, the Rubisco LSU degradation product appeared after 28 and 20 DAA under NSE and HSE, respectively, however, it appeared quite earlier in PBW343 (*i.e.*, after 21 and 15 DAA).

During NSE, Hindi62 exhibited higher endopeptidase activity as compared to PBW343 especially during the later stages of grain development (Fig. 4*A,B*). Both the genotypes exhibited lower endopeptidase activity under HSE as compared to NSE and this difference was more apparent in Hindi62. The acidic endopeptidase activity was found to be responsible for the loss in flag leaf nitrogen during grain development in both the genotypes (Hindi62, $r = -0.695$, $p<0.05$; PBW343, $r = -0.894$, $p<0.01$). Characterization of acidic proteases was done in the non-stressed and heat-stressed flag leaf of wheat genotypes by using specific inhibitors during grain development. It was observed that serine proteases were the most predominant proteases in wheat flag leaf during grain development under NSE as well as HSE, while other proteases like metallo- and aspartate proteases also played an important role during the later stages of senescence (Table 2). A similar protease profile was also evident by protease characterization gels (Fig. 5). Flag leaf of both the genotypes exhibited endoproteases of high molecular mass EP2 (78 kDa) and EP3 (67 kDa) isoforms up to 28 DAA and 20 DAA which were inhibited by specific serine protease inhibitor, while EP1 (90 kDa) appeared only at 35 and 25 DAA under NSE and HSE, respectively, which was consecutively inhibited by inhibitors of metallo-, serine- and aspartate proteases in both the environments.

Statistical analysis: Results are represented as mean ± SD, $n = 4$ for green flag leaf area and mean ± SD, $n = 3$ for other paramenters.

Discussion

In India, wheat productivity is the highest in fertile Indo-Gangetic plains of North India reaching 8 t ha^{-1} yield under irrigated conditions, while in Central India maximum yield ranged between 4–5 t ha^{-1} only. These differences in yield are mainly related to higher temperatures and longer day lengths prevailing in Central India during crop growth period as compared to North India (Ruwali and Prasad 1991). *Triticum aestivum* L. genotype Hindi62 is a heat-tolerant variety, a local selection by IARI Regional Station, Indore, Madhya

Pradesh, India which is suitable for cultivation in Central India. PBW343 is a high-yielding, medium-duration, heat-susceptible variety. In the present study, under normal sown environment (NSE), wheat genotypes experienced temperatures (T_{mean}) in the optimal range of 18–24°C during the post-anthesis phase (Table 1) (Porter and Gawith 1999). However, under heat-stress environment (HSE) wheat genotypes experienced severely higher temperatures in the range of 33–41°C (T_{max}). In field conditions, temperature increases gradually and thus, plants experience a gradual heat stress. Higher temperatures during HSE resulted in an accelerated growth and reduction in crop growth duration. Earlier heading under high temperatures is advantageous in the retention of more green leaves at anthesis, thus leading to a smaller reduction in the yield (Tewolde *et al.* 2006).

Senescence involves the active turnover and recapture of cellular materials for use in the growing parts such as developing seeds (Schippers *et al.* 2007). In our study, decline in green leaf area, Chl content, Cars, total soluble proteins, photosynthetic machinery, Rubisco activity and content and enhanced protein degradation are the common hallmarks of monocarpic senescence (Figs. 1–4). Plant aging and abiotic stresses including high-temperature stress accelerates the process of monocarpic senescence in wheat (Paulsen 1994; Reynolds *et al.* 2000). However, sensitivity to high temperature differed within the varieties (Plaut *et al.* 2004).

Low Chl content and pale green leaf colour were reported to be important traits in heat avoidance (Zaharieva *et al.* 2001). Low Chl content reduced the heating effect of high light intensities by decreasing the light absorbance and hence the lower leaf temperature as observed in Hindi62 (Fig. 1*A,B,C,D*). The rate of degradation of Car content was slower than the Chl content under both NSE and HSE conditions (Fig. 1*C,D,E,F*). Cars are involved in photoprotection dissipation process through the xanthophyll cycle (Gilmore 1997). High temperatures enhanced soluble protein concentrations in flag leaves upto 20 DAA. The total soluble proteins were more in PBW343 than in Hindi62 under both the environments (Fig. 1*G,H*). This was observed earlier in plant growth chamber studies in wheat and was attributed to enhanced nitrogen assimilation in leaves (Zhao *et al.* 2007).

High-temperature stress can reduce the photosynthetic source during grain filling both by accelerating leaf senescence and causing damage to the photosynthetic apparatus. Inhibition of photosynthesis by heat stress is a common occurrence for plants in tropical and subtropical regions. Under HSE, PBW343 showed drastic reduction in P_N and g_s (Fig. 2*B,D*). Higher g_s in Hindi62 under heat stress may be the consequence of paler leaves (Figs. 1*A,C* and 2*A,C*). Under high-temperature stress, F_v/F_m of flag leaves of both the genotypes reduced significantly with the progression of monocarpic senescence (Fig. 2*E,F*). In wheat, high temperatures and excessive light damaged different sites of PSII (Sharkova 2001). F_v/F_m of heat-susceptible genotype PBW343 was more affected by the heat stress than that of Hindi62. The decrease in F_v/F_m ratio indicates a reduction of maximum photochemical efficiency of the PSII complex, which could be due to an inefficient energy transfer from light-harvesting complex to the reaction centre and may result in photodamage to PSII by overreduction of reaction centers (Demmig-Adams and Adams 1992). At high temperatures, senescent leaves had an increase in q_N due to an increased thylakoid energization by the decreased use of ATP and NADPH caused by inhibition of Calvin cycle (Law and Crafts-Brandner 1999) (Fig. 2*G,H*). Higher q_N under HSE indicated a higher level of thermal dissipation involved in the xanthophyll cycle.

Inhibition of photosynthesis is reversible under moderate heat stress whereas under severe heat stress it is irreversible due to permanent damage of photosynthetic apparatus (Berry and Bjorkman 1980). Under high-temperature condition, CO_2 concentration becomes an increasingly greater limitation on Rubisco activity due to less carboxylation capacity of Rubisco. Decrease in P_N and reduction in Rubisco activity were severe in heat-susceptible genotype PBW343 as compared with heat-tolerant genotype Hindi62 under HSE (Figs. 3*A,B* and 4*A,B*). High-temperature-induced inhibition of P_N and g_s is associated with the faster rate of Rubisco inactivation (Morales *et al.* 2003). Activase kinetics and physical denaturation of activase appear to be causative factors contributing to the decrease in Rubisco activation at high temperatures (Salvucci and Crafts-Brandner 2004). The amount of Rubisco in leaves is controlled by the rate of its synthesis and degradation. Rubisco acts as a reservoir of leaf nitrogen besides fixing CO_2 and is rapidly and selectively degraded during natural and stress-induced senescence (Moreno *et al.* 1995). The flag leaf of wheat genotypes showed degradation of Rubisco LSU during monocarpic senescence, which was observed to be higher in the heat-susceptible genotypes PBW343 than the heat-tolerant genotype Hindi62 after 28 and 20 DAA under NSE and HSE, respectively (Fig. 3*C,D*). Rubisco LSU was degraded into a lower molecular mass of 52 kDa by-product during later stages of senescence as was revealed by immunoblot analysis using antibodies against Rubisco LSU (Fig. 3*E,F*). A 51 kDA fragment of Rubisco LSU formed in the stroma of chloroplasts under dark-induced senescence in wheat (Zhang *et al.* 2007).

The process of monocarpic senescence is accentuated by high sink strength and involves the mobilization of nitrogen derived from the proteolysis of proteins, especially Rubisco (Srivalli and Khanna-Chopra 1998). Endopeptidase activity increased significantly with progression of monocarpic senescence in wheat under NSE as well as HSE (Fig. 4). The relationship between loss of flag leaf nitrogen content and acidic endopeptidase activity showed a significant negative correlation for

heat-tolerant genotype Hindi62 (r = –0.695; p<0.050) and the heat-susceptible genotype PBW343 (r = –0.894; p<0.001). The increased endopeptidase activity could be responsible for the increased rate of Rubisco LSU degradation in PBW343 under HSE (Figs. 3*D,F* and 4*B*). Roberts *et al*. (2003) reported Rubisco degradation by endogenous chloroplastic serine proteases in wheat leaves. The vast majority of proteolytic enzymes related to senescent plant tissues are cysteine proteases (Buchanan-Wollaston *et al.* 2003) although an overall increase in the expression of genes representing the various classes of proteases, namely, serine-, aspartic-, metallo- and cysteine ones occurs during senescence (Guo *et al.* 2004). However, there is not much information indicating that plant serine proteases are associated with the senescence process except for those found in peroxisomes isolated from senescent pea leaves (Distefano *et al.* 1997). Two distinct substilisin-like serine proteases occur during dark-induced senescent wheat leaves (Roberts *et al.* 2006). The results obtained from endopeptidase activity using inhibitors showed predominance of serine proteases during monocarpic senescence, which was further confirmed by the protease inhibition gel assay, despite the substrates being different in both the cases (Table 2 and Fig. 5). Protease inhibition gels revealed two polypeptide bands corresponding to a molecular masses of 67 kDa (EP3) and 78 kDa (EP2), which were inhibited by specific serine protease inhibitor during monocarpic senescence. Thus, from the above findings it seems that serine proteases are predominantly involved during monocarpic senescence of wheat.

In conclusion, the results revealed that the studied genotypes behaved differently to heat stress by adapting different mechanisms like heat avoidance, heat tolerance and heat susceptibility. The accelerated phasic development in both the genotypes under HSE resulted in faster rate of senescence but the rate of senescence varied in both the genotypes. The tolerance for high temperature in Hindi62 was clearly evident in terms of pale green leaves with slower green leaf area degradation, higher g_s, higher stability in photosynthetic efficiency of PSII, Rubisco activity and Rubisco content. However, the heat-susceptible genotype PBW343 had small and dark green leaves with faster rate of green leaf area degradation and exhibited higher reduction in stomatal conductance and photosynthetic parameters under heat stress. The maximum PSII efficiency and Rubisco activity of PBW343 were found to be highly susceptible to heat stress, which may contribute to significantly faster rate of senescence and makes the genotype susceptible to heat stress. Serine proteases are the predominant proteases which played an important role in protein degradation of wheat during monocarpic senescence under normal as well as high-temperature environments.

References

Aggarwal, P.K., Sinha, S.K.: Performance of wheat and tritcale varieties in a variable soil water environment. IV. Yield components and their association with grain yield. – Field Crops Res. **17**: 45-53, 1987.

Al-Khatib, K., Paulsen, G.M.: Mode of high-temperature injury to wheat during grain development. – Physiol. Plant. **61**: 363-368, 1984.

Benbella, M., Paulsen, G.M.: Efficacy of treatments for delaying senescence of wheat leaves: II. Senescence and grain yield under field conditions. – Agron. J. **90**: 332-338, 1998.

Berry, J., Björkman, O.: Photosynthetic response and adaptation to temperature in higher plants. – Ann. Rev. Plant Physiol. **31**: 491-543, 1980.

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page, T., Pink, D.: The molecular analysis of leaf senescence – a genomics approach – Plant Biotechnol. J. **1**: 3–22, 2003.

CIMMYT.: CIMMYT/NARS Consultancy on ME1 bread wheat breeding. – Wheat special report No. 38. Mexico, DF 1995.

Demmig-Adams, B., Adams, W.W., III.: Photoprotection and other responses of plants to high light stress. – Ann. Rev. Plant Physiol. **43**: 599-626, 1992.

Distefano, S., Palma, J.M., Gomez, M., del Rio, L.A.: Characterization of endoproteases from plant peroxisomes. – Biochem. J. **327**: 399-405, 1997.

Ferreira, L.C.S., Bressan-Smith, R., Elias, T.F., Silva, F.F., Viana, L.H., Oliveira J. G. :Short-term responces of photosynthetic membrane lipids and photochemical effienciency in plants of *Phaseolus vulgaris* and *Vigna unguiculata* submitted to high irradiance. – Photosynthetica 45: 23-29, 2007.

Ferris, R., Ellis, R.H., Wheeeler, T.R., Hadley, P.: Effect of high temperature stress at anthesis on grain yield and biomass of field grown crops of wheat. – Plant Cell Environ. **34**: 67–78, 1998.

Gilmore, A.M.: Mechanistic aspect of xanthophylls cycle-dependent photoprotection in higher plant chloroplast and leaves. – Physiol. Plant. **99**: 197-209, 1997.

Gregersen, P.L., Holm, P.B.: Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). – Plant Biotechnol. J. **5**: 192-206, 2007.

Guo, Y., Cai, Z., Gan, S.: Transcriptome of *Arabidopsis* leaf senescence. – Plant Cell Environ. **27**: 521–549, 2004.

Hebeler, R., Oeljeklaus, S., Reidegeld, K.A., Eisenacher, M., Stephan, C., Sitek, B., Stühler, K., Meyer, H.E., Sturre, M.J.G., Dijkwel, P.P., Warscheid, B.: Study of early leaf senescence in *Arabidopsis thaliana* by quantitative proteomics using reciprocal $^{14}N/^{15}N$ labeling and difference gel electrophoresis. – Mol. Cell. Proteom**.** **7**: 108-120, 2008.

Jiang, C.Z., Rodermel, S.R. Shibles, R.M.: Photosynthesis, rubisco activity and amount, and their regulation by transcription in senescing soybean leaves. – Plant Physiol. **101**: 105-112, 1993.

Laemmli, U.K.: Cleavage of structure proteins during the assembly of the head of bacteriophage T_4. – Nature **227**: 680-685, 1970.

Law, R.D., Crafts-Brandner, S.J.: Inhibition and acclimation of photosynthesis to heat stress is closely correlated with activation of ribulose-1,5-bisphosphate carboxylase/oxygenase. – Plant Physiol. **120**: 173-181, 1999.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: Pigments of

photosynthetic biomembranes. – Methods Enzymol. **148**: 350-382, 1987.

Lim, P.O., Kim, H.J., Nam, H.G.: Leaf senescence. – Annu. Rev. Plant Biol. **58**: 115-136, 2007.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin-phenol reagent. – J. Biol. Chem. **193**: 265-275, 1951.

Mae, T., Kamei, C., Funaki, K., Miyadai, K., Makino, A., Ohira, K., Ojima, K.: Degradation of ribulose-1,5-bisphosphate carboxylase oxygenase in the lysate of the chloroplasts isolated mechanically from wheat (*Triticum aestivum* L.) leaves. – Plant Cell Physiol. **30**: 193-200, 1989.

Martínez, D.E., Bartoli, C.G., Grbic, V., Guiamet, J.J.: Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. – J. Exp. Bot. **58**: 1099–1107, 2007.

Morales, D., Rodríguez, P., Dell′Amico, J., Nicolás, E., Torrecillas, A., Sánchez-Blanco, M.J.: High-temperature preconditioning and thermal shock imposition affects water relations, gas exchange and root hydraulic conductivity in tomato. – Biol. Plant. **47**: 203–208, 2003.

Moreno, J., Penarubbia, L., Garcia-Ferris, C.: The mechanism of redox regulation of ribulose-1,5-bisphosphate carboxylase oxygenase turnover - a hypothesis. – Plant Physiol. Biochem. **33**: 121-127, 1995.

Mullarkey, M., Jones, P.: Isolation and analysis of thermotolerant mutants of wheat. – J. Exp. Bot. **51**: 139-146, 2000.

Noodén, L.D.: The phenomenon of senescence and aging. – In: Noodén, L.D., Leopold, A.C. (ed.): Senescence and Aging in Plants. Pp. 1-50. Academic Press Inc., New York 1988.

Patil, R.V., Chinnusamy, V., Chauhan, S., Ruwali, K.N., Pandey, H.N., Khanna-Chopra, R.: Heat stress tolerance in *T. aestivum* and *T. durum* wheat genotypes under continual and terminal heat stress environments. – J. Plant Biol. **35**: 1-10, 2007.

Paulsen, G.M..: High temperature responses of crop plants. – In: Boote, K.J., Bennet, J.M., Sinclair, T.R., Paulsen, G.M. (ed.): Physiology and Determination of Crop Yield. Pp. 365-389. ASA-CSSA-SSSA, Madion 1994.

Peoples, M.B., Dalling, M.J.: The interplay between the proteolysis and amino acid metabolism during senescence and nitrogen reallocation. – In: Noodén, L.D., Leopold, A.C. (ed.): Senescence and aging in plants. Pp. 181-217. Academic Press Inc., New York 1988.

Peoples, M.B., Pate, J.S., Atkins, C.A.: Mobilization of nitrogen in fruiting plants of a cultivar of cowpea. – J. Exp. Bot. **34**: 563-578, 1983.

Plaut, Z., Butow, B.J., Blumenthal, C.S., Wrigley, C.W.: Transport of dry matter into developing wheat kernels and its contribution to grain yield under post-anthesis water deficit and elevated temperature. – Field Crops Res. **86**: 185-198, 2004.

Porter, J.R., Gawith, M.: Temperatures and the growth and development of wheat: a review. – Eur. J. Agron. **10**: 23-36, 1999.

Porter, J.R.: Rising temperatures are likely to reduce crop yields. – Nature **436**: 174-174, 2005.

Reynolds, M.P., Balota, M., Delgado, M.I.B., Amani, I., Fisher, R.A.: Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. – Aust. J. Plant Physiol. **21**: 717-730, 1994.

Reynolds, M.P., Delgado, M.I.B., Gutiérrez-Rodríguez, M., Larqué-Saavedra, A.: Photosynthesis of wheat in a warm, irrigated environment. I. Genetic diversity and crop productivity. – Field Crops Res. **66**: 37-50, 2000.

Roberts, I.N., Murray, P.F., Caputo, C.P., Passeron, S., Barneix, A.J.: Purification and characerization of a substilisin-like serine protease induced during the senescence of wheat leaves. – Physiol. Plant. **118**: 483-490, 2003.

Roberts, I.N., Passeron, S., Barneix, A.J.: The two main endoproteases present in dark-induced senescent wheat leaves are distinct subtilisin-like proteases. – Planta **224**:1437–1447, 2006.

Ruwali, K.N., Prasad, L.: Effect of temperature stress on yield production of wheat. – In: Proc. Int. Conf. Plant Physiol. Pp. 271-277. BHU, India, Varanasi 1991.

Salvucci, M.E., Crafts-Brandner, S.J.: Inhibition of photosynthesis by heat stress: The activation state of Rubisco as a limiting factor in photosynthesis. – Physiol. Plant. **120**: 179-186, 2004.

Schippers, J.H.M., Jing, H.C., Hille, J., Dijkwel, P.P.: Developmental and hormonal control of leaf senescence. – In: Gan, S. (ed.): Senescence Processes in Plants. Pp. 145–170. Blackwell Publishing, Oxford 2007.

Schreiber, U., Bilger, W., Neubauer, C.: Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of *in vivo* photosynthesis. – In: Schulze, E.-D., Caldwell, M.M. (ed.): Ecophysiology of Photosynthesis, Pp. 49-70. Springer-Verlag, Berlin 1994.

Sharkova, V.E.: The effect of heat shock on the capacity of wheat plants to restore their photosynthetic electron transport after photoinhibition or repeated heating. – Russ. J. Plant Physiol. **48**: 793–797, 2001.

Sinha, S.K., Singh, G.B., Rai, M.: Decline in crop productivity in Haryana and Punjab: Myth or Reality? – Report of the fact finding committee, ICAR, India, New Delhi 1998.

Smart, C.M.: Gene expression during leaf senescence. – New Phytol. **126**: 419-448, 1994.

Srivalli, B., Khanna-Chopra, R.: Flag leaf senescence in relation to reduced reproductive sink strength in wheat. – Ind. J. Exp. Biol. **36**: 1013-1019, 1998.

Srivalli, B., Bharti, S., Khanna-Chopra, R.: Vacuolar cysteine proteases and ribulose-1,5-bisphosphate carboxylase/ oxygenase degradation during monocarpic senescence in cowpea leaves. – Photosynthetica **39**: 87-93, 2001.

Tewolde, H., Fernandez, C.J., Erickson, C.A., Wheat cultivars adapted to post-heading high temperature stress. – J. Agron. Crop Sci. **192**: 111-120, 2006.

Towbin, H., Staehelin, T., Gorgon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some application. – Proc. Natl. Acad. Sci. USA **76**: 4350-4354, 1979.

Viswanathan, C., Khanna-Chopra, R.: Heat shock proteins - Role in thermotolerance of crop plants. – Curr. Sci. **71**: 275-284, 1996.

Wahid, A., Gelani, S., Ashraf, M., Foolad, M.R.: Heat tolerance in plants: An overview. – Environ. Exp. Bot. **61**: 199-223, 2007.

Wardlaw, I.F., Wrigley, C.W.: Heat tolerance in temperate cereals - an overview. – Aust. J. Plant Physiol. **21**: 695-703, 1994.

Ye, Z.H., Varner, J.E.: Induction of cysteine and serine proteases during xylogenesis in *Zinnia elegans*. – Plant Mol. Biol. **30**: 1233-1246, 1996.

Zaharieva, M., Gaulin, E., Havaux, M., Acevedo, E., Monneveux, P.: Drought and heat responses in the wild wheat

relative *Aegilops geniculata* Roth: Potential interest for wheat improvement. – Crop Sci. **41**: 1321-1329, 2001.

Zhong-Hu, H., Rajaram, S.: Differential responses of bread wheat characters to high temperature. – Euphytica **72**: 197-203, 1993.

Zhang, L.-F., Rui, Q., Zhang, P., Wang, X.-Y., Xu, L.-L.: A novel 51-kDa fragment of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase formed in the stroma of chloroplasts in dark-induced senescing wheat leaves. – Physiol. Plant. **131**: 64-71, 2007.

Zhao, H., Dai, T.B., Jing, Q., Jiang, D., Cao, W.X.: Leaf senescence and grain filling affected by post-anthesis high temperatures in two different wheat cultivars. – Plant Growth Regul. **51**: 149-158, 2007.

Zivy, M., Thiellement, H., Devienne, D., Hofmann, J.P.: Study on molecular and cytoplasmic genome expression in wheat by two-dimensional gel electrophoresis. 1. 1st results on 18 alloplasmic lines. – Theor. Appl. Genet. **66**: 1-7, 1983.