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Comparative study of basal thermo tolerant attributes in tolerant and susceptible wheat cultivars

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Abstract
Two wheat cultivars, viz. C306 and HT41, have been evaluated for their responses to heat stress in terms of some biochemical and physiological attributes. During the exposure to high temperature (40°C for 6 and 12 h), a significant reduction in RWC and FW was recorded in HT41 which was also associated with heat susceptibility. The detrimental effects of high temperature were also apparent in terms of lipid peroxidation, chlorophyll content, H_2O_2 generation andelectrolyte leakage and these were more pronounced in HT 41. Accumulation of osmolytes like proline, total sugar increased due to heat injury and also varied between the two cultivars. In this present study evaluation and analysis of these biochemical and physiological characters under heat stress could unravel the mechanism of basal thermotolerance and thus might be useful as genetic stock to develop wheat tolerant varieties.

Keyword: HT 41, Lipid Peroxidation, H_2O_2.

Wheat is the most commonly grown cereal crop cultivated and consumed by people worldwide (Edgerton 2009). Importance of wheat as staple food is even more in context of growing world population and to meet the feeding demand of the we need between 840 and 1050 million ton production of wheat by 2020 (Kronstad, 1998). But in changing climate scenario declining wheat production is a big challenge to assure food security in India and other developing countries (Rosenzweig and Parry, 1994). Ideal temperature for wheat cultivation is between 17°C to 23°C (Porter and Gawith, 1999. Thus high temperature adversely affects wheat yield in many parts of world (Hameed *et al.*, 2010). Exposure of plants to heat causes imbalance of physiological and biochemical responses which have negative impact on respiration, water relations, photosynthesis, and membrane properties which can lead to break down in cellular organization (Wahid *et al.*, 2007). Heat stress enduring capacity of various crops is attributable to basal thermo tolerance level which helps the plants to survive exposure to temperatures above the optimal for growth (Bokszczanin *et a.l*, 2013). Differences in basal thermo tolerance level are thought to be correlated with various biochemical, physiological and molecular modulations responses to overcome heat stress (Kumar and Rai, 2014). The aim of the study was to compare heat tolerance capacity of two cultivars by heat susceptibility index and various physiological and biochemical alterations associated with heat stress and explore etiology of thermotolerance to identify possible means of detection and treatment approaches.

Materials and method

Plant material and experimental set up

Seeds of wheat (*Triticum aestivum* L.) cultivars, namely HT41 and C306 were selected for experimental purposes. The seeds were surface sterilized with 1% (w/v) sodium hypochlorite solution, washed with distilled water. Then the seeds were transferred to earthen pots containing soil with appropriate amount of compost and allowed to grow under optimum condition. One month old seedlings were then exposed to 40°C temperatures for 6 and 12 hours. Leaf samples from control and heat stressed seedlings were sampled.

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Relative water content (RWC) and Heat susceptibility index (HSI)

RWC was estimated by using formula given by Farooqui *et al.* (2000).

$$RWC\ (\%) = \frac{(\text{fresh weight - dry weight})}{(\text{fully turgid weight - dry weight})} \times 100$$

HSI was calculated by using following formula (Hameed *et al.* 2012)

$$HSI = \frac{(\text{reduction in seedling FW} + \%\ \text{reduction in RWC})}{2}$$

Cell viability and Leaf disc senescence assay

Cell viability percentage was determined by MTT assay (Chen et al, 1982). The degree of thermotolerance was examined using a chlorophyll bioassay (Fan et al, 1997) with some modifications. The healthy, fully expanded leaves from the plants were briefly washed in distilled water and 1 cm diameter leaf discs were subjected heat-shock treatment for 12 hours at 40°C. The effects of high temperature on leaf discs were observed by examining degree of bleaching (yellow color) and quantified by estimating their chlorophyll content Arnon (1949).

Structural changes in leaves were detected by light microscopic study. For that after heat treatment t.s of leaf was cut and stained with toluidine blue and observed under light microscope.

Quantification of osmolytes

Total sugar was extracted by following the method of Harborne (1973) by crushing of leaf samples in 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath and the aqueous fraction was re-suspended in distilled water which was then centrifuged at 5000 rpm for 10 mins. The supernatant was collected for estimation of total sugar. Estimation of total sugar was done by anthrone reagent following the method of Plummer (1978).

Extraction of free proline from the leaves was performed following the method of Caverzan et al. (2012). Leaf tissue was homogenized in 3% Sulfosalicylic acid and filtered through a Whatman No. 1 filter paper. The supernatant was used for estimation. Quantification of proline was done by the mixing filtrate with ninhydrin reagent. After that the mixture was kept for1 hr in a boiling water bath and chilled rapidly. The reaction mixture was then shifted to a separating funnel and 5 ml of toluene was added and mixed vigorously. The lower coloured layer was taken to measure absorbance at 520 nm in a colorimeter using toluene as blank and quantified from a standard curve of proline.

Quantification of H_2O_2 and in-situ localization

H_2O_2 within leaftissue were measured by following the method described by Jena and Choudhuri (1981). Study of H_2O_2 localization Washed leaf discs were placed in vial containing 3,3'-Diaminobenzidine (DAB) solution (pH3.8) and kept in dark overnight. Then leaf disc were boiled with ethanol: glycerol in the (4:1) until all the chlorophyll was removed. Then H_2O_2 localization was visible as dark brown patches of DAB - H_2O_2 polymers under light microscope (Thordal-Christensen and others 1997).

Determination of lipid peroxidation and electrolyte leakage

MDA was measured by following the method described by Heath and Packer (1968). It was measured by thiobarbiturate reaction where leaf tissue was homogenized in 0.1% (w/v) TCA. Absorbance of MDA content was then measured at 600 and 532 nm and MDA was quantified using extinction coefficient of 155 $mM^{-1}\ cm^{-1}$.

Electrolyte leakage was then measured by conductivity meter as described by Lutts and others (1996). Formula for calculating electrolyte leakage was

$$\textbf{Electrolyte leakage (\%)} = (L_t / L_0) \times 100$$

Where, L_t - Electrical Conductivity of the solution, L_0 - Final electrical conductivity (L_0)

Statistical analysis

Data were investigated statistically by the least significance test difference (LSD) at P d" 0.05 probability level. Values in figures and tables are represented as Mean ± SD. *designate significant differences (P d" 0.05).

Results

Evaluation of heat susceptibility index

Fresh weight and relative water content decreased in both wheat cultivars after exposure to 40°C for 6 hrs and 12 hrs. However decline in fresh weight and RWC was much more significant in HT41 than in C306 in respect to their controls (Fig. 1a and 1b). Maximum heat susceptibility index (HSI) which was calculated on the basis of changes in RWC and fresh weight was observed in case of HT 41 after 6 hrs and 12 hrs of heat stress (Fig. 1c).

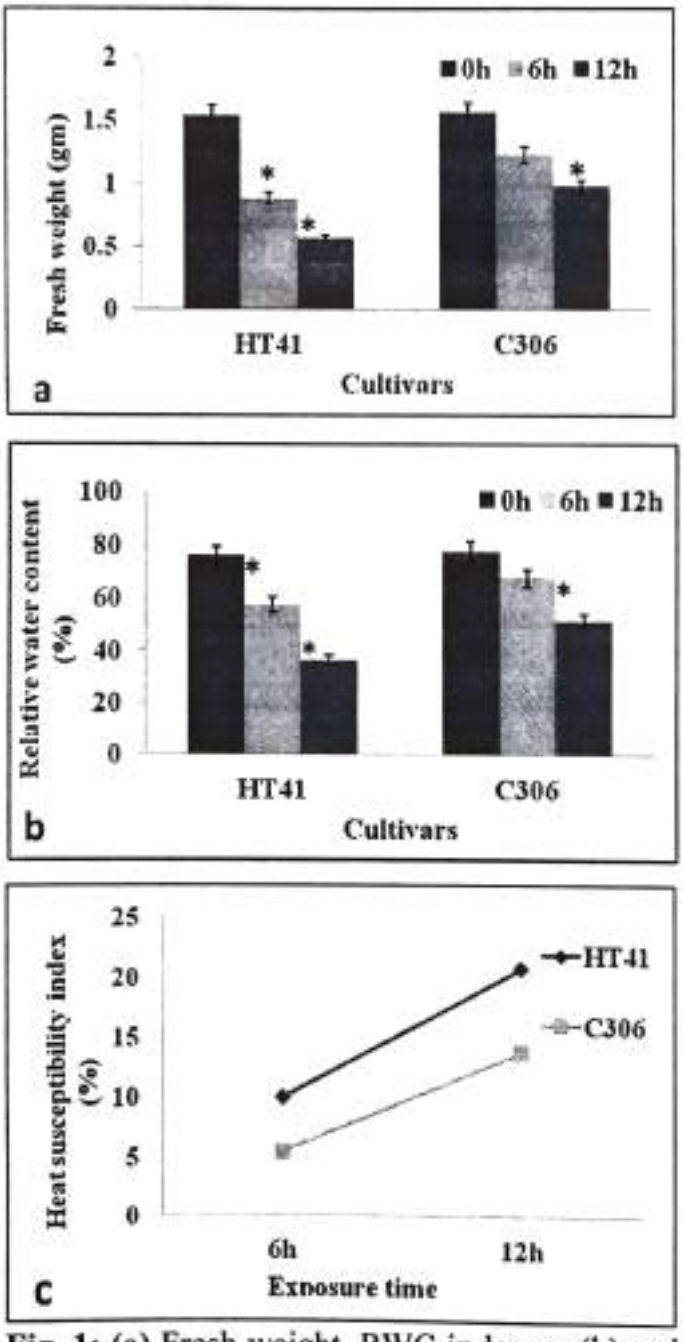

Fig. 1: (a) Fresh weight, RWC in leaves (b) and heat susceptibility index after 6 h and 12 h exposure to 40°C.

Leaf disc senescence assay and cell viability

From phenotypic changes and chlorophyll bleaching, it was evident that C306 plants are able to tolerate heat stress to some extent; while HT 41 plants cannot tolerate high temperature as heat induced loss of chlorophyll was more in HT 41 leaves in comparison to C306 after 12 hours of heat stress. In HT41 leaves chlorophyll leachate was 31.12 % higher after 6 hrs of heat stress and 29.23% higher after12 hrs of heat in comparison to C306 (Fig. 2a and 2b). Cell survival percentage significantly declined (Pd"0.05) in HT41 after 6 and 12 hours of heat stress where as in C306 seedlings cell survival percentage was noticeably high even after 12 hours of heat stress (Fig. 2c).

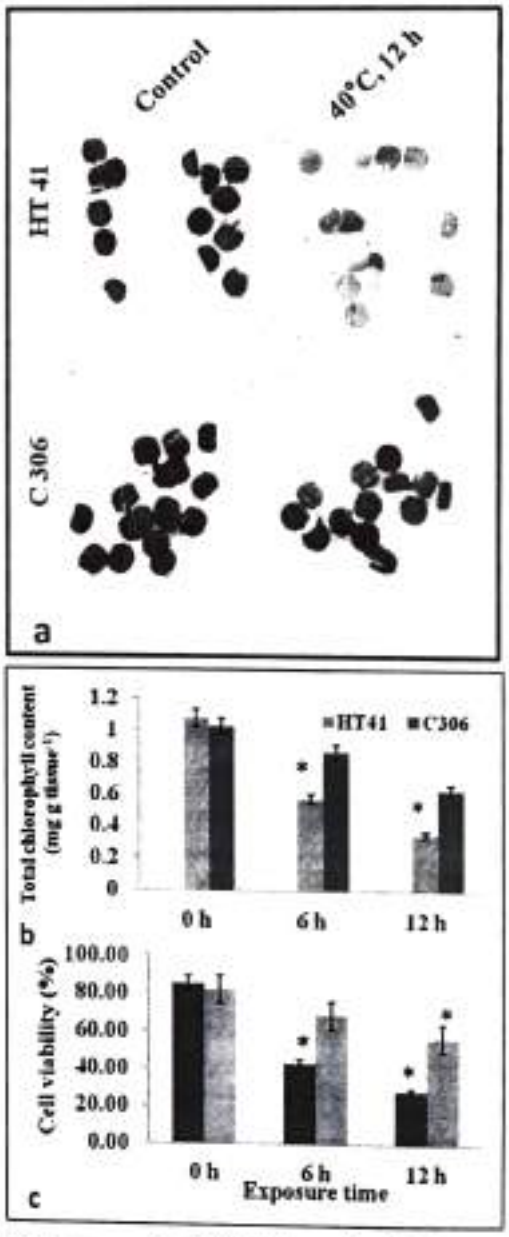

Fig. 2: Changes in (a) Chlorophyll bleaching (b) total chlorophyll content and cell viability after 6 h and 12 h exposure to 40°C.

Histological studies

Light microscopic studies (Fig. 3a and 3b) shows a transverse section of control leaf before imposition of heat stress. Epidermal cells leayer, mesophyll cells are clearly defined. In HT41 epidermal layer was distorted, mesophyll cells and vascular tissues were emaciated after 12 hours of heat stress. The width of the leaf section reduced due to loss of vacuoles mesophyll cells. Where as in C306 leaf mesostructure seems to be remained less affected (Fig. 3d) compared with the control (Fig. 3b). In C306 epidermal cell layer is clearly visible, compact vascular tissue and mesophyll cells containing vacuole chloroplasts in the parietal layer of the cytoplasm after 12 hours exposure at 40°C.

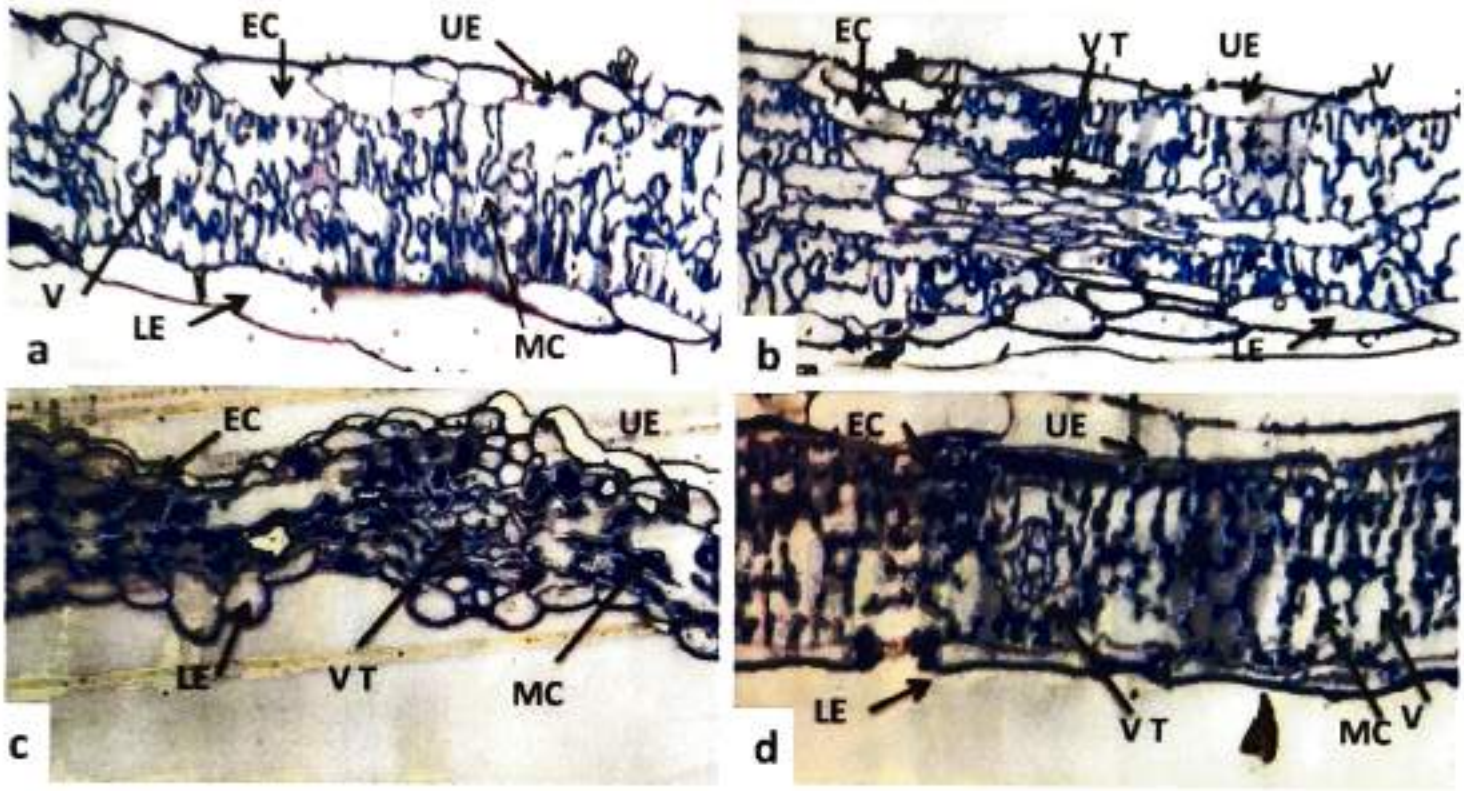

Fig. 3: Light microscopy of t.s of leaf showing alteration in various cell structure after heat treatement for 12 hours at 40°C. **EC** – Epidermal cell, **UP** – Upper epidermis, **VT** –Vascular tissues, **LE**- Lower epidermis, **MC** –Mesophyll cell, **V**- Vacuoles.

Biochemical response to elevated temperature

Lipid peroxidation and Electrolyte leakage

Temperature stress enhanced electrolyte leakage and membrane lipid peroxidation as reflected by elevated level of MDA (Table 1). In C306 and HT 41 cultivars MDA content gradually increased after 6 h and 12 h exposure to 40°C. Accumulation of MDA with elevated temperature in relation to control was highest in HT 41 and minimum in C306. Similar trend was also observed in case of Electrolyte leakage. Low level of lipid peroxidation and electrolyte leakage in C306 plants under heat stress clearly in indicate less plasma membrane injury.

H_2O_2 content and histochemical detection

Exposure to high temperature (40°C) for 12 hours accelerated accumulation of H_2O_2. Maximum accumulation of H_2O_2 after 6 and 12 hrs of heat stress was observed in HT 41 and minimum in case of C306 (Fig. 4a). Histochemical detection of H_2O_2 in the leaves was performed by visualizing dark brown patches of DAB- H_2O_2 complex with in leaf tissue. Microscopic examinations clearly showed that 12h after exposure to 40°C there was eruption of H_2O_2 in HT 41 leaf as indicated by dark brown patches. However, in C 306 dark brown area was less signifying less H_2O_2 accumulation after heat stress. (Fig. 4B).

Table 1. Changes in malonaldehyde (MDA) content and electrolyte leakage after 6 h and 12h exposure to 40°C.

Cultivars	MDA Content (μg g tissue^{-1})			Electrolyte leakage (%)		
	Exposure time (h)			Exposure time (h)		
	0	6	12	0	6	12
HT41	0.38 ± 0.02	1.64 ± 0.33*	2.48 ± 0.38*	11.09 ± 0.90	56.23 ± 1.57	78.58 ± 1.51*
C306	0.45 ± 0.04	0.88 ± 0.05	1.22 ± 0.24	11.96 ± 0.32	34.16 ± 1.48	54.49 ± 1.01*

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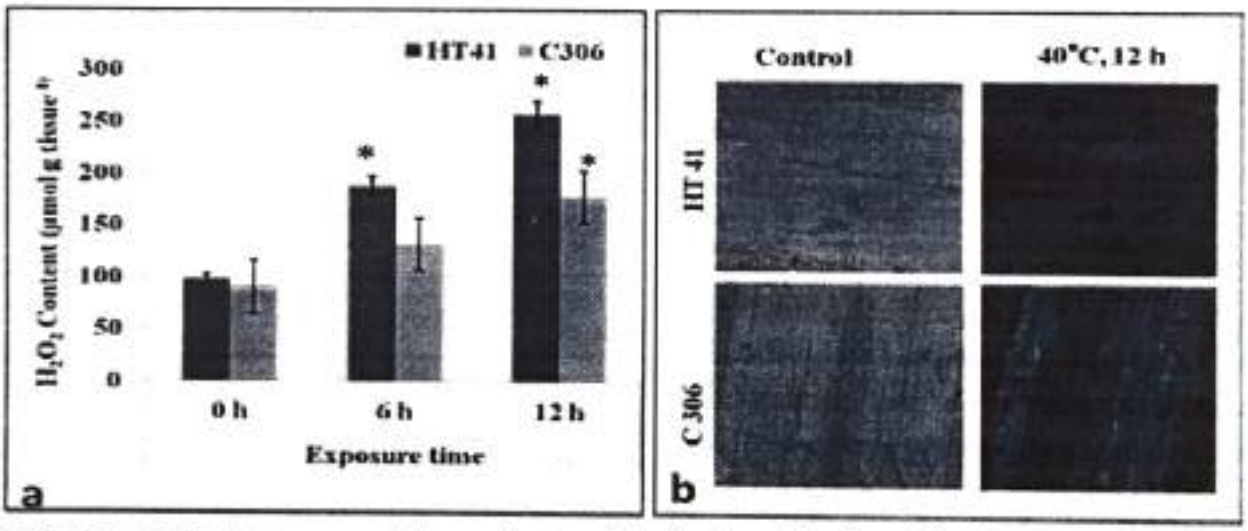

Fig. 4: Changes in hydrogen peroxide content and *in situ* localization of hydrogen peroxide after 6 h and 12 h exposure to 40°C.

Proline and Total sugar

Proline and total sugar content in both the cultivars gradually increased over time at 40°C upto 12 hours in relation to control. Maximum accumulation of proline was observed in HT 41 when exposed to heat stress for 12 hrs. On contrary total sugar level was significantly high in C 306 leaves after 12 hour incubation at 40°C (Table 2).

Table 2. Accumulation of proline and total sugar in leaf tissue after 6 h and 12 h exposure to 40°C.

Cultivars	Proline Content (mg g tissue^{-1})			Total sugar (mg g tissue^{-1})		
	Exposure time (h)			Exposure time (h)		
	0	6	12	0	6	12
HT41	1.34±0.06	6.64±0.43*	9.48±0.45*	6.08±0.60	9.89±0.27	16.56±0.45*
C306	1.43±0.12	3.87±0.25	5.12±0.28	5.45±0.12	18.87±0.28	25.34±0.21*

Discussion

Two wheat cultivars showed differential resistance to high temperature in terms of biochemical and physiological characteristics. Fresh weight (FW) and relative water content (RWC) provide an insight to state of water balance of plants (Kramer and Boyer, 1995). In our study RWC and FW decreased in both the cultivars under heat stress. The observed reduction in FW over the control with increasing incubation time at 40°C may be due to the loss of RWC and turgidity (Kesici *et al.*, 2013). Among the cultivars, C306 exhibited the highest RWC and FW while HT 41 had the lowest RWC after exposure to 40°C for 6 and 12 hours. As per Heat susceptibility index calculated using RWC and FW, HT 41 was found to be more heat susceptible than C306. Effect of heat induced dehydration was clearly seen on mesophyll cells and vascular bundles. In HT41, shrunken mesophyll cells and vascular bundles were noticed, whereas in C306 compact and less deformed mesophyll cells in flag leaves and vascular bundle was clearly visible. The dense and compact arrangement of mesophyll cells may help in reduction of dehydration and diffusion conductance in heat stressed C306. Comparative studies of cell viability and leaf disc secesence bioasaay indicate exposure to high temperature for 12 hours significantly reduced chlorophyll content and cell viability in HT 41 in comparison to C306 suggesting positive correlation of heat sensitivity to photosynthetic efficiency (Kumar et al., 2013). Least accumulation of MDA and H_2O_2 are considered favourable to confer thermotolerance (Wahid et al., 2007). Increased lipid peroxidation and H_2O_2 level inactivate cell membrane enzymes and proteins, consequently antioxidative (enzymatic and non enzymatic) machinery avert cell damage by regulating level of ROS (Ge *et al.*, 2005, Gill and Tuteja,2010). In present study, lipid peroxidation and H_2O_2 accumulation was less in C306 when exposed to heat stress. Results on electrolyte leakage revealed that stability of plasma membrane was least affected in C306 in comparison to HT41. Plants under stress accumulate various compatible osmolytes to maintain cell turgor and osmotic balance (Marijuan and Bosch 2013). In our observation in C306 proline accumulation was less and total sugar concentration was quite high in leaf tissues in comparison to HT41 after 12 hours of heat stress. Unlike other abiotic stresses, such as osmotic, salt, heavy metal and oxidative stress where proline play key role in enhancing tolerance (Hasanuzzaman et al., 2013) during high temperature stress, sugar accumulation appeared to play protective role in maintaining osmotic balance (Rizhsky et al., 2004).

In summary, thermo tolerance is a complex attribute and regulated by several biochemical and physiological parameters which helps plants to achieve normal physiological state. Considering all the above data and comparing with HSI it was found that among two cultivars taken for the study, C306 was able to endure the heat stress more efficiently than HT 41.

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