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Insights from morpho-physio-biochemical and molecular traits of hot pepper genotypes contrasting for heat tolerance

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ABSTRACT

The present study compared the morpho-physiological, biochemical, and molecular responses of heat-tolerant (DLS-161-1) and heat-susceptible (DchBL-240) genotypes of hot pepper under high-temperature conditions. The results showed that DLS-161-1 performed significantly better for various morphological parameters such as plant height (60 cm), number of fruits per plant (180.33), average fruit length (8.40 cm) and leaf parameters. While DChBL-240 has significantly higher malformed seeds per fruit (15.06) and average fruit diameter (12.56 cm). The physiological responses also differed significantly between the two genotypes, with the DLS-161-1 exhibiting higher membrane stability index (65.99%), pollen viability (92.10%), photosynthetic rate (20.73 µmol CO_2 m^2/sec.), stomatal conductance (0.31 mol H_2O m^2/sec.), and transpiration rate (8.85 mmol H_2O m^2/sec). Biochemical analysis revealed significantly increased activity of antioxidant enzymes such as guaiacol peroxidase (564.99 U/g FW) and catalase (711.90 U/g FW), increased protein content (13.38 mg/g F.W) and reduced malondialdehyde generation (13.42 nmol g^{-1} FW) in DLS-161-1 compared to heat susceptible genotype (DChBL-240). Further, two of the six heat shock protein genes, *CaHSP2271* and *CaHSP3*, were highly upregulated in the DLS-161-1. The observations suggest that DLS-161-1 could adapt to high-temperature conditions by regulating its morphological, physiological, biochemical and molecular mechanisms.

Key words: *Capsicum annuum* L., Antioxidant enzymes activity, High-temperature tolerance, Leaf gas exchange parameters, Heat shock proteins (*HSP*).

INTRODUCTION

Global warming, primarily due to the anthropogenic release of greenhouse gases, causes a severe threat to agricultural crop production. In its recent report, the Inter-governmental Panel on Climate Change (IPCC) recorded that average earth surface temperature will exceed 1.5°C over pre-industrial levels by 2040 and 2°C by 2050 and there will be more intense heat waves over Southeast Asia during the 21st Century (Lee *et al*., 15). Hot pepper (*Capsicum annuum* L.) is one of the major vegetable crop of the Solanaceae family. Though it is originated in Mexico, India is its largest producer, consumer and exporter. It is a tropical crop, but fails to set fruits if temperature rises above 40°C. Flower abscission is high at 32-38°C, while optimal fruit retention occurs at 16-21°C day temperature (Srivastava *et al*., 22). The period from early summer to early rainy season is considered as lean period for producing hot pepper. The pepper genotypes grown under high-temperature conditions exhibit reduced plant height (Usman *et al*., 23), decreased leaf area, dry mass, shoot and root length, fresh as well as dry weight, and number of leaves (Ali *et al.*, 2; Hussain *et al.*, 13). Their reproductive traits, such as fruit length, width, weight, number of fruits per plant, size, diameter, seed yield, and quality significantly decreased (Kaur *et al*., 14; Srivastava *et al*., 22). Furthermore, reduced membrane stability, pollen viability, altered photosynthetic rate, transpiration rate, relative chlorophyll content (RCC), and antioxidant enzyme activities were also observed to be altered in the pepper genotypes under heat stress (Usman *et al.*, 23; Kaur *et al.*, 14; Dobos *et al.*, 10; Ghai *et al.*, 11; Rajametov *et al*., 19; Hussain *et al.*, 13).

India is geographically situated in the Northern hemisphere. During the summer season, north India experiences very high temperatures. Additionally, global warming is expected to cause more intense, and frequent heat waves in the coming years, thus necessitating the development of heat tolerant hot pepper varieties. Continuous efforts are being made in the Division of Vegetable Science, IARI, New Delhi, to identify heat-tolerant lines in hot peppers. The present investigation compares morpho-physiological, biochemical, and molecular responses of two hot pepper genotypes with variable in heat tolerance.

MATERIALS AND METHODS

During the 2022-23 spring-summer season, we studied two *Capsicum annuum* L. genotypes,

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DLS-161-1 (tolerant) and DChBL-240 (susceptible), known for their distinct responses to high-temperature conditions. Seed sowing was done in February 2022 in 96-celled plastic trays (54 × 27 cm) filled with a mix of perlite, coco-peat, and vermiculite (1:2:1). After 35 days, seedlings were transplanted, and flowering commenced in May 2022. Each genotype was grown in three replications, with ten plants per replication, under field conditions at ICAR-IARI, New Delhi (28° 40'N latitude and 77° 13' E longitude), as shown in Fig. 1. Morphological, physiological, and biochemical traits were recorded for five randomly selected plants in each replication. The data were recorded on morphological traits such as plant height (cm), number of primary branches, number of fruits per plant, average fruit length (cm), average fruit diameter (mm), average fruit weight (g), average fruit yield per plant (g), number of malformed and healthy seeds per fruit, 100-seed weight (g), and fresh biomass (g). WinFOLIA software (Regent Instruments, Inc. Canada) was used to measure different leaf parameters, *viz*., leaf area (cm^2), length (cm), width (cm), perimeter (cm) and aspect ratio (ratio of width/ length of leaf) from top ten leaves of each plant.

The canopy temperature (CT) (°C) was recorded using a handheld infrared thermometer (Fluke-62-Max). In contrast, canopy temperature depression (CTD) was calculated as the difference between air and canopy temperatures. Normalized Difference Vegetation Index (NDVI) was measured using a green seeker (Handheld-505), stomatal density (SD) using a light microscope with a Magvision Imaging tool (Magnus Opto Systems, India) and the pollen viability (PV) (%) by observing the percentage of stained pollen following acetocarmine (2.5%) under observed the EVOS XL core microscope (Thermo and Fisher Scientific, Inc. USA). A conductivity meter was used to study membrane stability index (MSI) (%) and electrolyte leakage (EL) (%) using formula: and where, C_1 is the electrical conductivity (EC) of water containing fresh leaf disc after treatment at 40°C for 30 min and C_2 is the conductivity recorded after 100°C treatment for 10 min. In addition, leaf gas exchange parameters *viz*., net photosynthetic rate (µmol CO_2m^{-2}/sec.), stomatal conductance (mol H_2O m^{-2}/sec.), intercellular CO_2 concentration (Ci) (µmol CO_2/mol), transpiration rate (mmol H_2O m^{-2}/sec) were recorded using LI-6400 portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA) on a clear sunny day from the top leaf of the plants. Parameters such as instantaneous water use efficiency (µmol CO_2/mmol H_2O), intrinsic water use efficiency (µmol CO_2/mmol) and transpiration ratio (mmol H_2O/ µmol CO_2) were derived using following formulae:

$$\text{Instantaneous WUE} = \frac{A}{E}\ \text{Intrinsic WUE} = \frac{A}{g^s}\ ;\ \text{Transpiration ratio} = \frac{E}{A}$$

Where,
A = net photosynthetic rate, E = transpiration rate, gs = stomatal conductance.

Biochemical observations such as relative chlorophyll content (RCC) was measured using CCM-200 plus chlorophyll meter (Apogee Instruments Inc.) from the most recently matured leaves. The activities of malondialdehyde (MDA), Guaiacol peroxidase (GPX) (U/g FW), catalase (CAT) (U/g FW), superoxide dismutase (SOD)(U/g FW) and proline (µmol/g FW) were estimated following the protocols of Heath and Packer (12), Chance and Maehly (8), Aebi (1), Dhindsa *et al.* (9) and Bates *et al.* (5), respectively. CAT activity was calculated by plotting the standard curve, GPX and SOD were calculated using molar extinction coefficient and protein content was estimated using the Bradford reagent method (Bradford, 7).

The expression of six different heat shock protein genes *viz*., *CaHSP3*, *CaHSP703*, *CaHSP2271*, *CaHSP90*, *CaHSP2272*, and *CaCHSP* under ambient (27°C) and heat stress (44°C) conditions was studied as per the procedure established by Mangal *et al.* (18) with Light Cycler 96® (Roche LifeSciences) system using 2XSYBR Green master mix (Applied Biosystem, CA, USA) and ubiquitin as a reference gene for normalizing gene expression values. The relative expression level of candidate genes was determined also.

The differences in the morphological, physiological and biochemical observations between the two genotypes were compared by independent sample t-test using the t-test function (used both equal and unequal variance assumption based on F-test results).

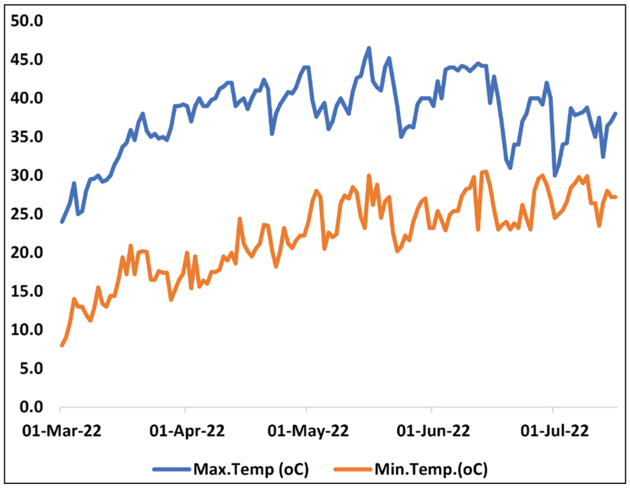

Fig. 1. Temperature variation during the study period.

RESULTS AND DISCUSSION

High temperature causes several morphological injuries, including inhibited root growth, scorching of leaves and fruits, and reduced yield. During the present investigation, it was observed that heat sensitive genotype (DchBL-240) showed significantly reduced plant height (48.53 cm), fresh biomass (362.33 g), leaf area (4.93 cm^2), perimeter (13.65 cm), leaf length (5.75 cm) and width (1.60 cm) as compared to tolerant one (Table 1). The observations indicated that elevated temperature had detrimental effects on both growth and productivity of the sensitive genotype, which is in line with the observations of Usman *et al*. (23) on chilli. However, the number of primary branches produced by both genotypes did not differ significantly (9.67 in tolerant and 7.33 in susceptible genotypes). These results appear to be in accordance with Srivastava *et al.* (22), who also reported that heat stress does not significantly affect vegetative stage in chilli. Numerous other studies have also demonstrated that the reproductive stage is the most susceptible to high-temperature. Present findings on reproductive parameters also indicated the same results. Number of fruits per plant (15), fruit yield per plant (49.87 g), average fruit weight (17.90 g), average fruit length (6.89 cm) (Fig. 2; Table 1), and hundred-seed weight (0.30 g) were significantly reduced in the susceptible genotype compared to the tolerant line (Table 1). The decline in values of all reproductive traits were the result of significantly reduced pollen viability (53.84%) in DchBL-240 (Table 2; Fig. 2). The results observed were consistent with past studies on chilli (Srivastava *et al*., 23; Kaur *et al*., 14). Higher fruit diameter observed in DchBL-240 seems to be genetically controlled. Further, no significant difference was observed in the number of seeds between susceptible (75) and tolerant genotypes (65.33) (Table 1; Fig. 2).

Table 1. Morphological observations recorded in heat-tolerant and susceptible hot pepper genotypes.

Trait	DLS-161-1	DChBL-240	Level of significance
Plant height (cm)	60.00	48.53	**
Primary branches per plant	9.67	7.33	NS
No. of fruits/ plant	180.33	15.00	*
Avg. fruit length (cm)	8.40	6.89	**
Avg. fruit weight (g)	19.02	17.90	NS
Avg. fruit diameter (mm)	8.48	12.56	*
Fruit yield per plant (g)	269.06	49.87	**
No. of seeds per fruit	65.33	75.00	NS
No. of malformed seeds	11.22	15.06	*
100-seed weight (g)	0.33	0.30	NS
Leaf length (cm)	7.69	5.75	**
Leaf width (cm)	2.10	1.60	***
Aspect ratio (W/L)	0.28	0.28	NS
Leaf area (cm^2)	8.81	4.93	***
Leaf perimeter (cm)	17.83	13.65	**
Fresh biomass (g)	490.67	362.33	*

*Significance at $P \leq 0.05$, ** at $P \leq 0.01$, *** at $P \leq 0.001$ level, NS - non-significance

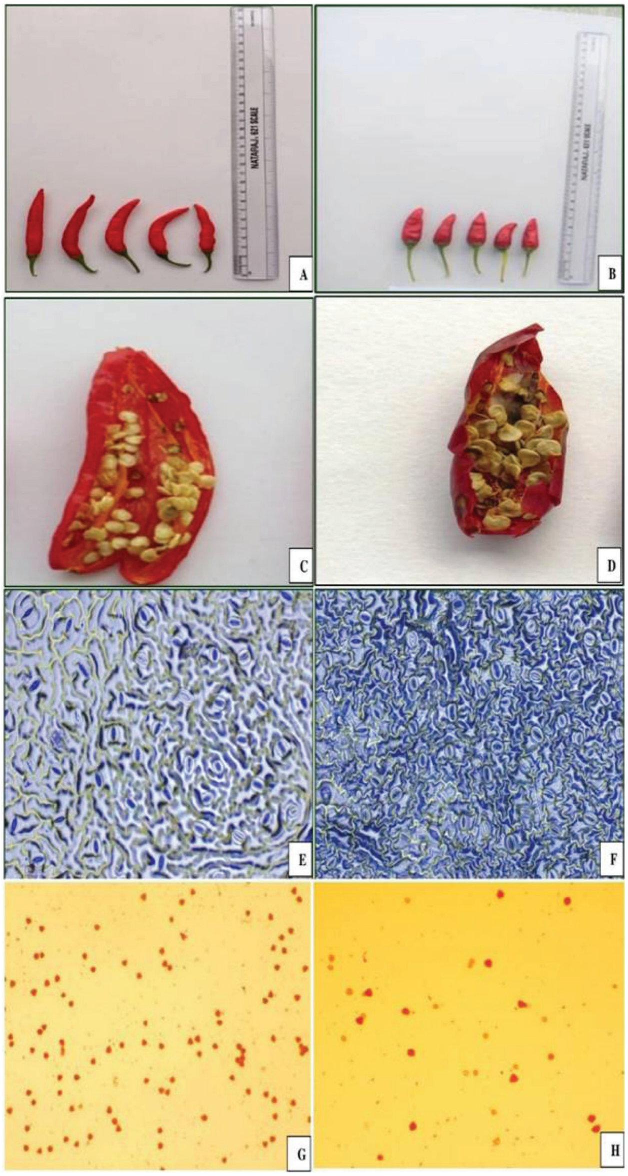

Fig. 2. Observations on heat tolerant (DLS-161-1) and heat susceptible (DChBL-240) genotypes, respectively; A-B: Fruit length; C-D: Seeds per fruit; E-F: Stomatal density; G-H: Pollen viability.

Table 2. Physiological parameters in heat-tolerant and susceptible genotypes.

Trait	DLS-161-1	DChBL-240	Level of significance
CT (°C)	33.09	35.53	***
CTD (°C)	3.34	1.09	**
NDVI	0.84	0.76	**
MSI (%)	65.99	55.67	**
EL (%)	34.01	44.33	**
PV (%)	92.10	53.84	**
SD (mm^{-2})	166.40	229.89	***
A (µmol CO_2 m^2/sec.)	20.73	14.27	***
gs (mol H_2O m^2/sec.)	0.31	0.24	*
Ci (mmol H_2O m^2/sec.)	267.67	291.67	NS
E (mmol H_2O m^2/sec)	8.85	5.83	***
A/E (µmol CO_2/mmol H_2O)	2.34	2.45	NS
A/gs (µmol CO_2/mmol)	67.70	59.83	NS
E/A (mmol H_2O/µmole CO_2)	0.43	0.41	NS

CT-Canopy temperature, CTD-Canopy temperature depression, NDVI- Normalized Difference Vegetation Index, MSI-Membrane stability index, EL- Electrolyte leakage, PV- Pollen viability, SD- Stomatal density, A- Net photosynthetic rate, gs-Stomatal conductance, Ci- Intercellular CO_2 concentration, E-Transpiration rate, A/E- Instantaneous water use efficiency, A/gs- Intrinsic water use efficiency, E/A- Transpiration ratio.

*Significance at P ≤ 0.05, ** at P ≤ 0.01, *** at P ≤ 0.001 level, NS - non-significance.

Table 3. Biochemical observations recorded in heat-tolerant and susceptible genotypes.

Trait	DLS-161-1	DChBL-240	Level of significance
RCC	46.47	36.9	NS
GPX activity (U/g FW)	564.99	404.68	***
CAT activity (U/g FW)	711.90	195.24	***
SOD activity (U/g FW)	520.79	307.92	NS
Protein (mg/g FW)	13.38	9.13	***
MDA (nmol g^{-1} FW)	13.42	18.02	**
Proline (umol/g FW)	5.86	8.32	NS

RCC- Relative chlorophyll content, GPX- Guaiacol peroxidase, CAT- Catalase, SOD- Superoxide dismutase, MDA-Malondialdehyde. *Significance at P ≤ 0.05, ** at P ≤ 0.01, *** at P ≤ 0.001 level, ns-non-significance.

However, the malformed seeds increased significantly in the susceptible genotype (15.06), indicating that heat stress also highly affected female reproductive organs.

Physiological parameters such as CTD (3.34°C), MSI (65.99%), PV (92.10%), NDVI (0.84), NPR (20.73 µmol $CO_2 m^{-2} sec^{-1}$), stomatal conductance (0.31 mol H_2O $m^{-2} sec^{-1}$) and transpiration rate (8.85 mmol H_2O $m^{-2} sec^{-1}$) were significantly high in the heat-tolerant genotype (DLS-161-1) as compared to the susceptible one (DChBL-240). These observations suggest an inherent physiological mechanism to combat heat stress in DLS-161-1. On the contrary, values of CT (35.33°C), EL (44.33%) and SD (229.9 per mm^2) were significantly higher in the susceptible genotype which accounted for the poor performance of DChBL-240 under heat stress. It was also observed that RCC, instant WUE, intrinsic WUE, and transpiration ratio were not significantly different among the two genotypes (Tables 2 & 3).

Heat tolerance is a complex phenomenon governed by multiple genes that affect both physiological and biochemical processes. The alterations in these processes under heat stress is an important criteria for selecting heat-tolerant genotypes. A significantly higher CT was observed in the susceptible genotype (35.5°C) compared to the tolerant line (33.0°C). The CT is reduced by transpiration cooling and keeps the leaves comfortably in the temperature range for optimum enzyme activity. Further, our investigation proves that the tolerant genotype had higher CTD (3.34°C) than the susceptible genotype (1.09°C) due to transpirational cooling mechanism. These observations also appear to align with earlier reports where high temperature caused significantly increased leaf/ canopy temperature in sensitive lines than tolerant lines of tomato (Bhattarai *et al*., 6). The observed significant increase in the NPR in the tolerant genotype could also be attributed to increased leaf area (Ashraf and Harris, 4), higher stomatal conductance and transpiration rate in the tolerant genotype (Rajametov *et al*., 19). These results also confirm that heat tolerance is a complex trait and is the cumulative effect of several traits leading to heat tolerance of a particular genotype. The WUE and transpiration ratio in this study were not significantly different between the tolerant and sensitive genotypes (Table 2), indicating that a heat tolerant genotype may not necessarily differ from sensitive genotype in all the heat tolerance contributing traits and same was also observed by Bhattarai *et al*. (5). High temperatures also resulted in increased cell membrane damage and leakage of solutes there by affecting the photosynthesis (Altenbach *et al*., 3). Heat-sensitive genotypes showed lower MSI (55.67%) and higher EL (44.33%) during the reproductive stage, which causes membrane dysfunction and poor genotype performance. These results are consistent with the previous results of Rajametov *et al.* (19) and Bhattarai *et al.* (5). The NDVI measures the health of green tissues and vegetation cover. We also observed a significant increase in NDVI in the heat-tolerant genotype (0.84). This finding conforms with earlier observations in *Capsicum annum* L. (Dobos *et al*., 10). Contrary to Shen *et al*. (21), we observed significantly reduced SD in the tolerant genotype (Table 2; Fig. 2), which appeared to be an adaptive mechanism in tolerant plants to minimise water loss under high-temperature..

The activity of antioxidant enzymes such as GPX (564.99 U/g FW), CAT (711.90 U/g FW), and protein content (13.38 mg/g FW) was significantly high in the DLS-161-1 suggesting that the tolerant genotype had an active biochemical mechanism to scavenge ROS generated due to heat stress (Table 3). When a plant is under stress, the peroxidation of membrane polyunsaturated fatty acids result in generation of malondialdehyde (MDA) and a high MDA content under stress represents increased sensitivity to the stress (Liu and Huang, 17; Rizhsky *et al*., 20). Heat-tolerant DLS-161-1 showed significantly lower accumulation of MDA than the DchBL-240, which further confirms an innate mechanism in DLS-161-1 to combat heat stress (Table 3).

To enhance thermotolerance, plants produce *HSPs* that preserve protein functionality, prevent aggregation, refold denatured proteins, eliminate harmful polypeptides, and restore cellular balance. Investigation on relative expression pattern of the six *HSP* genes did not reveal much difference in their expression pattern during ambient conditions. However, during heat stress conditions, *CaHSP2271* (26.8-fold) and *CaHSP3* (5-fold) were highly upregulated in the tolerant genotype (Fig. 3), which indicates that these stress proteins might be involved as a defence mechanism against the heat stress (Li *et al*., 16).

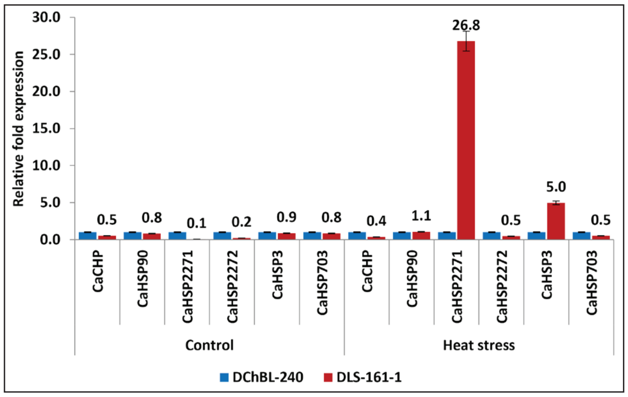

Fig. 3. Relative expression of different heat shock protein genes in *Capsicum annuum* L. heat-susceptible and tolerant genotypes under ambient (27°C) and heat stress (44°C) conditions.

Based on the results of the present study, it was concluded that plants evolve different physiological and biochemical mechanisms to better withstand heat stress. The identified heat-tolerant genotype can be further utilised in the breeding programme to develop heat tolerant varieties with superior agronomic performance.

AUTHORS' CONTRIBUTION

Conceptualization of research (MM, AS, PKJ); Designing of the experiments (AS, MM); Contribution of experimental materials (AS, BST, HY, AK, RP); Execution of the experiments and data collection (ATS, SS); Analysis of data and interpretation (ATS, MM, AS); Preparation of the manuscript (ATS, MM, AS, AK).

DECLARATION

The authors declare that they do not have any conflict of interest.

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