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Rapid induction of small heat shock proteins improves physiological adaptation to high temperature stress in peanut

K. Chakraborty* | S. K. Bishi* | A. L. Singh | P. V. Zala | M. K. Mahatma | K. A. Kalariya | R. A. Jat

ICAR-Directorate of Groundnut Research, Junagadh, Gujarat, India

Correspondence
K. Chakraborty, Department of Plant Physiology, ICAR-National Rice Research Institute, Cuttack, Odisha, India.
Email: koushikiari@gmail.com

Abstract

With the changing climatic scenario and increasing global mean temperature, heat stress became a major limiting factor for today's agriculture. To identify the underlying mechanism associated with heat tolerance in peanut, two experiments (field and growth chamber) were conducted with four genotypes (ICGS 44, GG 7, AK 159 and DRG 1) having differential high temperature stress sensitivity. Field grown plants under three different temperature (D_1, D_2 and D_3) regimes simulated three temperature treatment effects with a variability of 3–4/4–5°C in mean day/night temperature, respectively. In growth chamber, imposition of heat shock (10°C above ambient inside growth chamber) revealed not only rapid induction (within 0.5 hr) of HSPs, especially small HSPs (HSP 17, HSP 40) in tolerant genotypes, but also its sustenance for longer duration (2 hr), which might help them to have better physiological adaptation strategies under high temperature stress. This was evident from significant advancement in phenophases observed with increase in temperature by 15–18 days at physiological maturity, while pollen viability and membrane stability reduced below 50% and 41%, respectively in DRG 1 with increase in mean day/night temperature. Maintenance of higher photosynthesis and transpiration rate and stomatal conductance helped the tolerant genotype ICGS 44 to keep relatively cooler canopy and higher photosynthates, ensuring better physiological condition in this genotype under heat stress. Significant increase (~2.5-fold) in inositol and hexoses (glucose and fructose) content and reduction (>50%) in sucrose content in leaf tissues indicated degradation of storage carbohydrates for improved osmotic adjustment especially in tolerant genotypes under elevated temperature.

KEYWORDS
canopy temperature, groundnut, heat stress, leaf sugar profile, pollen viability, small heat shock protein

1 | INTRODUCTION

Being sessile in nature plants are more prone to external environment, which directly influences its growth and development. Due to rapid changes in climatic condition and unnatural rise in global mean temperature in recent past, today's agriculture has become vulnerable to number of abiotic stresses especially high temperature (HT) stress (Chakraborty, Bhaduri, Uprety, & Patra, 2014). The adverse effect of heat stress affects growth, development, metabolism and productivity of plants resulting in serious economic losses (Hasanuzzaman, Nahar, Alam, Roychowdhury, & Fujita, 2013). The projected 1.5–4.0°C rise in global mean temperature by the end of this century is expected to reduce agricultural yield by 15%–35% in Africa and Asia and by 25%–35% in the Middle East Asia (IPCC 2014; Ortiz et al., 2008). Peanut

*Both of these authors contributed equally for this work.

(also known as groundnut), mostly grown in tropical and sub-tropical semi-arid regions of the world, favours an optimal growth temperature in the range of 25–35°C (Cooper et al., 2009; Williams & Boote, 1995). A temperature rise beyond 35°C was reported to reduce pollen viability, fruit-set and thus yield of peanut crop (Vara Prasad, Craufurd, Kakani, Wheeler, & Boote, 2001).

Plants exposed to heat stress (at least 5°C above their normal growing condition) exhibit a characteristic set of cellular and metabolic responses required for the plants to survive under the HT conditions (Wahid, Gelani, Ashraf, & Foolad, 2007). High temperature in plants shortens the life cycle, increases senescence and severely affects the potential yields (Porter & Semenov, 2005). Limitation of plant growth under elevated temperature is reported due to physiological injuries viz. scorching of leaves, leaf senescence, pollen sterility, fruit damage and/or metabolic limitations such as reduction in C-assimilation, increased respiration and carbohydrate breakdown, increase in canopy temperature. (Bita & Gerats, 2013; Vollenweider & Günthardt-Goerg, 2005). Sensitivity of heat stress varies with intensity and duration of the stress, stages of development, species and genotypes with abundant inter- and intra-specific variations (Barnabás, Jäger, & Fehér, 2008; Sakata & Higashitani, 2008).

In most of the plants, ability to tolerate heat stress is directly associated with their ability to sustain leaf gas exchange characteristics by maintaining net photosynthetic rate and integrity of the cellular membranes as well as its pigment systems (Nagarajan et al., 2010; Scafaro, Haynes, & Atwell, 2010). The negative impact of heat stress in photosynthesis was characterized by the thermal injury to the enzyme Rubisco activase as well as altered partitioning of the net assimilates under stress condition (Ainsworth & Ort, 2010). Due to stomatal regulation of leaf gas exchange properties under heat stress, improved carbohydrate availability (both reducing and non-reducing) was reported to be a key physiological trait associated with HT stress tolerance (Liu & Huang, 2000). Efficient translocation from source to sink tissue through improved C-allocation and sugar signalling regulate HT tolerance (Roitsch & González, 2004). Cell wall and vacuolar invertase activity and increased sucrose import contributed to HT tolerance in tomato genotypes through increasing sink strength and sugar signalling activities (Li, Ji, Yang, Meng, & Guo, 2012). Dwivedi et al. (2017) reported efficient starch metabolism under heat stress regulates pollen viability and yield in wheat. Similarly, carbohydrate content of developing and mature pollen grains proved to be an important factor in determining pollen quality in heat-tolerant tomato cultivars (Firon et al., 2006).

Apart from the different physiological adaptive strategies, modulation of expression of different heat shock proteins (HSPs) was reported to be perhaps the most critical factors associated with heat tolerance in plants (Chen & Li, 2017). Based on molecular weight, heat shock proteins can largely be classified as large HSPs (60–110 kDa) and small HSPs (15–45 kDa) and it is thought to perform important physiological role in normal growing condition as well (Timperio, Egidi, & Zolla, 2008; Young, 2010). Under HT stress, misfolding or wrong aggregation of newly synthesized proteins and denaturation of existing proteins causes major setback to normal plant growth and development (Wahid et al., 2007). Thermo-stability of existing proteins and other macromolecular structures and prevention of newly synthesized proteins from denaturation were thought to be essential role of both small and large HSPs (Usman et al., 2014). In addition, larger HSPs, especially HSP 70 and HSP 90, were reported to act as molecular chaperone as they involved in up-regulation of several downstream genes associated with heat tolerance in plants (Kumar et al. 2016; Zhang et al., 2013).

In plants, increased production of these HSPs may be associated with abrupt rise (often referred to as heat shock) or gradual increase in temperature (heat stress) during growing period (Nover et al., 2001). Under HT stress, the expression pattern of these HSP genes with respect to time and intensity varies considerably not only between the species, but also within different genotypes of the same species (reviewed in Bita & Gerats, 2013). Although the basic responses of peanut genotypes in moderate and high temperature stress had been documented by previous workers, but that were mostly related to flowering behaviours, fruit-set and yield-related traits (Craufurd, Vara Prasad, Kakani, Wheeler, & Nigam, 2003; Vara Prasad, Boote, Hartwell Allen, & Thomas, 2003; Vara Prasad, Craufurd, & Summerfield, 1999, 2000; Vara Prasad et al., 2001). A comprehensive study on how these physiological traits are associated with expression of HSPs in terms of imparting HT tolerance in peanut still remains elusive. Hence, the objectives of this study were (i) to understand key physiological mechanism associated with HT tolerance in peanut, (ii) how the leaf level gas exchange traits and changes in leaf sugar profile contributes to HT stress tolerance and (iii) to understand the role of small and large HSPs in HT stress tolerance in peanut.

2 | MATERIALS AND METHODS

2.1 | Experimental setup

2.1.1 | Field experiment

To simulate three different temperature regimes (D_1, D_2 and D_3) during the crop growth period, more precisely at the time of sampling at flowering stage, the plant materials were sown in three different dates 20 January 2015, 10 February 2015 and 02 March 2015 by staggered sowing method, respectively. The experiment was conducted in the dry season from late winter to mid-summer (January–June 2015) in the research farm of ICAR-Directorate of Groundnut Research, Junagadh, India (21°31′N, 70°36′E), when an increasing pattern of temperature could be observed every year. The field experiment was conducted in two-factor split plot design with three replications and taking temperature regime as the main factor and genotype as the sub-factor.

2.1.2 | Growth chamber experiment

For growth chamber study, the same genotypes were sown in plastic pots having a mixture of soil, sand and perlite (1:1:1) and grown inside a walk-in type plant growth chamber (Model KEW/PGC-50;

Kaleidoscope Climatic Solutions, Bengaluru, India). The plants were grown in 12 hr day and 12 hr night cycle having day/night temperature and humidity of 35/25°C and 40/60% RH, respectively. At the time of imposition of heat stress (35 days after sowing), the temperature inside the growth chamber was elevated to 45°C (10°C above the normal day temperature) and the plants were exposed for a period of 2 hr.

2.2 | Plant material and sampling time

A group of popular peanut genotypes mostly grown in warmer region of India were screened for two successive dry seasons (2013 and 2014) and finally a panel of four differentially high temperature stress responsive (in terms of yield reduction under stress) genotypes viz. ICGS 44 (tolerant), GG 7 (moderately tolerant), AK 159 (moderately sensitive) and DRG 1 (sensitive) were selected (unpublished data) to study the mechanism and/or adaptive strategies in peanut genotypes under high temperature stress. To simulate three different temperature treatments, sampling was performed at a particular physiological stage (50% flowering stage) from each treatment of the temperature regimes. For growth chamber experiment, sampling was performed at 0, 0.5, 1.0 and 2.0 hr after imposition of stress (~10°C above ambient) and immediately proceeded for RNA extraction to study the induction pattern of heat shock proteins in leaves.

2.3 | Crop phenology and agronomic characteristics

Phenological observations viz. germination, flowering and physiological maturity for individual genotype × treatment combinations were recorded during the entire crop growth period. After physiological maturity, the economic yield was recorded and percentage yield loss was calculated for D_2 and D_3 condition in comparison with D_1.

2.4 | Pollen viability and membrane stability index

Fresh pollen grains were collected at 50% flowering stage and stained using freshly prepared 0.9% (w/v) MTT dye solution (thiazolyl blue (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl monotetrazolium bromide in 54% [w/v] sucrose). After 10 min of incubation, the viable cells reduced MTT to deep purple coloured formazan, which was scored under microscope (Model Olympus BX41, Tokyo, Japan). The data were expressed as mean values of at least 5 replications, where a single microscopic area (20×) was considered as a replication (Khatun & Flowers, 1995). Membrane stability index was estimated by measuring the electrical conductivity of 100 mg leaf tissue in 10 ml double-distilled water by heating at 40°C for 60 min (C_1) and 100°C for 10 min (C_2) as per the following formula (Chakraborty, Sairam, & Bhattacharya, 2012).

$$\text{MSI} = \left[1 - \left(\frac{C_1}{C_2}\right)\right] \times 100$$

2.5 | Canopy temperature, leaf gas exchange and chlorophyll fluorescence

Canopy temperature and gas exchange parameters viz. net photosynthetic rate (P_N), stomatal conductance (g_s) and transpiration rate (E) were measured using a portable photosynthesis system (Model LI-6400-XT; LI-COR, USA) between 09:30 and 11:30 hr (local time) in the third fully matured leaf on the main axis. Temperature was set at ambient and photosynthetically active radiation (PAR) was set at 1650 $\mu mol_{(photon)}\ m^{-2}\ s^{-1}$ inside the leaf chamber (composed of 90% and 10% red and blue light, respectively), and CO_2 was supplied artificially at a concentration of 400 $\mu mol\ m^{-2}\ s^{-1}$ inside the chamber with a stable flow rate of 400 μmol/s (Singh, Nakar, Chakraborty, & Kalariya, 2014). Chlorophyll fluorescence traits viz. maximum fluorescence (F_m), variable fluorescence (F_v) and maximum quantum efficiency of PS-II (F_v/F_m) were measured using plant efficiency analyzer (Model Handy PEA, Hansatech, USA) as per the method described by Havaux (1993). The selected leaves were dark adapted for at least 30 min using leaf clips before recording the observation. A saturating flash light (3,000 $\mu mol\ m^{-2}\ s^{-1}$) was applied to achieve the maximum fluorescence.

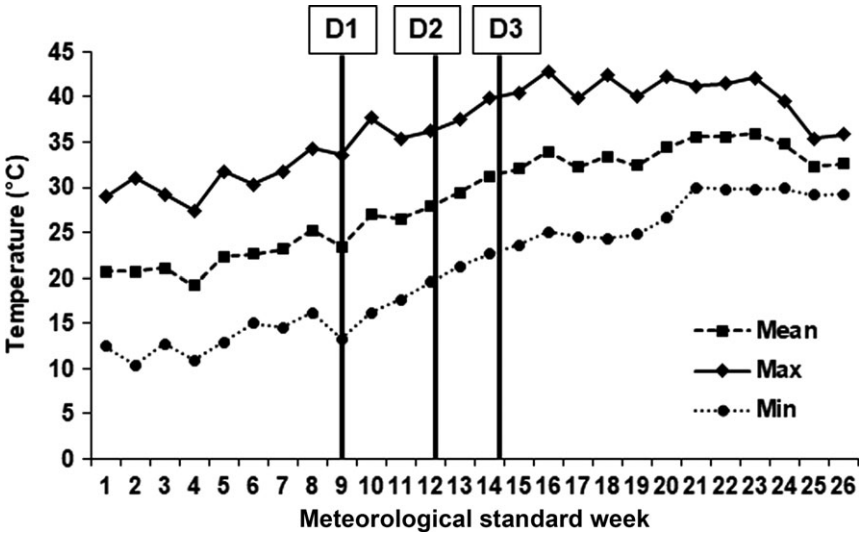

FIGURE 1 Changes in weekly mean air temperature during the crop growth period (2015). Here, the D_1, D_2, D_3 (as box and vertical bar) denote time of sampling at 50% flowering stage with respect to meteorological standard week for each temperature regime

2.6 | Leaf sugar profile by Ion Chromatograph

Quantification of oligosaccharide/sugars accumulation in leave tissues was estimated as described by Bishi et al. (2015). Leaf sugars were extracted in 80% ethanol from 500 mg fresh leaf tissues and the final volume was made up to 100 ml by MQ water. Sugars standards such as myo-inositol, glucose, fructose, lactose, sucrose, cellobiose, raffinose, stachyose and verbascose were obtained from Sigma Aldrich (USA) and Merck chemicals (India) and were adjusted to variable concentrations to get distinct peak height in the chromatogram. Lactose was used as internal standard during the analysis. About 25 μl of the membrane filtered samples were passed in a CarboPac PA10 Guard followed through the analytical column equipped with amino trap column in an Ion Chromatograph (Dionex, ICS 3000), using 150 mM NaOH as eluent with a flow rate of 1 ml/min. Data integration and interpretation were made with the help of the Chromeleon software provided with the equipment used.

2.7 | Gene expression studies

RNA isolation was made using RNeasy Kit (Qiagen, USA) following the manufacturer's protocol. Sample harvested at 0 hr was considered as control. The quality of the RNA was confirmed both in the agarose gel and by taking absorbance in Nanodrop Spectrophotometer (ND 1000). Three biological replicates were made to ensure the authenticity of the findings. Exactly 1 μg of total RNA from each biological replicate was used for cDNA synthesis using QuantiTech Reverse Transcription Kit (Qiagen). To confirm the cDNA synthesis, PCR amplification was made for each of the cDNA samples (~100 ng) as template and Ah-actin as primer. Further to ensure the specificity of the designed primers (see Table S1 for sequence details), PCR experiments were also conducted for each set of primers.

The relative transcript abundance of the HSP genes was determined by Real-Time quantitative PCR using QuantiFast SYBR Green PCR reaction kit (Qiagen) referring to the methodology followed by Chakraborty, Bishi, Goswami, Singh, and Zala (2016). The experiments were carried out in a StepOnePlusTM Real-Time PCR System (Applied Biosystem) following the reaction conditions such as: 95°C-5 min for 1 cycle; 95°C-10 s; and 60°C-30 s for 40 cycles. A melt curve analysis was carried out at the end of the PCR cycles to get the specificity of amplification. The relative fold changes in the transcript abundance of time-dependent heat responses in both tolerant and sensitive genotypes compared with their respective controls. The result was interpreted following the comparative $2^{-\Delta\Delta C_t}$ method, where the *Ah-actin* gene was used as internal control to normalize the PCR reactions (Schmittgen and Livak 2008).

2.8 | Statistical analyses

All the data recorded were the mean values of at least three independent experimental replications. The data were subjected to ANOVA appropriate to the experimental design, and differences at $LSD_{p = .05}$ were considered statistically significant (Gomez & Gomez, 1984). A two-tailed Duncan's multiple range test (DMRT) at 5% level of significance was carried out, and the statistically different values for each parameter were denoted by different alphabets.

3 | RESULTS

3.1 | Imposition of high temperature stress (HT) and effect on crop phenology

To compare the effect of high temperature stress at a particular growth stage, 50% flowering stage for each treatment (D_1, D_2 and

TABLE 1 Weekly mean temperature at the time sampling (at 50% flowering stage)

		Sampling period		
Treatments	Met. std. week	Max temp.	Min temp.	Mean temp.
D_1	9	33.6	13.3	23.5
D_2	12	36.2	19.5	27.9
D_3	14	40.4	23.0	31.7

Here, D_1, D_2 and D_3 represent three different temperature regimes created by three different sowing dates.

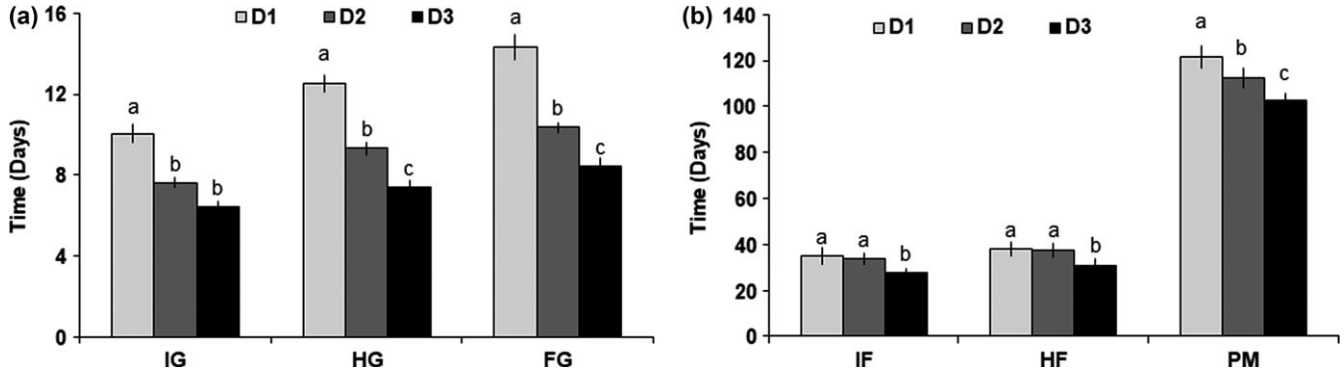

FIGURE 2 Changes in different phenophases (A, Germination and B, Flowering and maturity) of peanut genotypes when grown in three different temperature regimes (D_1, D_2 and D_3). Here, IG: initiation of germination; HG: half or 50% germination; FG: full or completion of germination; IF: initiation of flowering; HG: half or 50% flowering; PM: physiological maturity. Mean values sharing the same letter for each treatment, were not significantly different ($p \leq .05$) according to Duncan's multiple range test.

D_3, pointed out as vertical line and box in Figure 1) was considered. This resulted in a weekly mean day/night temperature variation of 33.6/13.3, 36.2/19.5 and 40.4/23.0°C, respectively for D_1, D_2 and D_3 condition at the time of sampling (Table 1). Growing plants in different temperature regime resulted in significant effect on germination process and the subsequent crop phenology (Figures 2 and 3). Mean time taken to initiate germination was reduced to 8 and 6 days, respectively in D_2 and D_3, as compared to 10 days in D_1. Similarly, number of days taken to attain 50% and full germination was also significantly hastened under higher temperature regime (Figure 2). At this stage, not much differences were observed between the studied genotypes exposed to HT stress (Table 2). Significant advancement in time of flowering was observed in D_3 as compared to D_1 and D_2 grown plants (Figure 3). The physiological maturity was hastened by 9 and 19 days, respectively in D_2 and D_3 as compared to D_1 grown plants. Least reduction in days to physiological maturity was observed in GG 7 (4 and 14 days, respectively, for D_2 and D_3), while for rest of the genotypes, the reduction was nearly same (Table 3).

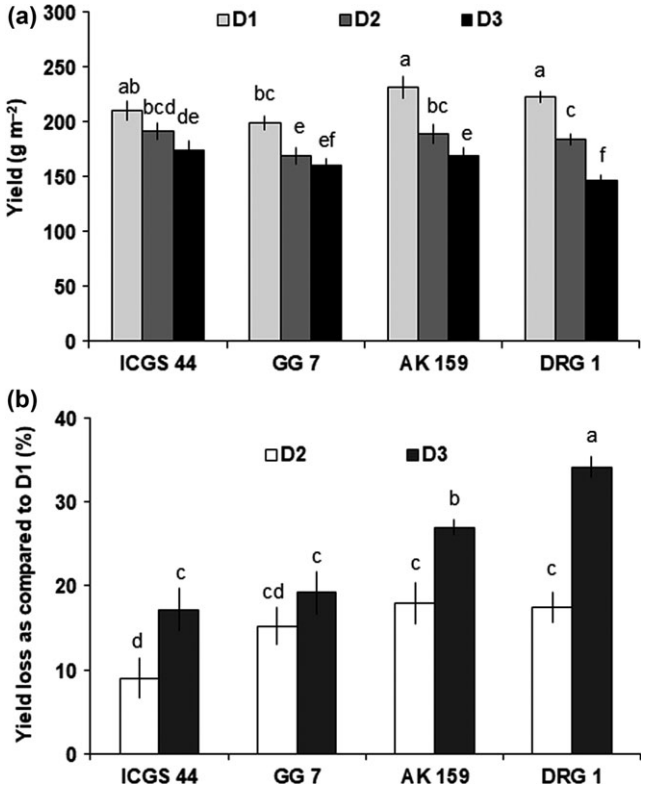

FIGURE 3 (A) Changes in pod yield of peanut genotypes grown in three different temperature regimes (D_1, D_2 and D_3). (B) Percentage yield loss with rising temperature taking D_1 as control. Mean values sharing the same letter for each treatment × variety combination, were not significantly different ($p \leq .05$) according to Duncan's multiple range test.

3.2 | Effect of HT on yield

Yield or economic biomass production in peanut genotypes was significantly affected by HT stress (Figure 3A). With ~7/10°C increase in mean day/night temperature (from D_1 to D_3), average yield drop recorded was as high as 15% and 25%, respectively in D_2 and D_3 condition in comparison with D_1 yield. Among the genotypes, ICGS 44 suffered least yield loss in the tune of 9% and 17% for respective temperature rise in D_2 and D_3 treatments (compared to D_1), while the loss was almost double (17% and 35%, respectively) in the most sensitive genotype DRG 1 under HT stress (Figure 3B).

3.3 | Effect of HT on pollen viability, membrane stability, plant canopy temperature and canopy-air temperature difference

Drastic reduction in pollen viability was observed with increase in mean day/night temperature. When stained with MTT dye, a distinctly visible alive (cells stained dark) and dead (cells stained light) pollen cells were observed under the microscope (Figure 4A). Pollen viability decreased with increase in temperature especially in AK 159 and DRG 1. More than 85% pollen viability was observed in all the genotypes in D_1, but in D_2 and D_3, it declined significantly (Figure 4B). Maximum decline was observed in DRG 1 (~24% and 43% in D_2 and D_3 condition, respectively), while the tolerant genotypes ICGS 44 and GG 7 showed <15% and <25% reduction in pollen viability in D_2 and D_3 condition, respectively.

TABLE 2 Genotypic differences in time of germination in peanut under D_1, D_2 and D_3 conditions

	Initiation of germination (days)			50% Germination (days)			Full germination (days)		
Cultivar	D_1	D_2	D_3	D_1	D_2	D_3	D_1	D_2	D_3
ICGS 44	10 (0.8)	8 (0.7)	6 (0.7)	13 (1.7)	9 (0.8)	7 (0.9)	14 (0.9)	10 (0.9)	8 (0.5)
GG 7	9 (0.6)	7 (0.9)	5 (0.8)	11 (0.9)	9 (1.1)	6 (0.5)	12 (1.1)	10 (0.7)	7 (0.6)
AK 159	10 (1.1)	7 (0.6)	6 (0.5)	12 (1.1)	9 (1.2)	7 (0.8)	13 (1.0)	10 (1.2)	8 (0.4)
DRG 1	9 (0.8)	7 (0.8)	5 (0.7)	12 (0.8)	8 (0.7)	6 (0.6)	13 (0.8)	9 (0.8)	7 (0.5)
$LSD_{p=.05}$ (V)	NS			NS			0.8		
$LSD_{p=.05}$ (T)	0.58			1.1			1.2		
$LSD_{p=.05}$ (V × T)	0.81			1.9			2.1		

Here, the values represent mean ± *SE*. Values in the parenthesis indicate standard error of mean (*SE*).

TABLE 3 Genotypic differences in time of flowering and physiological maturity in peanut under D_1, D_2 and D_3 temperature regimes

	Initiation of flowering (days)			50% Flowering (days)			Physiological maturity (days)		
Cultivar	D_1	D_2	D_3	D_1	D_2	D_3	D_1	D_2	D_3
ICGS 44	33 (1.7)	32 (1.9)	27 (2.1)	36 (1.2)	35 (0.9)	30 (1.2)	118 (3.4)	113 (1.9)	102 (4.3)
GG 7	35 (1.9)	35 (2.3)	29 (2.4)	38 (1.1)	38 (1.6)	31 (0.8)	120 (4.1)	116 (2.2)	106 (3.9)
AK 159	33 (1.8)	33 (2.0)	27 (1.9)	36 (2.0)	37 (1.1)	30 (1.1)	117 (2.9)	110 (3.6)	101 (2.7)
DRG 1	34 (1.6)	34 (2.2)	27 (1.3)	37 (1.8)	37 (1.4)	30 (0.9)	120 (3.1)	113 (2.9)	102 (3.1)
$LSD_{p=.05}$ (V)	NS			NS			4.2		
$LSD_{p=.05}$ (T)	2.1			1.9			6.1		
$LSD_{p=.05}$ (V × T)	3.2			2.8			9.8		

Here, the values represent mean ± *SE*. Values in the parenthesis indicate standard error of mean (*SE*).

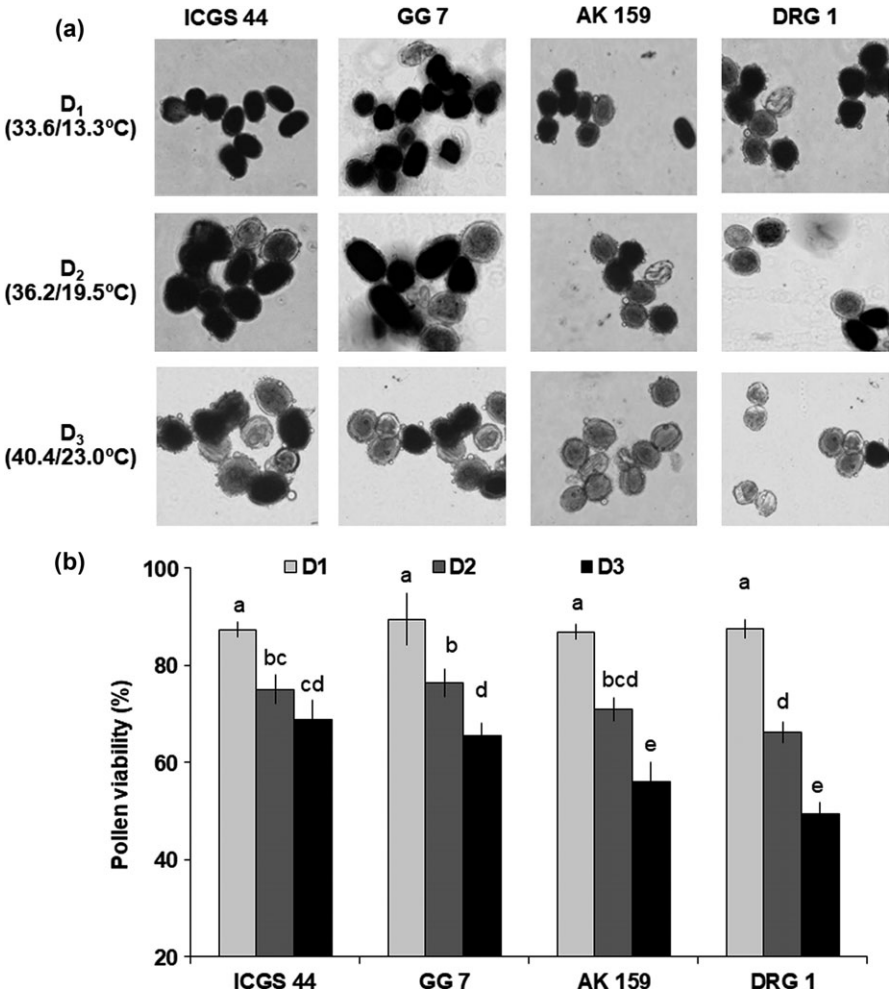

FIGURE 4 (A) Photomicrograph (20×) of pollen cells stained with MTT dye. Individual images shown are typical representation of one of five microscopic fields. The temperature indicated in the parenthesis denotes mean day/night temperature at the time of sampling. (B) Changes in pollen viability (%) under D_1, D_2 and D_3 temperature regimes. Mean values sharing the same letter for each treatment × variety combination, were not significantly different ($p \leq .05$) according to Duncan's multiple range test.

Membrane stability index (MSI), a key determinant of cell membrane integrity, was severely affected by HT stress (Figure 5A). The MSI reduced from 75% to 80% (in D_1 treatment) to <50% in all genotypes in D_3 treatment (except ICGS 44). The highest reduction in MSI was observed in DRG 1 (reduced to mere 41% in D_3), while least reduction (reduced to 54% in D_3) was observed in most tolerant genotype ICGS 44. With the increase in atmospheric temperature, both canopy temperature and canopy-air temperature difference (CTD) were increased in all the genotypes (Figure 5B,C). Among the genotypes, 5–9°C rise in plant canopy temperature was observed in D_3 condition as compared to D_1 condition. Heat-tolerant genotype ICGS 44 recorded least increase in canopy temperature (5.8°C), both AK 159 and DRG 1 showed the highest increase (>8.2°C). The CTD varied from 1.4°C to 2.5°C in D_1 across the genotypes, whereas it ranged from 3.5°C to 4.7°C in D_3. For individual genotypes, ICGS 44 showed the least increase in CTD (0.6°C and 1.6°C rise in D_2 and D_3,

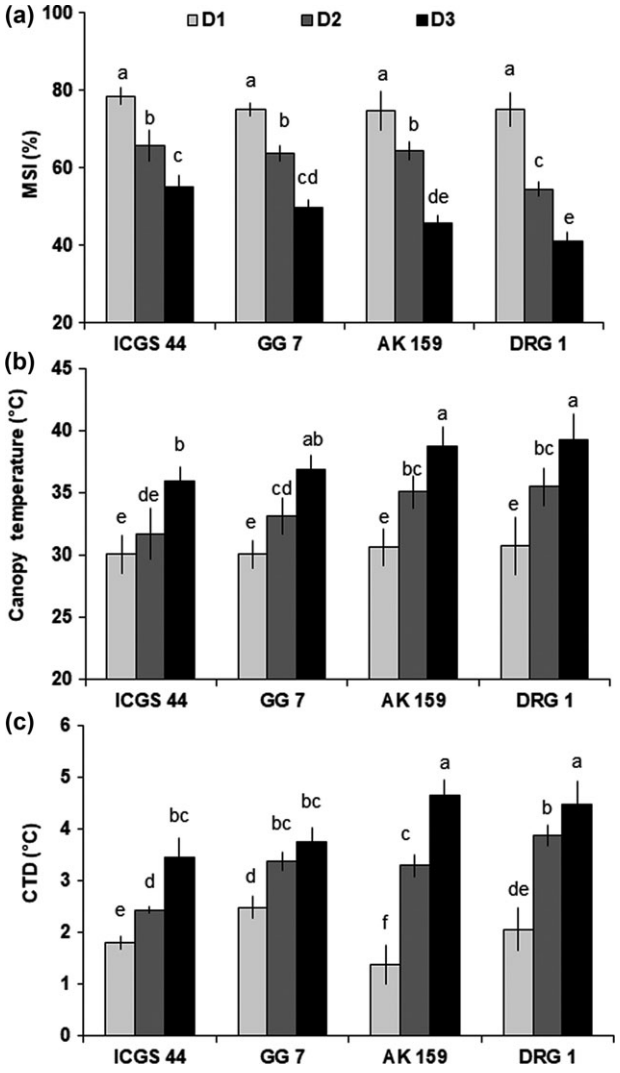

FIGURE 5 Changes in (A) membrane stability index (MSI) (B) plant canopy temperature (°C) and (C) canopy-air temperature difference (CTD) in peanut genotypes when grown in three different temperature regimes (D_1, D_2 and D_3). Mean values sharing the same letter for each treatment × variety combination, were not significantly different ($p \leq .05$) according to Duncan's multiple range test.

respectively) from 1.81°C in D_1 treatment, while the highest increase was observed in AK 159 (1.9°C and 3.3°C rise in D_2 and D_3, respectively) from a relatively lower CTD of 1.38°C in D_1 condition.

3.4 | Effect of HT on leaf gas exchange traits and chlorophyll fluorescence

Most of the leaf gas exchange parameters viz. net photosynthesis rate (P_N), stomatal conductance (g_s) and transpiration rate (E) were significantly affected by HT stress (Figure 6). Among the studied traits, P_N was the most severely affected trait by HT stress. Relatively sensitive genotypes DRG 1 and AK 159 showed >40% and ~75% reduction in P_N in D_2 and D_3, respectively as compared to D_1, while the reductions were significantly lower in ICGS 44 and GG 7 showing <25% and <50% reduction under similar conditions (Figure 6A). Like P_N, g_s also showed huge reduction under HT stress, especially in sensitive genotypes. The genotype AK 159 showed highest reduction (~45% and 80%, respectively for D_2 and D_3 condition) in g_s, while the other sensitive genotype DRG 1 recorded >40% and >70% reduction, respectively (Figure 6B). Least reduction in g_s was observed in GG 7, which showed <10% and <20% reduction under D_2 and D_3 condition, respectively.

Transpiration rate (E) also dropped significantly under the influence of HT stress (Figure 6C). Like g_s, the highest decline in E was also observed in AK 159 (32% and 56%, respectively for D_2 and D_3), the least decline was observed in ICGS 44 (<10% reduction in both D_2 and D_3 treatments). Significant changes in chlorophyll fluorescence characteristic (measured in terms of F_v/F_m) were observed under HT stress (Figure 6D). The genotypic differences were significant in D_1 and D_2, while the extent of reduction varied in tolerant and sensitive genotypes in D_3. ICGS 44 could maintain the highest F_v/F_m value (0.64), whereas it was reduced to 0.55 in DRG 1 under D_3 condition.

3.5 | Changes in leaf sugar profile

High temperature stress resulted in significant alteration in leaf sugar profile in all the peanut genotypes (Figure 7). Inositol content in the leaf tissues increased significantly in most of the genotypes (except, DRG 1). The increase was as high as ~1.5- and ~2.5-fold in ICGS 44 under D_2 and D_3, respectively in comparison with D_1 (Figure 7A). A moderate increase of ~30% and 50%, respectively, was observed for GG 7 and AK 159 under similar conditions. Unlike inositol content, the sucrose content in the leaf was reduced with increase in temperature (Figure 7B). Although similar extent of reduction was observed for all the genotypes, but ICGS 44 was found to have highest sucrose content (1,945 ppm) under D_3 condition, which was significantly higher compared to sensitive genotypes viz. DGR 1 (1,141 ppm) and AK 159 (1,239 ppm).

In contrast to the non-reducing sugar sucrose, its breakdown products (glucose and fructose) were increased under HT stress. Both the tolerant genotypes showed >60% increase in glucose content in both D_2 and D_3, whereas DRG 1 recorded mere 9% and 28% increase under similar condition (Figure 7C). On the other hand, highest increase in fructose content was observed in GG 7 (~155% and 180% for D_2 and D_3, respectively), whereas the increase was least in case of AK 159 (~20% and 45%, respectively for similar conditions) (Figure 7D).

3.6 | Expression of heat shock proteins (HSPs)

In the second set of experiments (inside growth chamber), the effect of heat shock on expression profile of different reported peanut HSPs was studied and it was found that rapid induction of HSPs (particularly HSP 17 and HSP 90) was correlated with long-term HT stress tolerance in peanut genotypes (Figure 8). The transcript abundance of HSP 17 significantly increased particularly in ICGS 44 (15.7-, 16.3- and 18.9-fold after 0.5, 1.0 and 2.0 hr of HT stress

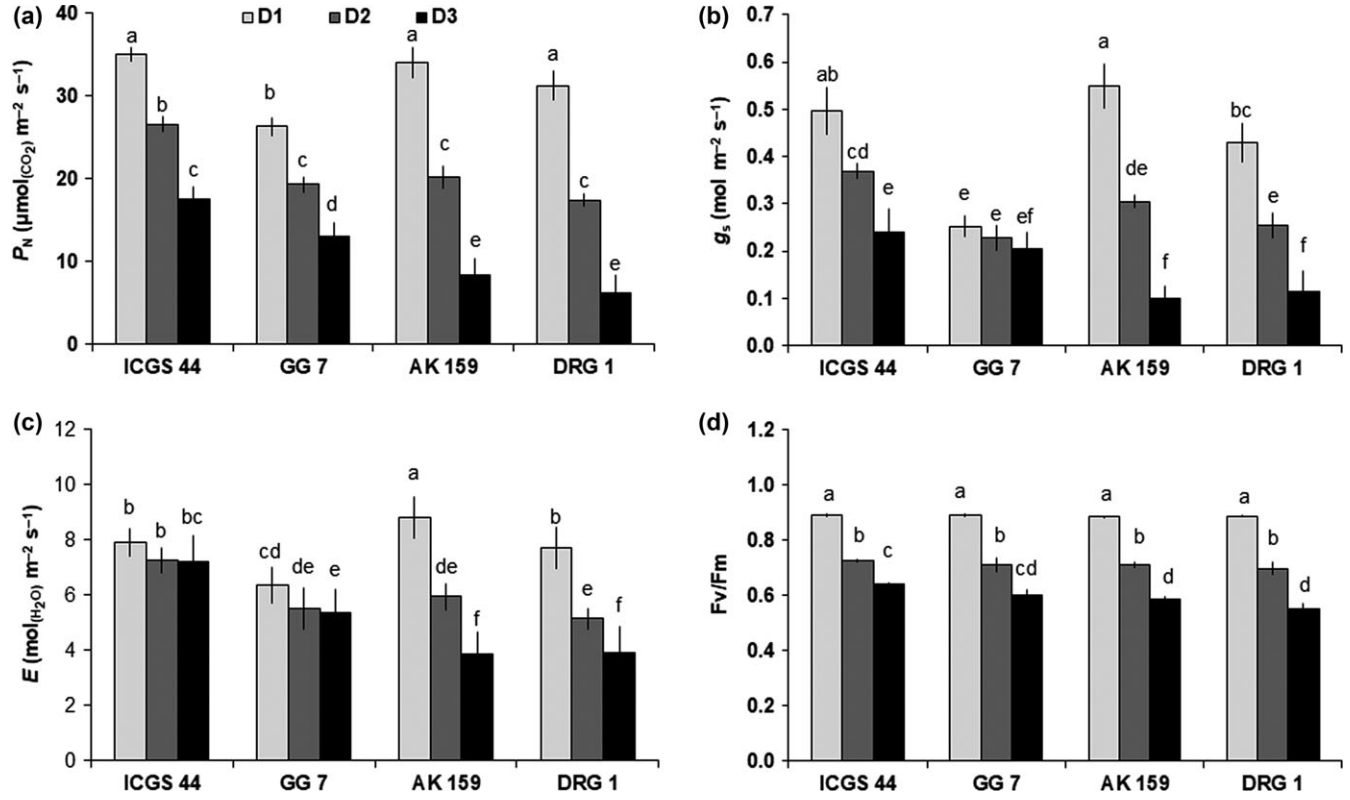

FIGURE 6 Changes in (A) net photosynthesis rate (P_N), (B) stomatal conductance (g_s), (C) transpiration rate (E) and (D) maximum quantum yield (F_v/F_m) in peanut genotypes when grown in three different temperature regimes (D_1, D_2 and D_3). Mean values sharing the same letter for each treatment × variety combination, were not significantly different ($p \leq .05$) according to Duncan's multiple range test.

imposition, respectively). An induction of much lesser extent was observed in rest of the genotypes with onset of stress (Figure 8A). Another small HSP, HSP 40 also showed similar pattern of induction (Figure 8B). Very rapid induction (within 0.5 hr of stress imposition) of HSP 40 was observed in ICGS 44, which went on increasing with duration of stress, but in other genotypes, it either showed an initial rise and sustained at that level (GG 7) or did not show much induction (AK 159 and DRG 1).

Unlike small HSPs, larger molecular weight HSPs viz. HSP 70 showed an initial rise (~3.0–3.5 fold) in transcript level, which remained stable in the most tolerant genotype ICGS 44 (Figure 8C). The induction was quite low (<2.0-fold) in both the sensitive genotypes (DRG 1 and AK 159). The transcript abundance of HSP 90 showed very high initial induction (>15-fold) within 0.5 hr of HT stress imposition, but the induction level came down significantly with longer duration. GG 7 showed an increasing induction pattern of HSP 90 till 1 hr of stress imposition, while the induction level was much lower (<2.0-fold) for both AK 159 and DRG 1 (Figure 8D).

4 | DISCUSSION

Increase in external atmospheric temperature beyond a certain threshold level causes impairment of plant growth and development alongside severe yield losses for agricultural crops (Porter, 2005). Threshold temperature for considering it as high temperature (HT) stress may vary significantly between the crop species (Allakhverdiev et al., 2008; Mittler, Finka, & Goloubinoff, 2012). In the present study, growing the plants in different temperature regime affected the whole process of plant growth and development, which resulted in advancement of all the phenological stages of peanut starting from initiation of germination to physiological maturity. Hence, the temperature effect was studied by sampling the plants at a particular growth stage (50% flowering stage in this case) and days to attain that stage varied significantly in different temperature regimes. Most of the physiological parameters including pollen viability and membrane stability affected significantly under heat stress, in which a differential response was observed between HT stress sensitive and tolerant genotypes. In the present study, we tried to explain the HT stress responses of peanut genotypes grown under field condition in terms of induction pattern of known HSPs by exposing them to heat shock in growth chamber condition, which are explained categorically in the following sections.

For most of the tropical C3 crops, a temperature range of 35–40°C is often considered to be moderate heat stress, while temperature above 40°C considered as severe heat stress (Larkindale, Mishkind, & Vierling, 2005). Peanut, a semi-arid warmer climate crop, has an optimum growing temperature of 25–35°C, but can tolerate a

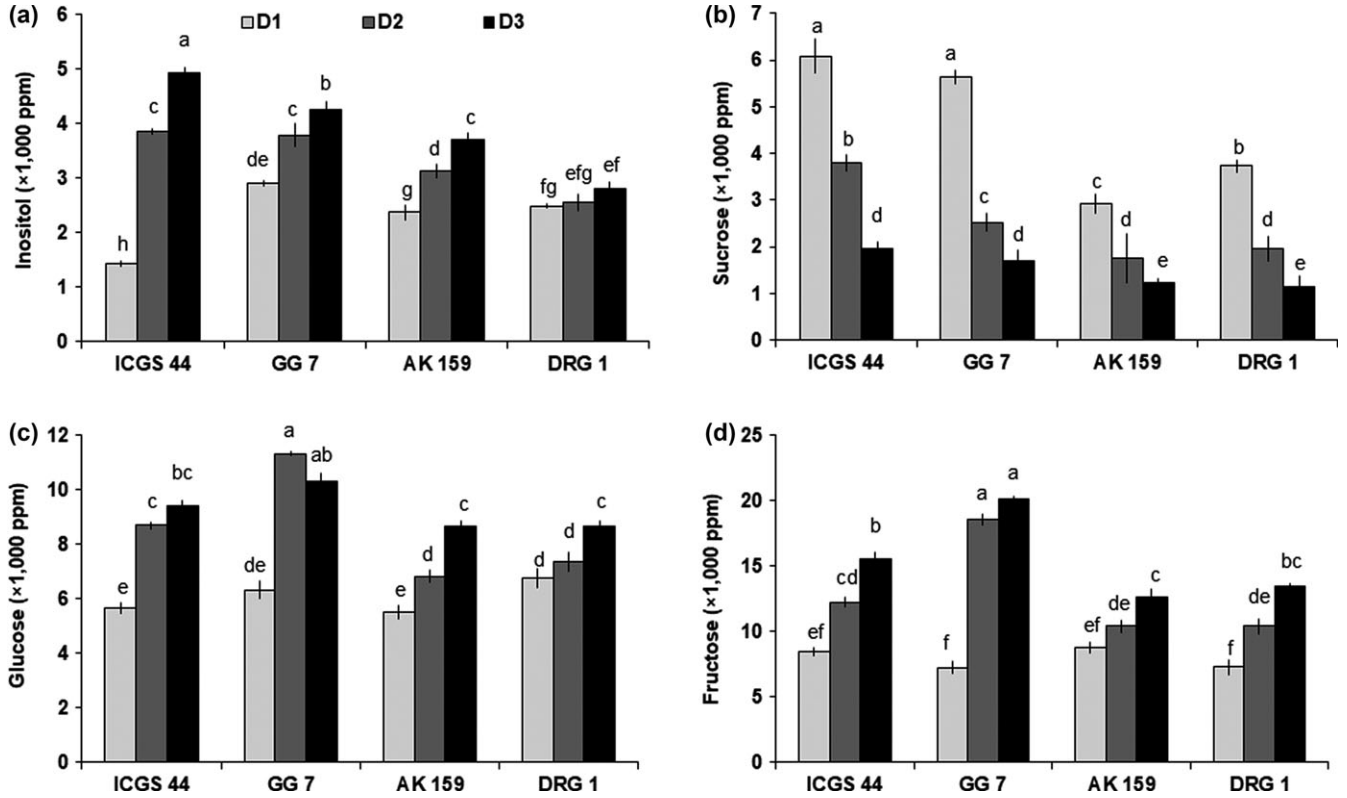

FIGURE 7 Changes in leaf carbohydrate profile (A) inositol, (B) sucrose, (C) glucose and (D) fructose in peanut genotypes when grown in three different temperature regimes (D_1, D_2 and D_3). Mean values sharing the same letter for each treatment × variety combination, were not significantly different ($p \leq .05$) according to Duncan's multiple range test.

temperature level as high as 40°C (Williams & Boote, 1995). Prolonged exposure to HT particularly at sensitive phases viz. germination and flowering results in reversible metabolic suppression and crop growth, ultimately leading to significant compromise in yield (Craufurd et al., 2003; Vara Prasad et al., 1999). In the present study, rise in temperature beyond 40°C (in D_3 condition) resulted in significant changes in crop phenology, which was evident from advancement of entire crop duration including germination, flowering and physiological maturity. As every crop species has a particular temperature requirement to complete each phenophases (Luo, 2011), hence it is quite obvious that we got shorter crop duration with subsequent rise in temperature regime during crop growth period in the present study. Shorter growing period and temperature-induced restriction in metabolic activities may have contributed to significant yield loss in peanut genotypes. HT stress at critical growth stages reported to show severe yield losses in peanut (Vara Prasad et al., 2001). Also, with shortening of the growth phases, more particularly, the vegetative phase results in lesser build-up of tissue reservoir and less source to sink translocation results in reduced pod yield in peanut (Akbar, Manohar, Variath, Kurapati, & Pasupuleti, 2017). Our results also showed similar reduction in yield especially in sensitive genotypes (DRG 1 and AK 159), when temperature went beyond 40°C at flowering stage. But, the tolerant genotypes showed quite less reduction in yield without much difference in phenophases.

Earlier studies on peanut reported temperature-induced male sterility as one of the key factors for yield loss (Craufurd et al., 2003; Vara Prasad et al., 2003). In the present study, we found significant reduction in pollen viability with increased temperature regime. Comparatively, much higher reduction was observed in the sensitive genotypes, which probably reflected in the significantly greater yield loss in those genotypes. Previous reports on peanut cv. Robut 33-1 suggested at least 50% reduction in pod yield when exposed to a mean day/night temperature regime of 36/25°C as compared to 27/17°C and an increase in day air temperature from 30/22°C to 35/22°C resulted in 33% reduction in pollen viability and fruit number (Ketring, 1984; Ong, 1984). Vara Prasad et al. (2000) suggested that critical floral bud temperature for fruit-set in peanut is 36°C, above which severe yield compromise could be observed. Increase in air temperature, particularly warmer night temperature, reduces pollen viability considerably in peanut (Vara Prasad et al., 2003). Indian peanut genotypes (ICGS 44 and GG 7) being more adapted to warmer climatic condition showed relatively less HT stress damage and showed moderate yield reduction (~20%) even when grown under highest temperature regime in the present study. Integrity of cell membrane is another key factor under HT stress. Increased kinetic energy of the molecules and changes in the lipid composition of the biological membranes results in increased permeability of the lipid bilayer of cell membrane, thereby promoting electrolyte leakage (Wahid et al., 2007). Considerable drop in MSI

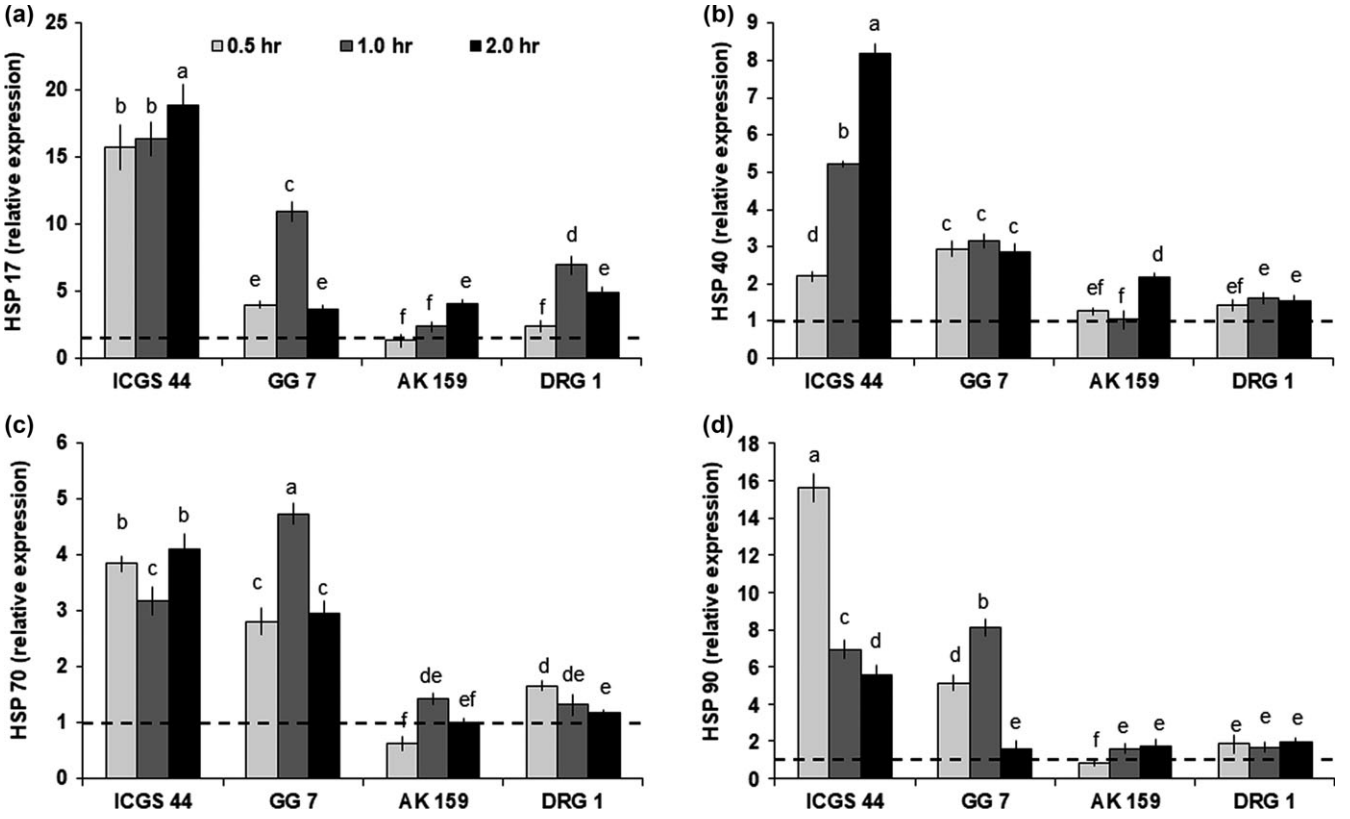

FIGURE 8 Temporal changes (0.5, 1.0 and 2.0 hr) in expression pattern of small and large HSPs in peanut genotypes exposed to 10°C above ambient (35°C) heat shock inside growth chamber. Relative expression level of (A) HSP 17, (B) HSP 40, (C) HSP 70 and (D) HSP 90 normalized using *Ah-actin* as internal control and compared with respect to basal expression level (0 hr) indicated as dotted line. Mean values sharing the same letter for each treatment × variety combination, were not significantly different ($p \leq .05$) according to Duncan's multiple range test.

was observed especially in DRG 1 and AK 159, and the reduction was significantly higher in these genotypes as compared to ICGS 44. Better maintenance of MSI in ICGS 44 under HT stress might consider to be one of the probable reasons for its lesser sensitivity to HT stress.

Under HT stress, net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (E) and F_v/F_m significantly reduced in peanut genotypes in the present study. Rate of photosynthesis and stomatal aperture under HT stress is known to be regulated by the rate of CO_2 assimilation in the mesophyll cell as well as the effectivity of photosystems (Wise, Olson, Schrader, & Sharkey, 2004). Due to decrease in the activation state of RUBISCO coupled with relative increase in oxygenase property of the enzyme, P_N and g_s showed inhibition under HT stress (Morales et al., 2003; Wahid et al., 2007). In the present study, we found drastic reduction in P_N, g_s and E under both 36.3/19.5 and 40.4/23.0°C temperature regimes, while the maximum efficiency of PSII also reduced significantly. Increased stomatal closure with the rise in surrounding air temperature might affect the overall plant water status, which led to reduction in transpiration rate in peanut genotypes. The tolerant genotype ICGS 44 not only maintained highest leaf gas exchange capacity, but also had maximum efficiency of PSII when grown beyond 40°C. It indicated that ICGS 44 had least stomatal as well as pigment system-mediated inhibition of gas exchange under HT stress. Due to maintenance of highest transpiration rate by ICGS 44 in D_3 condition, it could significantly cool down its canopy (as evident from lower CT and CTD data) and maintain a favourable metabolic status inside the leaf tissue under HT stress. Maintenance of cooler canopy temperature (low CT and CTD) is often thought as a mechanism to maintain better water status and escape heat stress as reported in other crop species (Fu et al., 2016; Rebetzke, Rattey, Farquhar, Richards, & Condon, 2013).

The potential of a genotype to synthesize organic osmolytes under HT stress is directly associated with its ability to maintain better water status and providing more membrane stability, thereby limiting stress to a certain extent (Rasheed, Wahid, Farooq, Hussain, & Basra, 2011). While analysing the sugar profile of the leaf, we observed sharp rise in inositol and soluble hexoses content (glucose and fructose), known to play key role in osmotic adjustment especially in the tolerant genotypes. On the other hand, sucrose content was decreased with temperature stress. This could well be due to (i) limitation of P_N and lesser photoassimilate production under HT stress or (ii) breakdown of carbohydrate reserve of mesophyll tissue for biosynthesis of other organic osmolytes under resource-limiting condition. HT-induced increase in organic osmolyte was reported in many crops (Ashraf & Foolad, 2007; Wahid &

Close, 2007), while some reported stress-induced reduction in sucrose content mostly due to photosynthetic limitation in plants (Hussin, Geissler, & Koyro, 2013; Sassi-Aydi, Aydi, & Abdelly, 2014).

Although, the heat shock proteins (HSPs) are normal feature of plant cell due to its requirement in different physiological and developmental processes, but altered expression pattern of it had been reported under various abiotic stresses, especially under HT stress (Pratt, Krishna, & Olsen, 2001; Usman et al., 2014; Young, 2010). In the second experiment, when the plants were exposed to 45°C heat stress (10°C above the ambient growing condition), sharp increase in both small (HSP 17 and HSP 40) as well as larger molecular weight (HSP 70 and HSP 90) HSPs was observed. Both ICGS 44 and GG 7 not only showed considerably higher induction just after stress imposition, but also sustained higher induction pattern for longer duration of time as compared to DRG 1 and AK 159. This ability of the tolerant genotypes for prompt induction of HSPs probably helped them to counteract HT stress better by binding to the thermolabile proteins and macromolecules and preventing them from misfolding or other heat injuries. Definite role of small HSP for preventing wrong aggregation or irreversible unfolding of partially denatured proteins was established in many crops under heat stress (Goswami et al., 2016; Lee et al., 2007; Xu, Zhan, & Huang, 2011). Besides, both HSP 70 and 90 play key role as molecular chaperon and known to modulate downstream gene expression under heat stress (Duan et al., 2011; Zhang et al., 2013).

5 | CONCLUSION

Taken together, the present study gives an in-depth report on physiological and molecular mechanism of HT stress tolerance in peanut genotypes. Comparatively, lesser temperature-induced male sterility and better membrane stability under HT stress at flowering stage found to be one of the key determinants of yield stability in peanut. Improved plant water status through osmotic adjustment as well as leaf gas exchange traits enables the tolerant genotypes to maintain cooler crop canopy and thereby better physiological and metabolic condition in tolerant genotype under HT stress. Early induction and longer sustenance of both small and large HSPs resulted in lesser heat damage to the key macromolecules inside the cell and might also promote the downstream expression of important thermo-tolerance genes/proteins in tolerant peanut genotypes. How each HSP is functioning and what are the most important mechanism affected by these HSPs under HT stress in peanut will be our future course of study.

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ORCID

K. Chakraborty http://orcid.org/0000-0001-7081-4681

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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