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RESEARCH ARTICLE

Impact of heat stress on physiological characteristics and expression of heat shock proteins (HSPs) in groundnut (*Arachis hypogaea* L.)

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Abstract

The current climate change has a profound impact on agricultural production. Despite the unanimous efforts of several nations to prevent further increase in global temperatures, developing adaptive strategies by imparting heat tolerance in crop plants is essential to ensure global food security. This study demonstrates the impact of heat stress on the morphological, physiological and biochemical properties of different groundnut genotypes derived from a recombinant inbred line (RIL) population (JL 24×55–437). The plants were grown in controlled conditions and a high-temperature stress of 45 °C was gradually imposed by placing the plants in an environmental chamber during peak reproductive stage [25 days after sowing (DAS) to 60 DAS]. Heat tolerant genotypes had better biochemical machinery to withstand the heat stress-induced oxidative burst with higher activity of catalase and peroxidase. Also, the tolerant genotypes had lesser membrane damage as indicated by lower malondialdehyde levels. Greater expression of heat shock proteins (*HSP17*) transcripts alongside elevated levels of both enzymatic and non-enzymatic antioxidant activity was observed when exposed to high temperature, indicating their potential association with heat stress tolerance in groundnut.

Keywords Peanut · Global warming · High temperature tolerance · Environmental chamber · Heat stress responsive genes · Malondialdehyde (MDA) · HSPs

Introduction

Global warming is driving climate change at a significant rate (Lorenz et al. 2019). The Intergovernmental Panel on Climate Change (IPCC) projected a 1.5 °C rise in global temperature between 2021 and 2040. Approximately 90% of global groundnut production occurs in semi-arid regions, characterized by persistent high temperatures and water scarcity. Crop modelling studies, particularly in the southern states of India, have indicated that these regions face significant challenges not only in terms of yield but also in maintaining crop quality and nutritional value, particularly under heat stress (Prasad et al. 2010; Yang et al. 2020; Kadiyala et al. 2021). The availability of cultivated groundnut genome sequence (Bertioli et al. 2019) has enabled researchers to identify genes and favourable alleles for improved stress adaptation, enhanced nutritional value, and increased productivity, thereby contributing to global food security and addressing malnutrition in low-income countries (Aravind et al. 2022). The ideal temperature range for groundnut

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growth is between 28 and 30 °C, with a base threshold temperature of 10 °C. Temperatures beyond the optimal range negatively impact seed germination and seedling emergence (Prasad et al. 2000) and morphological traits, such as plant height and root growth, leading to yield loss in groundnut (Dash et al. 2020). The reproductive stages including flower initiation, flowering, peg formation and early pod formation are particularly sensitive to high-temperature stress in groundnut leading to direct yield losses (Prasad et al. 1999; Craufurd et al. 2003; Puppala et al. 2023).

Studies on the parameters measuring solute leakage, chlorophyll fluorescence (Chauhan and Senboku 1997) and membrane thermostability (Talwar et al. 1999) during heat stress serve as effective screening tools and indicators of heat tolerance. Chlorophyll Stability Index (CSI) stands as the direct measure of thermotolerance in terms of chlorophyll stability, generally genotypes with high CSI are tolerant to heat stress (Kohila and Gomathi 2018). Heat stress can disrupt the lipid bilayer structure of cell membranes, leading to the displacement of membrane-associated proteins. Therefore, measuring cell membrane stability under heat stress conditions in terms of membrane injury index (MII) gives the relative injury percentage and is a useful trait in screening genotypes for heat stress tolerance. A stable membrane supports uninterrupted photosynthesis and efficient translocation of photosynthates, contributing to high yield under heat stress conditions. For efficient photosynthesis, the leaf area is also very important to harvest maximum sunlight. Hence, high specific leaf area (SLA) is indicative of widespread foliar display to absorb more light which naturally increases evaporative demand and is considered as an important indicator of yield under normal conditions (Wright et al. 2004; Cheng et al. 2016). Specific leaf weight (SLW) is the inverse of SLA, in turn represents the thickness of the leaf (Nageswara et al. 2001; Songsri et al. 2008). Leaf thickness and leaf weight were significantly reduced at the plant's maturity under high-temperature conditions (Bala and Sikder 2018).

Heat stress induces oxidative burst by production of highly reactive oxygen species (ROS) which causes injury to protein and nucleic acid (Choudhury et al. 2017). ROS acts as a signalling molecule, triggering stress response mechanisms in plants to adapt under stressful conditions (Choi et al. 2017). Decrease in activity of enzymatic antioxidants viz., catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) increases ROS accumulation in plant cells (Suzuki et al. 2011; Das and Roychoudhury 2014). Malondialdehyde (MDA), generated through lipid peroxidation due to ROS, serves as a reliable indicator of oxidative damage to biomembranes (Savicka and Skute 2010; Djanaguiraman et al. 2018). MDA content is found to increase in thermo-sensitive genotypes in comparison to thermo-tolerant genotypes and has a significant correlation with electrolyte leakage (Wilson et al. 2014; Sharma et al. 2023). Carotenoids play a vital role in protecting chlorophyll pigments from oxidation by quenching the surplus light quanta by acting as a non-enzymatic antioxidant (Camejo et al. 2006). Temperature Induction Response (TIR) is yet another parameter to evaluate genotypes for thermotolerance at the germination stage. The seeds to be screened are exposed to lethal temperatures, followed by recovery under optimum temperature to measure and categorize thermotolerance based on recovery percentage. In groundnut, temperature-induced seedlings demonstrated better recovery compared to those directly exposed to lethal temperatures (Gangappa et al. 2006; Kokkanti et al. 2019).

The production of heat shock proteins (HSPs) in plants is induced by high temperatures (Nakamoto and Hiyama 1999; Ellis 2006). Heat stress induces the formation of misfolded aggregated proteins, which are subsequently ameliorated by chaperones, particularly large HSPs. Additionally, newly synthesized proteins are protected from denaturation by the combined action of small and large HSPs (Usman et al. 2014). HSP17 (a 17 kD small HSP) interacts with vulnerable proteins, preventing their aggregation under heat stress conditions (Port et al. 2004) and increases the CAT activity upon interaction (Li et al. 2017) which indirectly improves membrane stability (Török et al. 2001). HSP17, HSP70 and HSP90 are also a part of heat shock transcription factor autorepression cycle (Guo et al. 2001; Kim and Schöffl 2002) and HSP60 exhibits a specialized ATP-dependent folding mechanism (Baniwal et al. 2004). Rapid induction of small HSPs (*HSP17* and *HSP40*) under heat stress (35 °C) in tolerant genotypes of groundnut was reported by Chakraborty et al. (2018).

Understanding the physiological and biochemical responses of groundnut genotypes to heat stress is crucial for developing heat-tolerant crops. While numerous physiological studies have elucidated heat tolerance mechanisms in various crops, there is limited information available on groundnut to date. In this context, a recombinant inbred line (RIL) population (JL 24×55–437) was developed and phenotyped at three locations for agronomic, phenological, and physiological traits under heat stress. A genetic map was constructed with 478 single-nucleotide polymorphism (SNP) loci spanning a map distance of 1,961.39 cM, leading to the identification of 45 major main-effect QTLs for productivity and heat tolerance-related traits (Sharma et al. 2023). From this population, 11 heat-tolerant and 10 heat-sensitive groundnut genotypes were selected based on the pod yield under heat stress and extensively evaluated.

Materials and methods

Plant materials and experimental setup

The RIL population (JL 24 × 55–437) was initially screened for yield and other heat-tolerance related traits across two different locations (UAS, Dharwad, India and ICRISAT, Hyderabad, India) during post-rainy (summer) seasons in the years 2017–2020 (Sharma et al. 2023). Based on the preliminary findings, 11 heat-tolerant and 10 heat-sensitive RILs were selected from the JL 24 × 55–437 mapping population (Table 1), for a detailed analysis conducted in this study. Two sets of the plants (control and treatment) were initially grown in a 'All weather greenhouse' at the Department of Microbiology, UASD. One set (treatment) was moved to 'Environmental chamber' (Kaleidoscope Climatic Solutions, Bangalore) at the Institute of Agri-Biotechnology (IABT), Department of Biotechnology, UASD during the reproductive stage to mimic the natural heat stress conditions by providing heat stress (45/30 ± 1 °C; 60 ± 5% RH) during the reproductive stage (25–60 DAS) of the crop considered as Heat stress condition (Treatment, T) (Fig.S1). After heat stress, pots were transferred to 'Greenhouse' at IABT, UASD and were maintained at warm (40/28 ± 2 °C; 50 ± 5% RH) conditions until harvest (Fig.S2). Another set of plants maintained at a controlled greenhouse under optimum temperature (25/20 ± 3 °C; 60 ± 5% RH) levels was considered as ambient condition (Control, C).

Phenotypic observations

Phenotypic observations viz., SPAD chlorophyll meter reading (SCMR), SLA, SLW, leaf moisture content (LMC, %) and leaf thickness (LT, cm) were recorded after a recovery period (80 days) from heat stress initiation. At the time of harvest, morphological parameters including, the plant height (PH, cm), number of primary branches (NPB), number of secondary branches (NSB) and haulm weight (HW, g) were recorded.

Table 1 Genotypes used in the study from JL 24 × 55–437 mapping population

Category	Genotype
Parents	JL 24 × 55–437
HT RILs	ICGR 151918, ICGR 151956, ICGR 151978, ICGR 151998, ICGR 152007, ICGR 152008, ICGR 152014, ICGR 152015, ICGR 152039, ICGR 152097, ICGR 152134
HS RILs	ICGR 151901, ICGR 151923, ICGR 151943, ICGR 151974, ICGR 151993, ICGR 151996, ICGR 152020, ICGR 152040, ICGR 152042, ICGR 152090

HT: Heat tolerant genotypes; HS: Heat sensitive genotypes, RILs: recombinant inbred lines

Physiological parameters

The leaf area parameter was measured using a leaf area meter (Biovis PSM, India) by taking four separated leaflets at 80 days after heat stress initiation. The leaf samples were then oven-dried and the dry weight was recorded in grams (g). Specific leaf weight and specific leaf area were calculated. The amount of moisture content present in a freshly collected leaf sample is considered as leaf moisture content (LMC) expressed in percentage (%). The Chlorophyll Stability Index (CSI, %) was calculated as the difference in light transmission percentage between treated (kept at 65 °C water bath for an hour) and untreated samples (room temperature, 25 °C) for an hour (Murthy and Majumdar 1962). Fresh plant leaf material (0.5 g) was taken and 10 ml of 100% dimethyl sulfoxide (DMSO) was added to the control sample and 0.5 g of fresh plant leaf material was taken in a test tube and heated in a water bath for 1 h @ 65 °C and after heating 10 ml of 100% DMSO was added to the treated sample and both treated and untreated samples were incubated overnight. Chlorophyll extracted into the DMSO solution was collected from the test tubes and concentrations of chlorophyll a, b, and total chlorophyll were quantified in both treated and untreated samples by reading the absorbance at A_{665} nm, A_{649} nm and A_{480} nm. The amount of chlorophyll and carotenoids (CAR) present in the extract (µg chl/ml) were calculated by taking the ratio of total chlorophyll of the heated sample to total chlorophyll of untreated sample and was expressed in percentage (Lichtenthaler 1987).

$$\text{Ca} = \text{Chla}\left(\mu\text{g ml}^{-1}\right) = \left[\left(12.9 \times A_{665}\right) - \left(3.45 \times A_{649}\right)\right]$$

$$\text{Cb} = \text{Chlb}\left(\mu\text{g ml}^{-1}\right) = \left[\left(21.9 \times A_{649}\right) - \left(3.45 \times A_{665}\right)\right]$$

$$\text{Total Chl(a + b)}\left(\mu\text{g ml}^{-1}\right) = \text{Ca} + \text{Cb}$$

$$\text{CSI\%} = (\text{Chlorophyll in heated sample} / \text{Chlorophyll in non} - \text{heated sample}) \times 100$$

$$\text{Total CAR} = \left(1000 \times A_{480} - (2.14 \times \text{Ca}) - (70.16 \times \text{Cb})\right)/220$$

SPAD chlorophyll meter reading (SCMR, SPAD units) was measured from the third completely expanded leaf from the apex by using Konica Minolta chlorophyll meter (Japan) in the morning hours between 9 am to 12 noon (Castelli et al. 1996). The cell membrane thermostability was measured by estimating the membrane injury index (MII, %) 20 days after heat stress initiation by using a modified protocol of

electrolyte leakage test (Chauhan and Senboku 1997). Electrical conductivity (EC) was measured by taking discs of a second completely expanded leaf from the top as samples in test tubes. The covered test tubes were kept at room temperature for an hour and then incubated at 45 °C for 30 min in a water bath and autoclaved at 100 °C for 10 min and the respective EC were recorded at room temperature (EC_a), 45 °C (EC_b) and 100 °C (EC_c) with Conductivity Meter 306 (Systronics, India). The membrane injury index was determined by calculating the ratio of $EC_b - EC_a$ to EC_c and expressed in percentage (Sullivan 1972). Lipid peroxidation assay/malondialdehyde assay (MDA, µmols g^{-1}FW) was performed to measure oxidative damage from photo-inhibition by measuring lipid peroxidation in leaves by using the Thiobarbituric acid (TBA) method by recording the absorbance at A_{532} nm and A_{600} nm (Heath and Packer 1968).

Biochemical parameters

The leaf samples were collected 20 days after heat stress initiation for the estimation of CAT and peroxidase (POX) activity. The extraction buffers for CAT and POX were prepared as per Sadasivam and Manikam(1992) and the pH of the solution was adjusted to pH 7 and pH 6.1, respectively. The snap-frozen leaf samples were pulverized using liquid nitrogen and suspended in 500 µl of the respective ice-cold extraction buffers independently. The tubes were centrifuged at 15,000 g and the supernatant obtained was collected and used as the crude enzyme extracts of CAT and POX, respectively. CAT activity (µmols g^{-1}FW) was estimated at 25 °C by adding 50 µl of crude enzyme extract in 2.95 ml of pH 7 phosphate buffer (containing 0.05% H_2O_2) and absorbance (A_{240} nm) recorded against blank (buffer containing the same amount of enzyme without H_2O_2) over 5 min for stabilization (Beers and Sizer 1952). The activity of guaiacol peroxidase (POX, µmols g^{-1}FW) was determined at 25 °C by adding 50 µl of crude enzyme extract in 2.95 ml of pH 6.1 phosphate buffer (containing 50 µl of 0.042% H_2O_2 and 50 µl of 20 mM guaiacol) and absorbance (A_{436} nm) recorded against blank (buffer containing same amount of enzyme without guaiacol and H_2O_2) over 5 min for stabilization (Chance and Maehly 1955).

Statistical analysis of observed parameters

Data obtained from phenotypic, physiological, and biochemical traits were subjected to Analysis of variance (ANOVA) to partition the variation due to different sources. Independent T-test was performed using respective open source codes in Python v.3.9 for all observations recorded among the parents and RILs by different combinations for comparative study viz., C *vs* T, JL 24 (P1) (C) *vs* 55–437 (P2) (T), P1(T) *vs* P2(T), P1(C) *vs* P1(T), P2(C) *vs* P2(T), HT(C) *vs* HS(C), HT(T) *vs* HS(T), HT(C) *vs* HT(T) and HS(C) *vs* HS(T) at 5%, 1% and 0.1% significance levels. Correlation analysis was performed for different traits using Python v.3.9 for both control and treatment conditions. To find the effect of other parameters on SLA under heat stress, stepwise multiple linear regression (MLR, lasso) was performed with SLA as a dependent variable by using Python v.3.9.

Temperature induction response (TIR)

The thermal tolerance of seeds was estimated for the groundnut genotypes along with the parents using the temperature induction response (TIR) method (Fig. S3). Seeds were treated with 2% Bavistin solution for 15 min and washed well with sterile water followed by soaking the seeds for 2 h. The imbibed seeds were transferred to a petri dish with water-soaked germination paper and kept for germination over 48 h in a growth chamber (Environmental Chamber, Kaleidoscope Climatic Solutions, Bangalore) at 30 °C with 50% relative humidity. Later, uniform seedlings were transferred to three different petri plates labelled as induced, non-induced and control (with replicates) and the initial length of seedlings (cm) was recorded. One set (induced) was subjected to high-temperature stress through induction of 35 °C for one hour, 40 °C for one hour, 45 °C for two hours and 55 °C for three hours, another set (non-induced) was exposed to 30 °C for four hours and a lethal temperature of 55 °C for three hours and an experiment control setup was maintained at 30 °C throughout the experiment for seven hours. After temperature induction, the seeds were left to recover for 3 days at 30 °C and 50% relative humidity and the final length of seedlings (cm) was recorded at the end of the recovery period (Gangappa et al. 2006) and the TIR was calculated as follows,

$$\begin{aligned}&\text{Growth Differential Response } (GDR)\\&= \text{Growth at end of recovery (cm)}\\&\quad -\text{Growth at end of induction (cm)}\end{aligned}$$

$$\begin{aligned}\text{TIR} &= (\text{GDR of induced} - \text{GDR of non} - \text{induced})\\&\quad /\text{GDR of non} - \text{induced}\end{aligned}$$

Expression analysis of heat stress-responsive genes

The leaf samples were collected and snap-frozen in Invitrogen™—RNaseZap™ treated aluminium foil and later transferred to a -80 °C deep freezer. For isolation of RNA, the collected leaf tissue was powdered in diethyl pyrocarbonate (DEPC) treated pestle mortar using liquid nitrogen. 100 mg of the powdered leaf tissue was suspended in 1 ml of

Ambion® TRIzol® reagent in DEPC treated 2 ml centrifuge tubes and RNA was isolated by following the manufacturer's protocol. The genomic DNA contamination was removed by using the Ambion® TURBO DNA-*free*™ kit. Further, the purified RNA was quantified using NanoDrop™ 2000 and checked for integrity using EtBr-stained 1% Formaldehyde, 1.2% Agarose gel electrophoresis run in 1X MOPS buffer. Equal concentrations of RNA samples from heat-sensitive (HS) and heat-tolerant (HT) groups were bulked independently as HT and HS bulks. The bulked RNA was used for cDNA synthesis using Invitrogen™ SuperScript® III First-Strand Synthesis System, following the manufacturer's protocol. The genes were selected based on previous studies (Chakraborty et al. 2018; Kokkanti et al. 2019). *ADH3* was used as the reference gene/housekeeping gene, as it showed the most stable expression in all conditions in groundnut (Reddy et al. 2013). The primer sequences are presented in Table S1. The annealing temperatures (Ta) were standardized by performing gradient PCR. The gene expression studies were conducted in QuantStudio™ 5 Real-Time PCR—Applied Biosystems, by using Power SYBR Green PCR Master Mix (Thermo Fisher). An equimolar concentration of cDNA was used as a template for expression analysis of all the genes. Quantitative real-time PCR (qRT-PCR) profile conditions were: 95 °C for 5 min followed by 40 cycles of 30 s at 95 °C, 30 s at respective Ta and 30 s at 72 °C with fluorescent signal recorded at extension. A melt curve analysis was performed post 40 cycles of PCR amplification by gradually increasing (0.038 °C s^{-1}) temperature from 60 °C to 95 °C with constant fluorescent signal recording. The experiment designing and data acquiring was performed with Design and Analysis v.2.5 software, from the manufacturer. The relative gene expression levels were calculated following the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001).

Results

Heat-sensitive and heattolerant genotypes exhibit distinct physiological response to heat stress

In this study, groundnut genotypes were grown under control and heat stress conditions and were screened for various morphological, physiological, biochemical and molecular responses. ANOVA was performed, and most traits showed significant variation among the genotypes under both control and heat stress conditions (Table S2 & S3, Figs. 1 and 2). Among parents, JL 24 had high levels of MDA content (0.98 µmols g^{-1} FW) than the tolerant plant, which recorded significantly lower levels (0.63 µmols g^{-1} FW). Under heat stress,

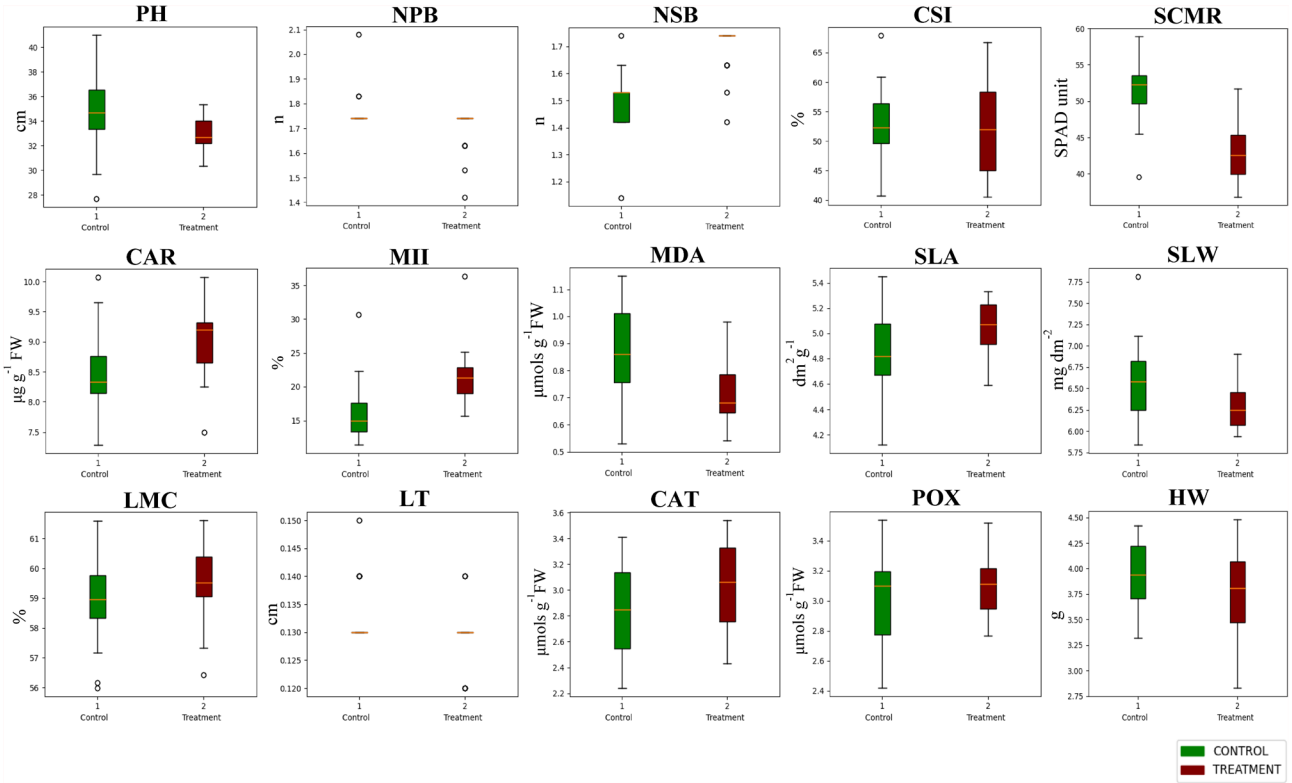

Fig. 1 Frequency distribution of morphological, physiological and biochemical traits for heat tolerance among HT and HS genotypes derived from JL 24 and 55–437

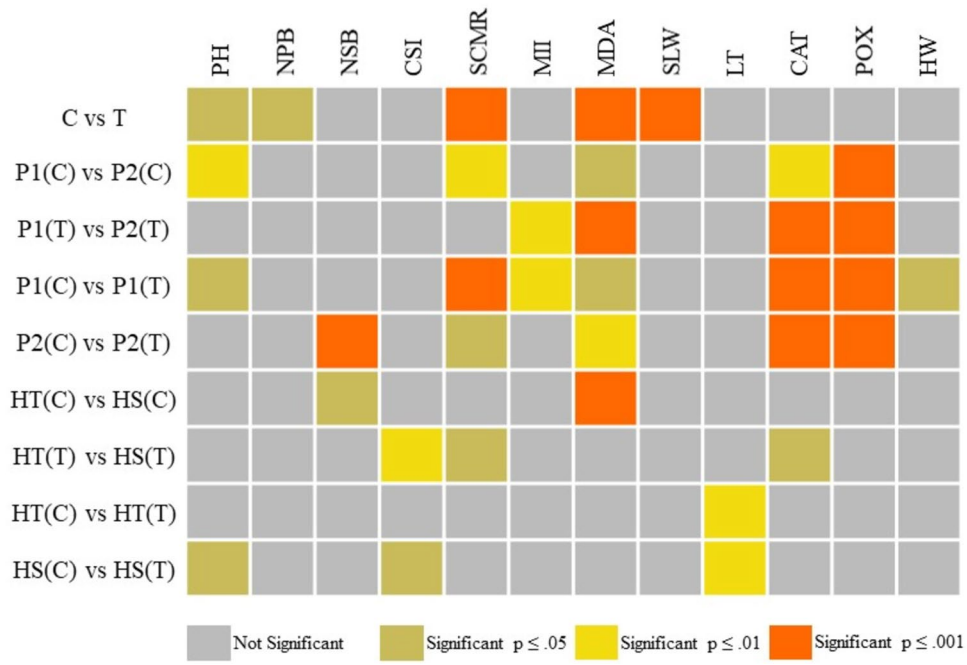

Fig. 2 Independent T-test for morphological, physiological and biochemical traits for heat tolerance among/ between parents and HT and HS genotypes under/ between both control (C) and treatment (T) conditions

MDA was relatively higher in the HS genotypes (0.78 µmols g^{-1} FW) than HT genotypes (0.63 µmols g^{-1} FW). In this study, The HT and HS mean showed significant difference in CSI (HT- 56.4% and HS- 46.7%) and SCMR (HT- 43.54 SPAD units and HS- 40.51 SPAD units). HT genotypes had more chlorophyll stability with CSI of 55.8% which was notably higher than the HS genotypes (46.7%). The membrane injury in terms of MII was measured in both control and treatment conditions and was found to be relatively increased in the treatment (21.3%) over control (16.2%). Both HT and HS genotypes showed significant reduction in LT under heat stressed condition over control condition. Traits like CHLa, CHLb, CAR, SLA, and LMC did not have any significant differences among the selected genotypes. SLA was recorded higher in treatment condition (5.046) over control condition (4.842). Under heat stress ICGR 151993 (HS genotype) had the highest SLA (5.33 dm^2g^{-1}) and ICGR 152134 (HT genotype) recorded the lowest (4.59 dm^2g^{-1}). SLW decreased under heat stress (6.281) compared to control (6.568). A significant reduction of HW in sensitive parent (JL 24) under heat stress (3.68 g) over control condition (4.19 g) was recorded. Whereas, 55–437 was unaffected under heat stress in terms of HW. ICGR 152014 (HT genotype) showed highest HW (4.48 g), whereas ICGR 151974 (HS genotype) recorded the lowest (2.83 g) under heat stress (Table S4-S8, Fig. 2).

Heat stress tolerance is modulated by enzymatic and non-enzymatic antioxidants

The plants maintained under a heat stress environment were subjected to study the activity of enzymatic antioxidants viz., CAT and POX. The parent 55–437 showed significantly higher CAT (3.18 µmols g^{-1}FW, 28.8% higher) and POX (3.42 µmols g^{-1}FW, 16% higher) activity over the sensitive parent (JL 24). Among RILs, highest CAT activity was observed in HT mean (3.30 µmols g^{-1}FW) than HS mean (2.73 µmols g^{-1}FW). POX activity was found insignificant between the HT and HS genotypes. Yet, ICGR 152039 of the HT group had the highest POX activity (3.52 µmols g^{-1}FW). Similarly, ICGR 151918 of the HT group showed highest CAT activity (3.54 µmols g^{-1}FW). Carotenoids, a non-enzymatic antioxidant, were found to increase in heat-stressed plants (9.015) compared to those grown under control conditions (8.444), in both HS or HT genotypes (Fig. 2).

Correlation of heat stress tolerance-related traits in heat sensitive and heat tolerant genotypes

Under control conditions, SLA showed a negative correlation with SLW and positively correlated with LMC. LT was positively correlated with CAR, MII, and SLW, but negatively with SLA at 1% significance. Under heat stress, NPB and NSB were positively correlated. SLA showed a negative correlation with SLW and positively correlated with LMC. CAT was negatively correlated with MDA at 1% level of significance. (Table S9 & S10, Fig. 3).

Impact of other parameters on specific leaf area

Specific leaf area (SLA), defined as the ratio of total leaf area to total leaf dry mass has been shown one of the leaf traits best reflecting the whole plant growth (Cheng et al.

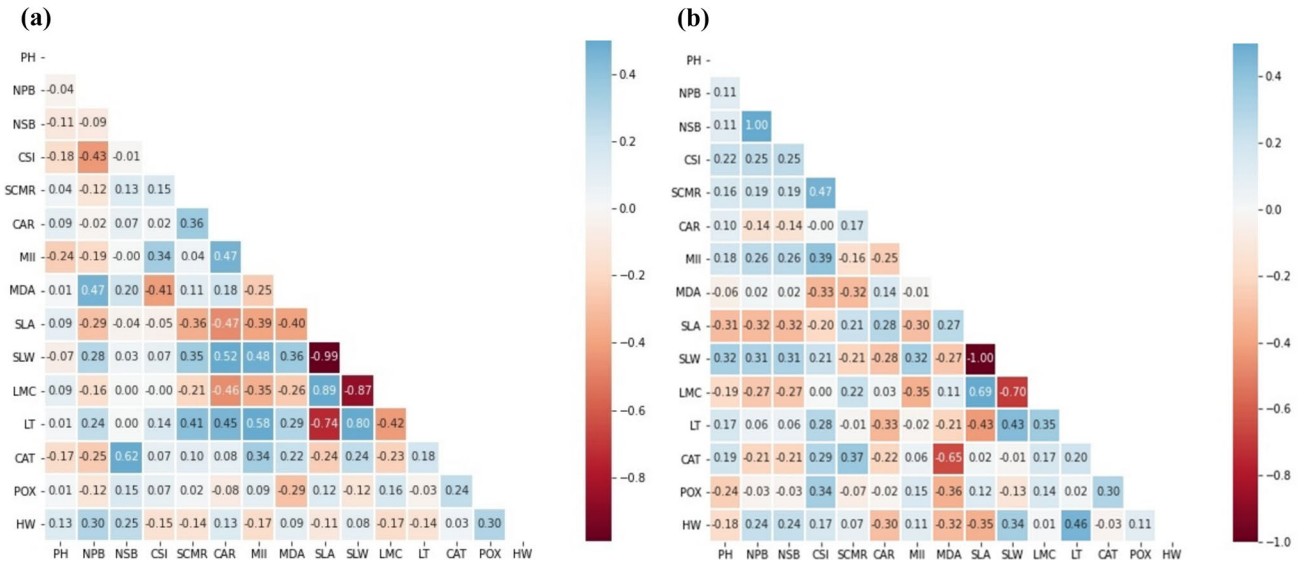

Fig. 3 Correlation of morphological, physiological and biochemical traits for heat tolerance among HT and HS genotypes along with its parents under both **a** control and **b** treatment conditions

2016). In this study, to find the effect of other parameters over SLA under heat stress, step-wise multiple linear regression (MLR) (Lasso) was performed with SLA as a dependent variable. To fit a linear regression model with the parameters, the selected parameters must not be collinear with each other. Hence, all parameters were screened for the variation inflation factor (VIF) and parameters that showed multicollinearity (Fig. 4a) with high VIF were excluded from the developing model. The parameters selected were CSI, SCMR, CAR, MDA, LT, CAT and POX with VIF ranging from 1.2–2.5. White's test and Breush-Pagan's test were performed to check the values for heteroscedasticity. The p-value observed for both tests was more than 0.05 which proves the data is not heteroscedastic. Ljung-Box test showed higher p-value more than 0.05 ensuring no autocorrelation within the parameters. The mean residual (the vertical distance between the regression line and the data point) was found to be very less (4.4^{e-14}) which ensures the values follow normal distribution as plotted in QQ-plot (Fig. 4b). The errors were calculated viz., mean squared error (MSE, 0.0021), mean absolute error (MAE, 0.038) and root mean squared error (RMSE, 0.045) which were found to be negligible ensuring good data quality. The dataset was fitted to a linear regression model (OLS). The machine learning model generated by the algorithm showed 95.2% accuracy which explained 51.9% (R^2 value) of variation in SLA in terms of contribution of other independent variables. To visualize the interaction of the parameters, a LM-plot was plotted (Fig. 4c). The plot showed the interaction of SLA with CSI, SCMR, CAR, MDA, LT, CAT and POX. Among the model traits, CAR, MDA, CAT and POX showed constant increase under treatment conditions with an increase in SLA. SCMR was increasing with an increase in SLA under heat stress. CSI was found to be reducing with increasing SLA. LT values ranged from 0.12 cm to 0.14 cm, whereas the plot had a much wider range in X-axis making visualisation harder for the decreasing LT. Hence, SLA is significantly affected by leaf thickness. It is also a proof that thinner leaves (with high SLA) will have increased lipid peroxidation under stress (increased MDA) and a decrease in chlorophyll stability (lower CSI). To study the individual interaction of all the RILs and parents pair plot was plotted and observed that, HT genotypes showed high levels of carotenoid content under heat stress (Fig. 5). The frequency distribution of HS genotypes and HT genotypes were differentiated in diagonally arranged histogram plots for the model traits. The interaction of RILs among various traits across MDA gave a clear visualization of grouped genotypes based on their diverse performance. The scatter plot plotted for MDA against CAT showed JL 24's position where it had the lowest CAT and POX activity and higher MDA content.

Screening of groundnut genotypes for temperature induction response (TIR)

Temperature induction response (TIR) was carried out to examine the performance on germination of the genotypes under induced heat stress conditions. A pilot experiment conducted with 21 RILs and the parents showed a significant difference among the RILs at 1% for growth during recovery (GDR) -induced, non-induced, and control (Fig. 6). Among 10 RILs from the HS group, ICGR 151993 had very low germination and was excluded from the experiment. The TIR of HT mean was 3.54 *i.e.* heat induced seeds have 3.54 times

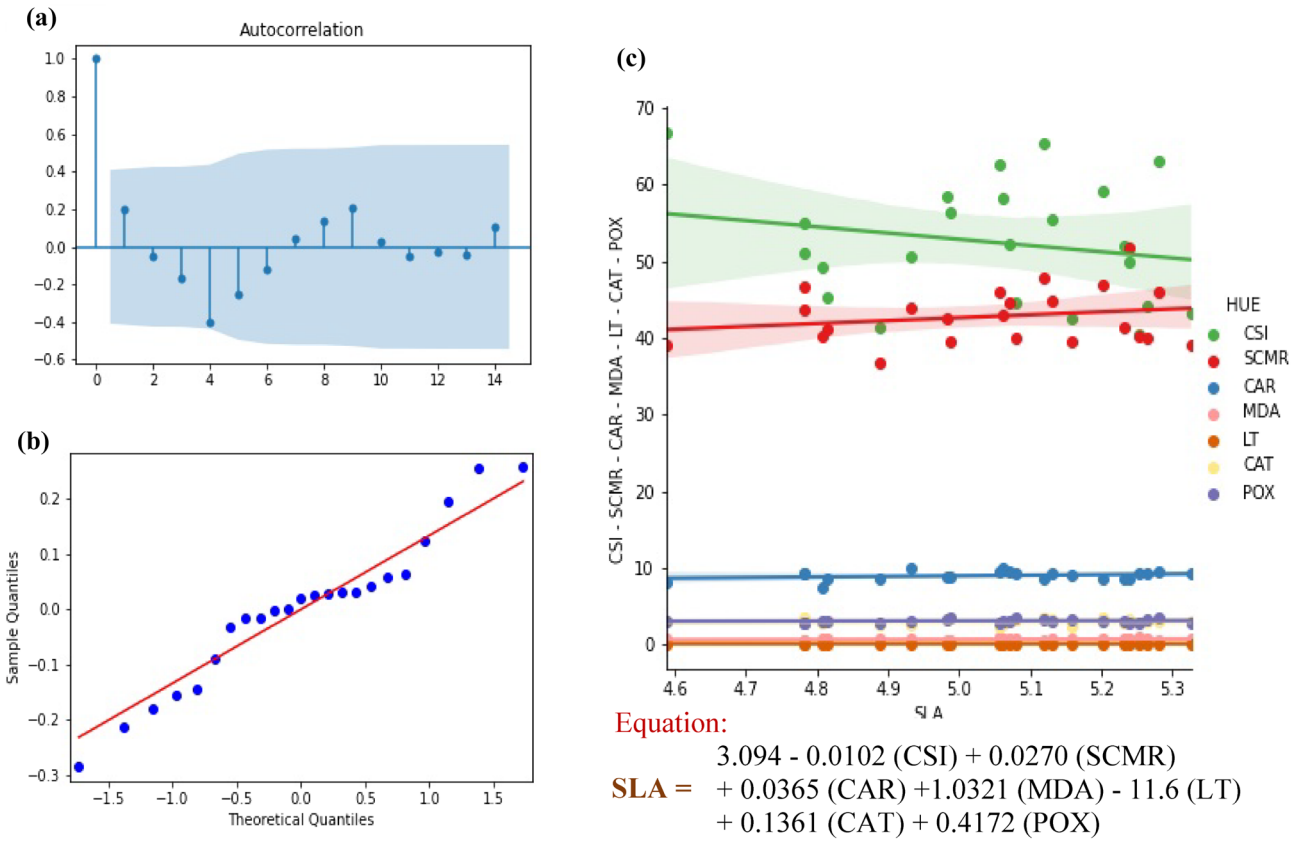

Fig. 4 Multiple Linear Regression for the traits included in the MLR model among HT and HS genotypes along with its parents under heat stress. **a** Autocorrelation plot, **b** QQ–plot and **c** LM–plot (regression model)

better growth than the non-induced ones. HS means showed only 2.31 times increase in induced over non-induced. JL 24 was found to be better than both the HT and HS means (5.19 times) yet significantly lesser than the tolerant parent 55–437 which showed 8.03 times better growth in induced over non-induced. It was observed that both the parents performed well when compared to the HT and HS mean however, 55–437 showed good growth in induced over non-induced seedlings. ICGR 152014 (HT genotype) showed 9.04 times increased seedling growth in induced over non-induced seedlings under heat stress (Table 2).

Relative gene expression of heat stress-responsive genes

The genes selected for differential expression were *AhHSP17*, *AhHSP40*, *HSP60*, *AhHSP70*, *AhHSP90*, *DREB2A* and *LEA4-2*. *ADH3* was used as an internal control/reference gene. Among seven genes assayed, *AhHSP17* showed manifold increased expression (> 1979 fold) in HT bulk under heat stress condition over control condition (Fig. 7). *AhHSP70* and *AhHSP90* also showed upregulation in treatment over control in HT bulk sample. HS bulk had 897.2 folds increase in expression of *AhHSP17*, yet significantly less than HT bulk (1979 folds). *DREB2A* and *LEA4-2* were upregulated highly in HS bulk and not in any other samples (Table S11). *AhHSP40* was found to have upregulated only in the heat sensitive parent and HS bulk, whereas, *HSP60* was upregulated only in HS and HT bulks (Table S11).

Discussion

Elevated temperatures disrupt physiological processes in plants, and the stress response varies considerably. Therefore, an integrated study encompassing physiological, biochemical, phenological parameters, and associated molecular pathways is quite essential to understand heat stress response. In this context, 21 RILs with diverse phenotypes (heat tolerant, 11 genotypes and sensitive, 10 genotypes) along with the parents (JL24 and 55–437) grown under both control and heat stress conditions were screened for various morphological, physiological, biochemical and molecular responses. Traits were assessed at various stages of crop growth under both control and treatment conditions. A

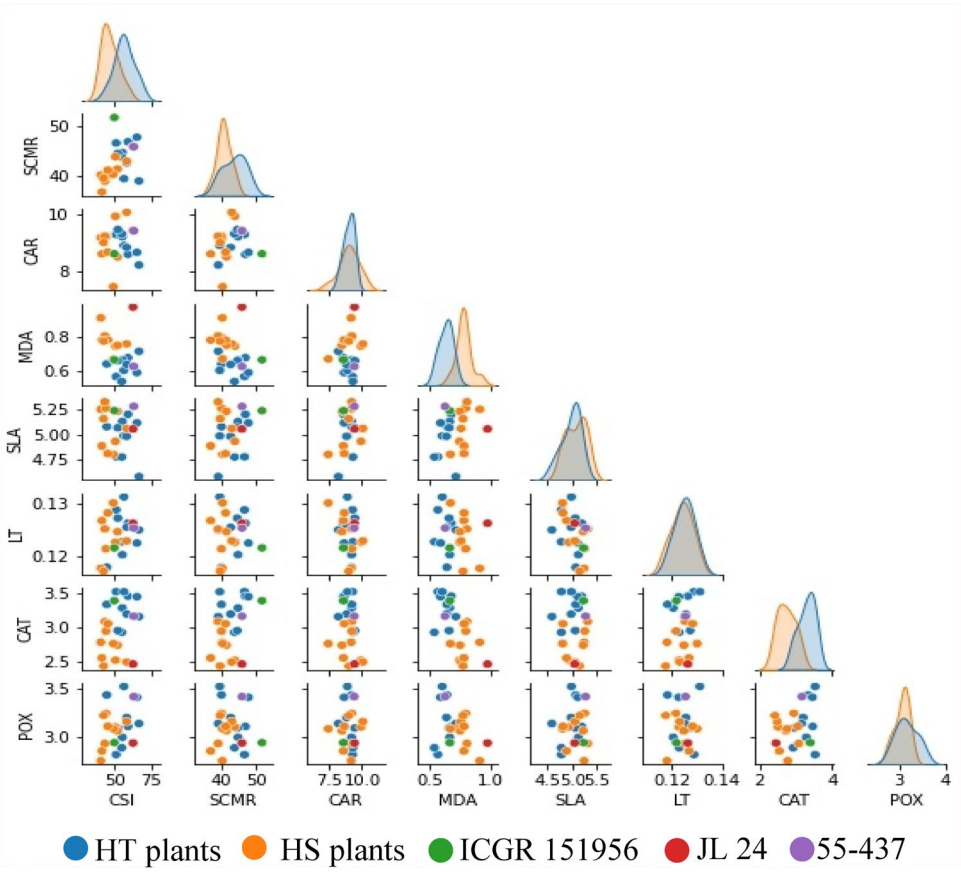

Fig. 5 Pair plot for the traits included in the MLR model among selected HT and HS genotypes along with its parents under heat stress

significant reduction in plant height and NPB was observed under heat stress conditions compared to control conditions. Plant height is controlled by the interaction of genotype with the environment. Heat stress accelerate plant growth and development. Hence, the given plant under stress is provided less amount of time to acquire nutrients which results in reduced plant height and number of primary branches (Dash et al. 2020). Plants under heat stress conditions exhibited delayed flower initiation compared to those in control conditions. This may be due to the adverse effect of heat (45 °C) on the reproductive growth physiology increasing flower abortion (Talwar et al. 1999).

The chlorophyll stability index directly indicates the extent of stress tolerance. The photosynthetic rate and dry matter production will be unaffected when chlorophyll remains stable under stress conditions. In our study, HT genotypes had more chlorophyll stability with CSI of 55.8% which was significantly higher than the HS mean (46.7%). Heat stress affects various plant processes; successful adaptation requires a resilient cell membrane system that remains functional under stress (Raison 1980). Membrane injury in terms of MII was measured in both control and heat stress conditions and the results indicate MII is relatively higher in treatment conditions over control. This lack of significant differences may be attributed to the heat acclimatization process (Singh et al. 2016). Though there was no difference in MII among the parents under control condition, the HT and HS genotypes differed with HS showing 13.8% more solute leakage. Notably, ICGR 151956, an HT genotype while performing well in terms of flowering and appearance, showed the highest MII (30.7%), as an exception in control conditions.

The cell membrane is primarily a lipid bilayer that protects the cell organelles. ROS production in response to heat stress significantly damages the cell membrane leading to lipid peroxidation (Jain et al. 2001). The resulting oxidative stress is overcome by antioxidant defense mechanisms involving enzymatic antioxidants viz., catalase and peroxidase. Under heat stress conditions, the tolerant parent 55–437 showed significantly higher CAT (3.18 µmols g^{-1}FW, 28.8% higher) and POX (3.42 µmols g^{-1}FW, 16% higher) activity over the sensitive parent (JL 24) (Table S6). Among RILs, highest CAT activity was observed in HT mean (3.30 µmols g^{-1}FW) than HS mean (2.73 µmols g^{-1}FW). There was no significant difference in POX activity between the HT and HS genotypes (Table S7). Yet, ICGR 152039 of the HT group had the highest peroxidase activity (3.52 µmols g^{-1}FW), whereas, ICGR 151918 of the HT group showed highest CAT activity (3.54 µmols g^{-1}FW) thereby, protecting the plant from the adverse effects of

Trait	F value	S Em	CD (5%)	CV
GDR of induced	74.71**	0.14	0.39	5.41
GDR of non-induced	27.47**	0.14	0.41	17.32
GDR of control	75.39**	0.17	0.48	4.9

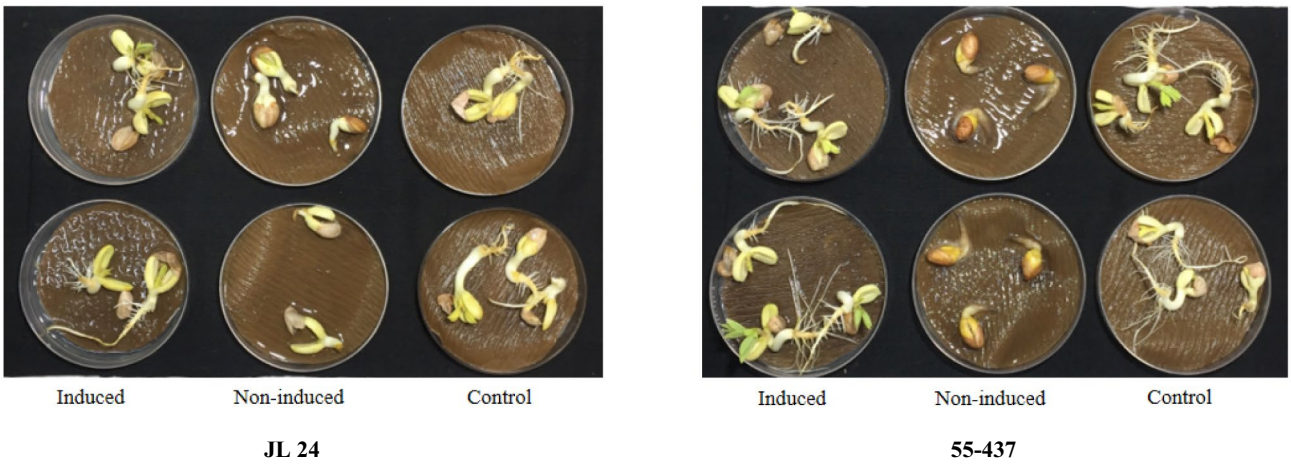

Fig. 6 Temperature induction response studies in grouped diverse genotypes and parents of JL 24 × 55–437 RIL population

Table 2 Temperature induction response (TIR) of groundnut genotypes

HT RILs	Increase in growth of induced over non-induced/ lethal seedlings	HS RILs	Increase in growth of induced over non-induced/ lethal seedlings
ICGR 151918	6.85	ICGR 151923	2.16
ICGR 151978	2.04	ICGR 151943	0.59
ICGR 151998	7.77	ICGR 151974	5.75
ICGR 152007	2.81	ICGR 151996	−0.13
ICGR 152008	2.54	ICGR 152020	3.26
ICGR 152014	9.04	ICGR 152040	1.97
ICGR 152015	0.98	ICGR 152042	1.31
ICGR 152039	1.28	ICGR 152090	5.69
ICGR 152097	2.48	ICGR 151901	0.22
ICGR 152134	2.67	**HS mean**	**2.31**
ICGR 151956	0.48	JL 24	5.19
HT mean	**3.54**	55–437	8.03

ROS. Malondialdehyde (MDA), a by-product of lipid peroxidation serves as an indicator of oxidative stress-induced damage (Savicka and Skute 2010; Tommasino 2012). In this study, under heat stress, MDA was relatively higher in the HS mean (0.78 µmols g^{-1} FW) than HT mean (0.63 µmols g^{-1} FW) (Table S7). Among parents, JL 24 had higher levels of MDA content (0.98 µmols g^{-1} FW) than the tolerant plant which recorded significantly lower levels (0.63 µmols g^{-1} FW) (Table S6). Several studies have showcased similar results where heat-sensitive plants recorded higher levels of MDA than tolerant plants under heat stress (Han et al. 2013; Jin et al. 2020; Markovićet al. 2020). Carotenoid (a non-enzymatic antioxidant) content increases with an increase in temperature and influences heat stress tolerance (Kumar

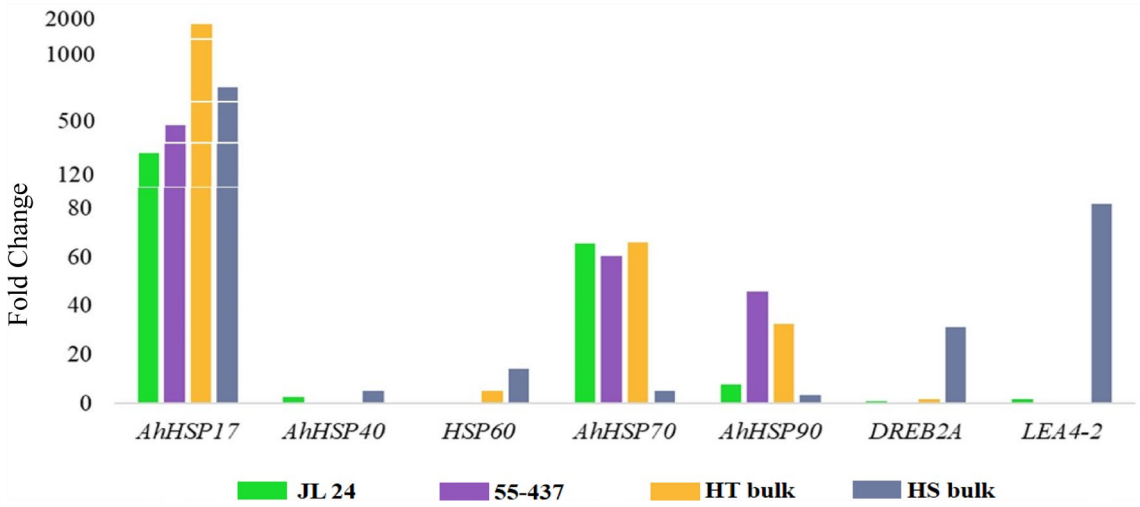

Fig. 7 qRT-PCR expression profile for heat stress responsive genes in treatment over control in terms of fold changes

et al. 2020). Carotenoid content under treatment conditions was higher than in the control. This observation was similar to the reports of Dash et al. (2020). Higher levels of antioxidants were observed in tolerant plants, along with lower MDA content, strongly suggesting that the antioxidant system effectively prevented the cell membrane damage by counteracting the ROS produced under heat stress.

SLA plays an important role in linking plant carbon (C) and water cycles because it describes the distribution of leaf biomass relative to leaf area, and thus refers to carbon gain relative to water loss, within a plant canopy. The plants grown at higher temperatures have thinner leaves due to fewer cell layers, which leads to higher SLA. In contrast, a decrease in SLA has been reported at elevated CO_2, which leads to extra palisade layer development, increased mesophyll cell size, increase in internal surface area for CO_2 absorption (Pilumwong et al. 2007) and it affects the photosynthesis, in turn, yield of the crop. SLA was recorded higher in treatment conditions than control. Under heat stress, ICGR 151993 from (HS genotype) had highest SLA (5.33 dm^2g^{-1}) and ICGR 152134 (HT genotype) recorded the lowest (4.59 dm^2g^{-1}). This finding aligns with earlier reports by Nautiyal et al. (2002, 2008), where tolerant genotypes had low SLA and sensitive genotypes had high SLA. SLW decreased under heat stress compared to control. Continuous heat stress from flowering to harvest significantly impacted source-sink transitions, reducing total dry matter production and other yield components (Craufurd et al. 2003). Hence, higher SLW or lower SLA reduces the chances of membrane injury, which tends to impart thermotolerance (Nautiyal et al. 2008). The lack of an apparent variation in leaf area implies that changes in SLA were caused by thicker leaves. This statement is partially supported by the recorded SLW and LT data, wherein the HT mean is higher than the HS mean (not significantly) under heat stress. The generated MLR model elucidates the magnitude of the relation between the independent and the dependent variable. SLA is highly influenced negatively by LT and CSI, i.e., with an increase in SLA, a significant decrease in LT and CSI is observed with relatively low thermotolerance. MDA tends to increase with the increase in SLA, clarifying the membrane injury. Since, more SLA under treatment induced membrane injury, an increase in CAT and POX activity can be observed as a counteraction from the above equation. This is as per the observations of Nautiyal et al. (2002, 2008) where the tolerant genotypes had lower SLA and more LT while the sensitive genotypes showed higher SLA.

Correlation among morphological, physiological and biochemical traits for heat tolerance among HT and HS genotypes along with parents JL 24 and 55–437 under control (Table S9) and treatment (Table S10) conditions were studied. Under control conditions, a strong positive correlation between leaf thickness (LT) and membrane injury index (MII) was observed (Table S9 and S10), indicating that thicker leaves sustain greater membrane injury upon sudden heat shock, likely due to increased solute leakage. However, this correlation became negligible under heat stress, suggesting that plants acclimated to high temperatures effectively mitigated the adverse effects of LT on MII. Thus, while thicker leaves may enhance heat tolerance (Nautiyal et al. 2008), our findings indicate that the acclimatization process is crucial for reducing membrane injury in this population. CSI represents the stability of chlorophyll pigment under heat stress. More stability indicates better tolerance. This correlation suggests that higher chlorophyll stability followed by active chlorophyll accumulation during recovery period has increased the SCMR values. Chlorophyll accumulation is considered as an important factor for

thermotolerance (Selvaraj et al. 2011; Yeh et al. 2012) and such phenotype can be promising in finding genes that are related to thermotolerance. With the higher levels of chlorophyll content, an upsurge in carotenoid content was observed that possibly accounts for collective thermotolerance. A significant negative correlation was observed between CAT and MDA, as increase in antioxidants (CAT activity) prevented lipid peroxidation under heat stress (Savicka and Skute 2010). Multiple linear regression was performed with parameters that satisfied the assumptions for a perfect linear regression model. SLA being an important trait that could significantly affect the yield and yield-related traits was considered as the dependent variable to study the effects of other related traits. The generated model elucidated the magnitude of the relation between the independent and the dependent variables. SLA was highly influenced negatively by LT and CSI, i.e., with a decrease in LT and CSI, SLA increases significantly with relatively low thermotolerance. MDA tends to increase with an increase in SLA, leading to higher membrane injury. Since, higher SLA under heat-stress conditions induced membrane injury, increase in CAT and POX activity can be observed. These results were analogous with Nautiyal et al. (2008).

Temperature induction response (TIR) is a conventional approach for identifying thermotolerant variations for germination among cultivars and even within an inbred population (Kokkanti et al. 2019). Plants exposed to gradually elevated yet non-lethal temperatures tend to acquire thermotolerance and this protects the plant beyond the levels of inherent thermotolerance (Senthil-Kumar et al. 2007; Rani et al. 2018). This is due to several stress-responsive physiological and biochemical pathways being triggered like HSP induction that stays over a time offering heat stress tolerance. It was observed that both the parents performed well when compared to the HT and HS mean yet, 55–437 showed better growth in induced over non-induced seedlings. ICGR 152014 (HT genotype) exhibited a 9.04-fold increase in growth in heat-induced seedlings compared to non-induced under heat stress (Table 2). Similar reports suggest that plants that were acclimatized to gradually increasing temperatures, survived the heat stress than the non-induced plants (Senthil-Kumar et al. 2007 and Gangappa et al. 2006). It's important to note that this strategy only affects the plant's capacity to germinate in the presence of heat stress and has no bearing on the plant's general capacity to withstand heat. It may not accurately forecast the plant's heat tolerance during other phases of its life cycle.

Apart from several physiological and biochemical adaptive strategies involved, the regulation of HSPs is one of the most important aspects of heat stress tolerance in plants (Young 2010; Usman et al. 2014; Chen and Li 2017). Among seven genes assayed, *AhHSP17* exhibited a striking 1979 fold upregulation of expression in the heat tolerant (HT) bulk under heat stress conditions, compared to control conditions (Table S11, Fig. 7). *AhHSP17* is a 17 kD protein (micro protein) that is classified as small HSPs (Chakraborty et al. 2018) that kept accumulating under heat stress. Under control conditions, the expression of *AhHSP17* transcripts was very meagre. Increased expression of *AhHSP17* in the tolerant parent and tolerant bulk over sensitive parent and sensitive bulk suggests, that the small HSPs probably facilitated survival under heat stress by binding to heat-sensitive proteins, lipids, and macromolecules offering protection to its vulnerable sites from ROS or other heat stress injuries (Port et al. 2004). Under oxidative stress, the activity of enzymatic antioxidants like CAT was found to be increased upon interaction with small HSPs. This suggests that, the decreased levels of MDA and increased CAT activity in tolerant genotypes under heat stress might be due to induction of HSPs under heat stress. Several reports in other crops showed that small HSPs have a role in preventing irreversible unfolding or wrong aggregation of partially denatured proteins (Lee et al. 2007; Xu et al. 2011; Goswami et al. 2016). Unlike *AhHSP17*, HSPs with higher molecular weight (*AhHSP70* and *AhHSP90*) showed relatively less upregulation under heat stress in this study. This might be possibly due to the acquired thermotolerance (Kokkanti et al. 2019) or attaining saturation after certain levels as observed by Chakraborty et al. (2018) using real-time PCR. AhHSP70 and AhHSP90 play an important role in the nascent folding of the aggregated polypeptide to provide stability (Kumar et al. 2016; Yamada et al. 2007) by acting as molecular chaperones in retaining intracellular damaged proteins to their form (Gupta et al. 2014). Besides chaperone activity, they are known to regulate several downstream gene expressions under heat stress (Duan et al. 2011; Zhang et al. 2013). HSPs tend to function efficiently when the plant is under stress in aiding other molecules, because of their loss of cysteine amino acid responsible for the disulfide bond during evolution, hence are not themselves affected by heat stress (Fu et al. 2003).

Upregulations of HSPs suggest an efficient upstream regulatory mechanism with competent signal perception and transduction that could have led to the expression of several transcription factors (TFs). TFs are vital for the modulation of gene expression and signal transduction among the stress response regulatory proteins and deliver protection under abiotic stress (Fragkostefanakis et al. 2015). *DREB2A* plays a major role in plant growth and metabolism especially under drought conditions (Lata and Prasad 2011). *LEA4-2* proteins are generally upregulated under drought stress and very few reports are available for its upregulation under heat stress (Kokkanti et al. 2019). Both *DREB2A* and *LEA4-2* genes were upregulated only in HS bulk under heat stress. However, the selective upregulation of these TFs and

AhHSP40 only in sensitive bulk and sensitive parent is not understood completely.

Conclusions

This study offers a detailed analysis of the physiological, biochemical and molecular mechanisms underlying heat stress response in heat tolerant and sensitive groundnut genotypes. Among the physiological and associated biochemical parameters screened, it is evident that the levels of enzymatic (CAT, POX) and non-enzymatic (CAR) antioxidants play a vital role in conferring thermotolerance subsequently contributing to increased chlorophyll stability expressed as CSI. In this study, the HT genotypes under heat stress showed similar patterns of thermotolerance mechanism. The HT genotypes exhibited thicker and darker leaves as evidenced by SLA and SCMR values. Tolerant genotypes under heat stress exhibited reduced levels of MDA thereby validating reduced membrane injury. Additionally, *HSP17* was found to have upregulated (1979 folds) in HT genotypes, which along with the oxidative enzymes, collectively confers thermotolerance. The prolonged accumulation of these HSPs safeguarded vital stress-responsive enzymes and other macromolecules enhancing survival under stress conditions. Hence, these parameters (CAT, POX, CAR, MDA, SLA and HSP17 expression) show potential importance and can be used as indicators for further screening of genotypes for heat stress tolerance in groundnut. Furthermore, the insights gained from the RIL population used in this study, including the identification of key QTLs linked to heat tolerance traits, provide a valuable resource for future breeding programs aimed at enhancing heat tolerance in groundnut. This foundational knowledge can guide future research efforts, particularly in applying advanced functional genomics approaches to unravel complex signalling pathways and their cross-talks during stress tolerance, necessitating comprehensive studies through advanced and precise functional genomics approaches for deeper insights.

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Author contributions SNN conceptualized idea, planned experiment and coordinated with co-authors and finalized the MS. AB conducted the experiments and data analysis. PR and DBR provided technical help to conduct the experiment. AB, SRJ and SNN wrote and revised the manuscript. PUK, BB, VPC, KKM provided lab facility and support to the experiments. PJ, MKP, RKV provided the material, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

Data availability All data are available in the manuscript or Supplementary files. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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