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Research Paper

Evaluation of heat stress response in pepper (*Capsicum annuum* L.) seedlings under controlled environmental conditions using a high-throughput 3D multispectral phenotyping

Yoonah Jang [a,b,*], Roland Schafleitner [b], Derek W. Barchenger [b], Ya-ping Lin [b], Junho Lee [a]

[a] Vegetable Research Division, National Institute of Horticultural and Herbal Science, RDA, Wanju-gun, Jeollabuk-do, 55365, Republic of Korea
[b] World Vegetable Center, Shanhua, Tainan, 74151, Taiwan

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ABSTRACT

Climate change-driven heat stress presents a significant threat to global pepper production, highlighting the urgent need for efficient methods to assess heat tolerance in breeding programs. This study presents a robust approach for assessing heat stress responses in pepper integrating high-throughput phenotyping and multivariate analysis. Twenty pepper genotypes were evaluated under controlled temperature conditions (40/35 °C day/night) for 14 days using the TraitFinder system equipped a pair of 3D multispectral scanner. Principal component analysis (PCA) of morphological and spectral traits revealed progressive divergence between control and heat-treated groups, with the maximum separation observed at day 10 ($\Delta C = 2.05$). Three distinct response groups were identified based on Euclidean distances in the PCA space: low response (five genotypes), moderate response (nine genotypes), and high response (six genotypes). The PC-based distance metric showed strong correlations with conventional stress tolerance indicators, including biomass retention ($r = 0.66$) and root system maintenance ($r = 0.48$). Notably, genotype 'Pep17 (GPC121710)' demonstrated enhanced growth under heat stress (26 % increase in 3D leaf area), while 'Pep06 (GPC003350)' showed marked growth reduction (27 % decrease). This study validated the integration of high-throughput phenotyping with PCA-based metrics for the quantitative assessment of heat stress responses. The method offers an efficient tool for identifying heat-tolerant pepper genotypes and holds potential for application to other crops and stress conditions, supporting climate resilience breeding programs.

1. Introduction

Extreme weather events driven by the climate change, particularly high temperatures, pose a significant threat to the stability of global crop production (Gornall et al., 2010). Pepper (*Capsicum annuum* L.), is one of the most important vegetable and spice crop worldwide. Global chili pepper production reached about 40 million tons in 2022, with an estimated economic value of approximately 15 billion USD (FAOSTAT, 2024). However, peppers are sensitive to high temperature, which adversely impacts both their productivity and quality (Rajametov et al., 2021). Consequently, developing heat-tolerant pepper varieties has become a priority for ensuring stable production in the face of rising temperatures.

Peppers have an optimum temperature range of 25–30 °C day and 18–19 °C night (Jang et al., 2008). However, exposure to temperatures exceeding 30 °C induces a range of physiological and morphological changes that compromise their growth and productivity. Heat stress disrupts key physiological processes leading to cell membrane damage, accumulation of reactive oxygen species (ROS), chloroplast degradation, reduced photosynthetic efficiency, and increased respiration (Hu et al., 2010; Usman et al., 2015, 2020; Rajametov et al., 2021; Sachdev et al., 2021; Padilla et al., 2024). These changes result in morphological alterations including stunted growth, particularly in the root system, flower abscission, and fruit malformation, further diminishing crop quality and yield (Lee et al., 2014; Oh and Koh, 2019). Heat stress during the flowering stage is particularly detrimental because it reduces pollen viability and germination rate, resulting in decreased fruit set (Erickson and Markhart, 2002; Kaur et al., 2016; Lin et al., 2022). It has been reported that heat stress during this critical phase can drastically reduce pepper yields (Rosmanina et al., 2022).

* Corresponding author.
E-mail address: limejya@korea.kr (Y. Jang).

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Heat-tolerant peppers have developed various strategies to adapt to high-temperature stress. These include the enhancement of antioxidant systems, increased expression of heat shock proteins (HSPs), and accumulating osmolytes and other osmotic adjustment substances (Usman et al., 2015; Ghai et al., 2016; Bello et al., 2023; Preet et al., 2023). Recent research has suggested that heat-tolerant peppers maintain constant photosynthesis via increased transpiration under heat stress condition (Sachdev et al., 2021). These complex responses to heat stress contribute to the survival and maintaining productivity of pepper under challenging environmental conditions.

Traditionally, heat tolerance evaluation has relied on measuring physiological and morphological traits or conducting visual assessments (Ghai et al., 2016; Ali et al., 2020; Rajametov et al., 2021; Shi et al., 2023). However, these approaches are often time- and labor-consuming, expensive, destructive and subjective. Advances in imaging technologies now enable rapid, non-destructive, continuous, objective, and high-throughput phenotyping, offering a more precise and efficient way to evaluate crop stress responses (Walsh et al., 2024). Various imaging techniques including RGB, thermal, multispectral, hyperspectral, and fluorescence imaging, have been employed in plant stress research to enhance accuracy and efficiency.

Heat stress in plants leads to reduced growth, altered canopy architecture, wilting, and changes in pigmentation due to chlorophyll degradation and anthocyanin accumulation (Bita and Gerats, 2013). These stress-induced alterations manifest as both structural and spectral changes, which can be effectively captured and analyzed using advanced imaging and 3D laser scanning techniques. 3D Multispectral laser scanning, especially RGB and near-infrared (NIR) imaging, has emerged as a powerful and non-destructive tool to assess plant responses to stress (Aneley et al., 2023; Fumia et al., 2023; Agarwal et al., 2024). This technique allows for the calculation of various vegetation indices, such as the normalized difference vegetation index (NDVI) and the normalized pigment chlorophyll ratio index (NPCI), which serve as indicators of plant health and photosynthetic efficiency under stress. In addition, 3D laser scanning techniques have proven effective in monitoring structural changes in plants, such as alterations in leaf angle, plant height, and biomass, providing valuable insights into how plants adapt to stress (Kjaer and Ottosen, 2015).

Screening for heat tolerance in the field is challenging due to interactions with confounding environmental factors and uncontrollable conditions, although a wide variety of measurable traits are available for successful selection in the field (Bita and Gerats, 2013). The inherent variability of field environments often complicates the ability to isolate the effects of heat stress from other stress factors such as drought, nutrient deficiencies, or pest pressure. This variability can mask the true heat tolerance potential of different genotypes, making the selection process more difficult and less precise. Uncontrollable environmental conditions in the field often fail to provide consistent and reproducible results, making it challenging to interpret plant responses to heat stress accurately. However, controlled environmental studies that employ imaging techniques can complement field trials by offering more precise and reproducible data on how plants respond to heat stress. These advanced technologies enhance the ability to detect and quantify stress responses, providing a high-throughput, scalable approach for screening tolerant cultivars and understanding the underlying mechanisms of tolerance (Chawade et al., 2019). This approach allows for the evaluation of a large number of genotypes under standardized conditions, potentially accelerating the breeding of heat-tolerant varieties.

The objective of this study was to develop a rapid and accurate method for evaluating heat tolerance in pepper genetic resources using 3D laser scanning. Our goal is to provide a comprehensive understanding of how pepper respond to heat stress and offer an efficient tool for selecting heat-tolerant entries. By integrating controlled environment studies with advanced 3D laser scanning, we aimed to address the limitation of field screening and generate more reliable data for heat tolerance assessment. Furthermore, this methodology could potentially be applied to evaluate stress tolerance in other crops, making a valuable contribution to crop breeding research aimed at mitigating challenges of climate change.

2. Materials and methods

2.1. Plant material and growth conditions

This experiment was conducted in a growth chamber at the World Vegetable Center (WorldVeg), Taiwan from June to August 2024. A total of 20 pepper (*Capsicum annuum* L.) genotypes from the global *Capsicum* core collection were used (Tripodi et al., 2021). The genotypes had previously been evaluated for heat tolerance under field conditions by Fumia et al. (2023) and were selected for their performance to high temperature stress in the field (Table 1). Seeds of each genotype were sown in 70-cell plug trays (L 550 × *W*353 × *H* 48 mm, 7 × 10 cells) filled with a commercial media (King Root, Dayi Agritech Co., Ltd., Taiwan). Thirty-five seeds were sown per genotype and germinated in a growth chamber (L 150 × *W*90 × *H* 150 cm, two layers with 8 LED lamps, custom-made) set at 28 °C and dark conditions for 5 days after sowing.

After germination, the growth chamber with the trays was set to a temperature of 25/20 °C (day/night) and a photoperiod of 12/12 h (day/night). The actual day and night air temperature, relative humidity, and photosynthetic photon flux (PPF) were 26.6 ± 0.9/21.5 ± 1.5 °C, 77.2 ± 1.5/78.4 ± 2.2 %, and 174.3 ± 10.5 μmol·m^{-2}·s^{-1}, respectively. The trays were bottom-irrigated twice a week over four weeks after germination. From the 5th week on, a nutrient solution (Autopot power nutrients, N-P-*K* = 4.5–1–5.6, Autopot Australia Pty., Ltd., Australia, EC 1.2 dS·m^{-1}) was supplied twice a week, with approximately 2 L of solution per plug tray. Additional water was provided as needed when the growing medium appeared dry based on plant condition.

2.2. Heat stress treatment and measurements

2.2.1. Heat stress treatment

Seven weeks after sowing, 34 seedlings per genotype were divided into the control and heat stress treatment groups with 17 plants each. A walk-in chamber (L 4 × W3 × H 3 m, H-6400, Taiwan Hipoint Corporation, Taiwan) set to 50 % relative humidity and a light intensity of approximately 200 μmol·m^{-2}·s^{-1} was used for the heat stress treatment. The temperature in the heat stress treatment group was set to 40/35 °C day and night temperature and 12h/12 h photoperiod, while the control group was maintained at 25/20 °C day and night temperature and 12h/

Table 1
Pepper genotypes used in the experiment.

Code	Genotype	Scientific name	Heat tolerance[z]
Pep01	GPC035430	*Capsicum annuum*	Highly Tolerant
Pep02	GPC042150	*Capsicum annuum*	Highly Tolerant
Pep03	GPC017800	*Capsicum annuum*	Highly Tolerant
Pep04	GPC003010	*Capsicum annuum*	Tolerant
Pep05	GPC003090	*Capsicum annuum*	Tolerant
Pep06	GPC003350	*Capsicum annuum*	Tolerant
Pep07	GPC004160	*Capsicum annuum*	Tolerant
Pep08	GPC005480	*Capsicum annuum*	Tolerant
Pep09	GPC003040	*Capsicum annuum*	Intermediate
Pep10	GPC014630	*Capsicum annuum*	Intermediate
Pep11	GPC007080	*Capsicum annuum*	Intermediate
Pep12	GPC018160	*Capsicum annuum*	Intermediate
Pep13	GPC008380	*Capsicum annuum*	Intermediate
Pep14	GPC095130	*Capsicum chinense*	Intermediate
Pep15	GPC001740	*Capsicum chinense*	Intermediate
Pep16	GPC121620	*Capsicum annuum*	Sensitive
Pep17	GPC121710	*Capsicum annuum*	Sensitive
Pep18	GPC122310	*Capsicum annuum*	Sensitive
Pep19	GPC123750	*Capsicum annuum*	Sensitive
Pep20	GPC127520	*Capsicum annuum*	Sensitive

[z] Results of field evaluation by the breeder (Fumia et al., 2023).

12 h photoperiod. The heat stress treatment was applied for 14 days. The light intensity of the LED was adjusted by controlling the height of the LED light sources. The air and root temperatures, relative humidity, and PPF data were collected at 30-mintute intervals using environmental sensors (LightScout Quantum Light sensor and external (soil)) temperature sensor, Spectrum Technologies, Inc., USA) and a data logger with a built-in temperature and humidity sensor (WatchDog 1000 Series Micro Stations, Spectrum Technologies, Inc., USA) (Fig. 1). The actual average day and night air temperatures were 25.0 ± 0.3/17.7 ± 0.3 °C in the control and 35.7 ± 0.3/31.5 ± 0.1 °C in the heat treatment. Root-zone temperatures were maintained 2–3 °C lower than the air temperatures, with average day and night values of 22.3 ± 0.3/14.8 ± 0.4 °C and 33.5 ± 0.6/29.9 ± 0.4 °C for the control and heat treatment, respectively. The average day and night relative humidity was 70.0 ± 1.5/73.2 ± 1.6 % in the control and 59.5 ± 2.0/65.3 ± 0.8 % in the heat treatment. The PPFs measured 195.1 ± 5.7 and 226.8 ± 9.9 μmol·m^{-2}·s^{-1} in the control and heat treatment, respectively.

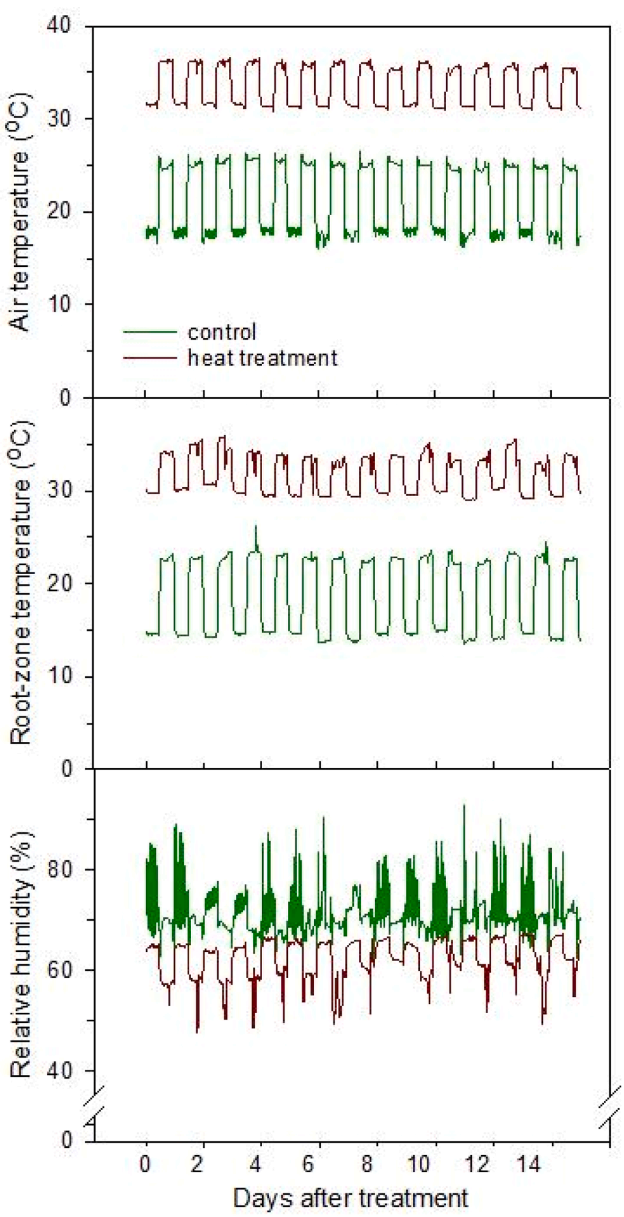

Fig. 1. Air and root-zone temperature and relative humidity in the walk-in chamber during heat treatment.

2.2.2. Phenotyping using 3D multispectral scanner

Phenotypic data for 20 pepper genotypes were acquired from both heat treatment and control groups using a portable phenotyping workstation on wheels (L 2.5 × W 0.6 × H 2.0 m, TraitFinder, Phenospex, The Netherlands) equipped with a sensor head consisting of a pair of multispectral 3D laser scanners (PlantEye F600, Phenospex, The Netherlands). The TraitFinder was located in the same indoor research facility as the walk-in chambers used for heat stress treatment, with an ambient temperature maintained at approximately 25 °C during measurements. Scanning was performed at 2-day intervals over a total period of 14 days of heat treatment. Four plug trays, with four plants per genotype in each tray were scanned maintaining a distance of approximately 0.7–0.8 m between the scanner and the plants, with the scanners moving at a constant speed of 6.7 cm/s. The PlantEye F600 comprises a near infrared (NIR) and RGB light and corresponding sensors for red (R; λpeak = 624–634 nm), lime green (G; λpeak = 530–540), blue (B; λpeak = 465–485), and NIR (λpeak = 720–750 nm) spectral bands, along with a LiDAR laser source (λpeak = 935–945 nm) for 3D data cloud construction. Scanning data were immediately processed by the built-in HortControl software, generating point clouds containing spatial (X, Y, Z) and spectral (R, G, B, NIR) information for each data point. The traits automatically analyzed by the HortControl software included 3D leaf area, projected leaf area, digital biomass, plant height, plant height average, canopy light penetration depth, NDVI ((NIR– R) / (NIR + R), −1 to 1), NPCI ((R – B) / (R + B), −1 to 1), plant senescence reflectance index (PSRI, (R – G) / NIR, −1 to 1), and green leaf index (GLI, (2 × G – R – B) / (2 × G + R + B), −1 to 1).

2.2.3. Root growth phenotyping by image data analysis

Root growth phenotyping was performed according to the method described by Kim et al. (2024), with slight modifications. Three pepper seedling root balls per treatment from each genotype at the end of the experiment, 14-days after the onset of the heat treatment were placed in a photobox (L 63 × W63 × H 63 cm, Z60 LED, ZENITH, Taiwan) with LED lighting installed at the top. A surface image of each root ball was captured using a digital camera under consistent light conditions. The images were then processed and analyzed using Image J Fiji software to quantify root growth (Fig. 2). The processed images were used to calculate the two main parameters for quantitative assessment of root growth. The root surface area was determined by converting the number of pixels corresponding to the root tissue in the actual area using a scale marker present in each image. Additionally, the root to root ball ratio was computed as the proportion of root pixels to the total root ball pixels as expressed as a percentage, providing a measure of root density within the root ball.

2.2.4. Manual phenotyping

At 14 days after the heat stress treatment, at the end of the experiment, physical measurement of the pepper seedlings was conducted. The above-ground parts of three seedlings per treatment were separated and weighed. After measuring the fresh weight, the shoot samples were oven-dried at 80 °C (DKN613, Yamato Scientific Co., Ltd. Japan) for three days and weighed. The stem diameter was measured at 1 cm below the cotyledon node. Leaf chlorophyll content was estimated using a SPAD meter (SPAD-502Plus, Konica Minolta, Japan). Measurement were taken from three fully expanded leaves from the top of each plant. Three readings were taken for each leaf and averaged to obtain the representative SPAD value for each seedling.

2.2.5. Visual assessment of heat stress

A visual assessment was conducted to evaluate the impact of heat stress on pepper seedlings. The assessment was carried out on day 2, 6, 10, and 14 after the initiation of heat treatment. A 5-point scale (visual score: 5 healthy ~ 1 dead) was used to rate the visual appearance of the seedlings, following the method described by Shi et al. (2023) (5 class, class 0 normal with no damage ~ class 4 severe damage) and Jang et al.

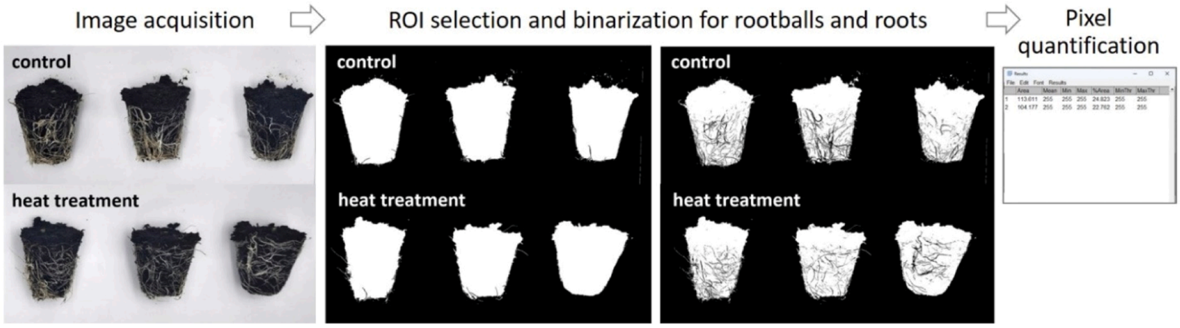

Fig. 2. Workflow depicting the region of interest (ROI) selection, binarization, and quantification of root ball and root areas using Image J software.

(2024) (6-point scale, 0 healthy ~ 5 dead) .

2.3. Statistical analysis

Statistical analyses were performed using R software (version 4.4.1, R Foundation for Statistical Computing, Vienna, Austria). Principal component analysis (PCA) was conducted on centered and scaled. The distance between the treatment group centroids (ΔC) at each timepoint was calculated as follows:

$$\Delta C = \sqrt{[(x_1 - x_2)^2 + (y_1 - y_2)^2]}$$

where (x_1, y_1) and (x_2, y_2) are the coordinates of the control and heat-treated group centroids in the PC1-PC2 space.

The mean Euclidean distance (D) between the control and heat-treated samples for each genotype across the treatment period was calculated as follows:

$$D = \sqrt{[(PC1_h - PC1_c)^2 + (PC2_h - PC2_c)^2 + (PC3_h - PC3_c)^2]}$$

where $PC1_h$, $PC2_h$, $PC3_h$ represent the mean PC scores for heat treatment and $PC1_c$, $PC2_c$, $PC3_c$ represent those for control conditions.

K-means clustering was performed with $k = 3$, with the optimal number of clusters determined using the elbow method by plotting the within-cluster sum of squares against different k values (Fig. S1). Correlation between phenotyping and actual measurements at 14 days after heat treatment were analyzed using Pearson correlation coefficients (r) in R Software. The graphical representations for growth and spectral trait data were created using the SigmaPlot software (version 14, Grafiti, Palo Alto, CA, USA). Statistical significance for the effects of heat treatment was determined using Student's *t*-test with $\alpha = 0.05$.

3. Results

This study evaluated twenty pepper genotypes, including of eighteen *Capsicum annuum* and two *C. chinense* genotypes selected for their varying levels of reported heat tolerance (Table 1). Morphological and physiological responses to heat stress were monitored non-destructively over the 14-day treatment period using the TraitFinder system. The analysis revealed significant genotypic variation in response to heat stress. Among the genotypes, 'Pep17 (GPC121710)' stood out, exhibiting robust vegetative growth under heat stress, with increases of 26 % in 3D leaf area and 46 % in digital biomass compared to the control. In contrast, 'Pep06 (GPC003350)' showed a marked growth reduction, with declines of 27 % in 3D leaf area and 21 % in digital biomass under heat stress (Fig. 3, S2-S7).

PCA of morphological and spectral traits of pepper genotypes revealed progressive differences between the control and heat-treated groups over time. The separation was minimal at day 2 (ΔC = 0.47), but became increasingly distinct, with clear clustering patterns emerging by day 4 (ΔC = 1.69). The Euclidean distance in the PCA space showed distinct phases: initial response (days 2–4), adaptation phase (days 6–8, ΔC = 1.81 and 1.54), maximum separation (day 10, ΔC = 2.05), and stabilization (days 12–14, ΔC = 1.61 and 1.97) (Fig. 4).

The first three principal components consistently explained approximately 80 % of the total variance (Fig. 5). PC1 explaining 31–37 % of the variance, was primarily associated with morphological traits, while PC2 (24–29 %) captured spectral parameters. PC3, accounting for 19–24 % of the variance, reflected complex trait interactions. K-means clustering analysis based on PCA coordinates identified three distinct groups, with noticeable transitions between clusters during the heat treatment period (Fig. S8). Further analysis of heat stress response patterns through calculation of mean PC distances between control and heat-treated conditions for each genotype (Fig. 6) identified three response groups: a low response group (5 genotypes: 'Pep15 (GPC003090)', 'Pep17 (GPC121710)', 'Pep12 (GPC018160)', 'Pep3 (GPC017800)', 'Pep18 (GPC122310)'), a moderate response group (9 genotypes), and a high response group (6 genotypes: 'Pep2 (GPC042150)', 'Pep6 (GPC003350)', 'Pep5 (GPC003090)', 'Pep8 (GPC005480)', 'Pep19 (GPC123750)', 'Pep16 (GPC121620)').

Temporal dynamics analysis based on changes in PC distances (Fig. 7) showed distinct response patterns between groups. The high response group exhibited maximum deviation from control conditions around day 10, while the low response group maintained relatively stable patterns throughout the treatment period. Further analysis of the PC-wise response distribution over time (Fig. S9) highlighted variations in trait dynamics. PC1 distance demonstrated a fluctuating pattern with initial positive values, a mid-treatment decline, and a subsequent increase in the later stages (day 10–14). PC2 distance exhibited a progressive shift from negative to positive values throughout the treatment period, with maximum positive changes observed in the final days. PC3 distance displayed a U-shaped pattern, decreasing to its lowest values during mid-treatment before increasing again in the later stages, particularly on day 14.

Correlation analysis of phenotypic parameters at day 14 (Fig. 8) showed meaningful relationships between traditional destructive measurements and TraitFinder-based assessments. Notably, 3D leaf area correlated positively with fresh weight ($r = 0.66$) and dry weight ($r = 0.48$). Terminal evaluation after the 14-day heat treatment revealed significant genotype-dependent variations in conventional growth parameters (Fig. 9). Shoot fresh weight ranged from 65 % to 120 % of control values, reflecting diverse responses to heat stress. Root system analysis showed that most genotypes either maintained or increased their root area under heat stress, with only two genotypes ('Pep06 (GPC003350)', 'Pep07 (GPC004160)') showing reductions exceeding 15 %. SPAD values showed modest variations (85–105 % of the control), indicating that chlorophyll content was maintained under heat stress conditions.

Visual scoring and flowering pattern analysis revealed distinct heat stress responses among pepper genotypes (Fig. S10 and S11). Visual scores in control plants remained consistently high (4–5), while heat-treated plants showed genotype-specific decreases. Notably, 'Pep06

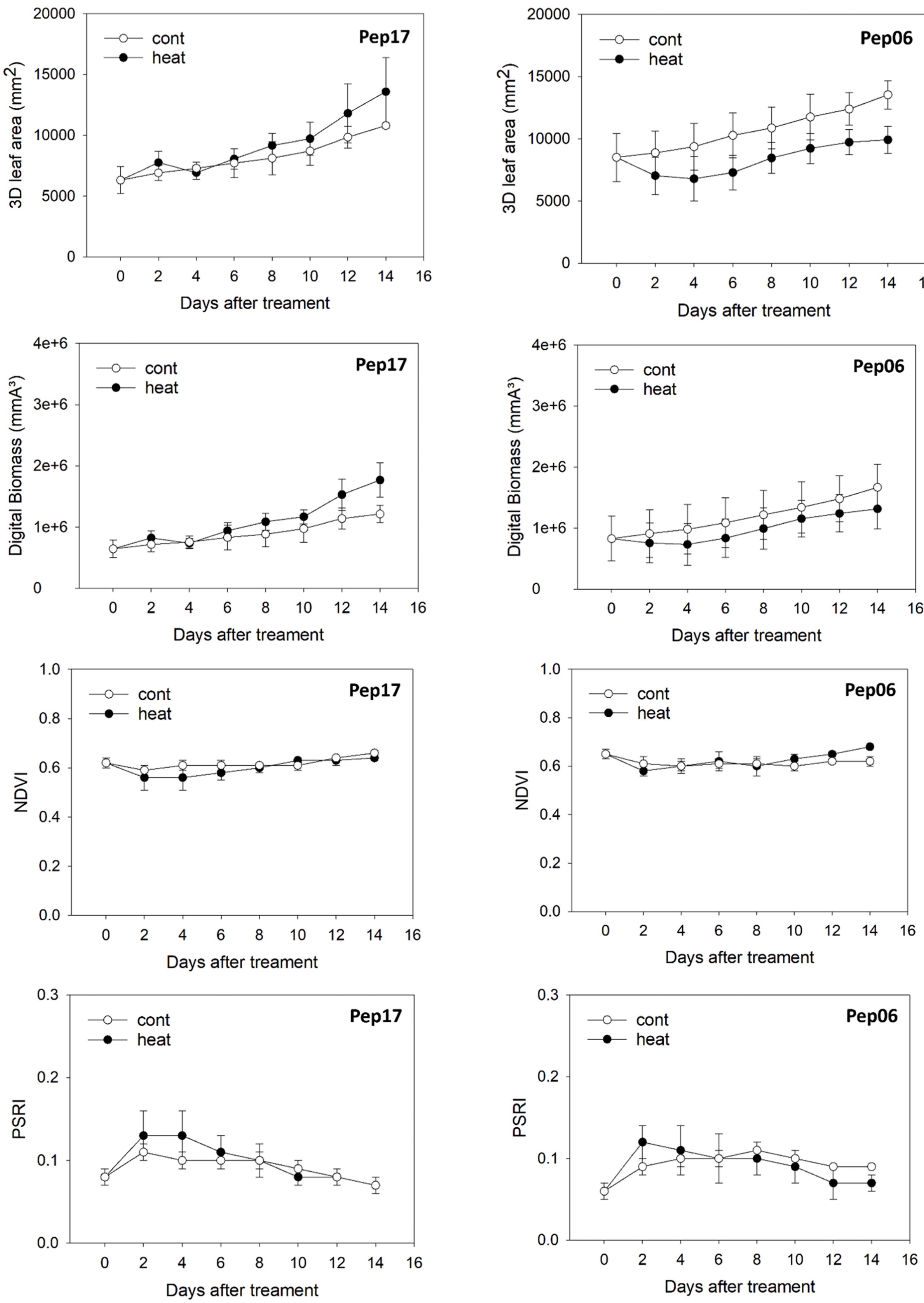

Fig. 3. 3D leaf area, digital biomass, NDVI, and PSRI of pepper genotypes 'Pep17' (left) and 'Pep06' (right) influenced by heat treatment. Values have been expressed as mean ± SD.

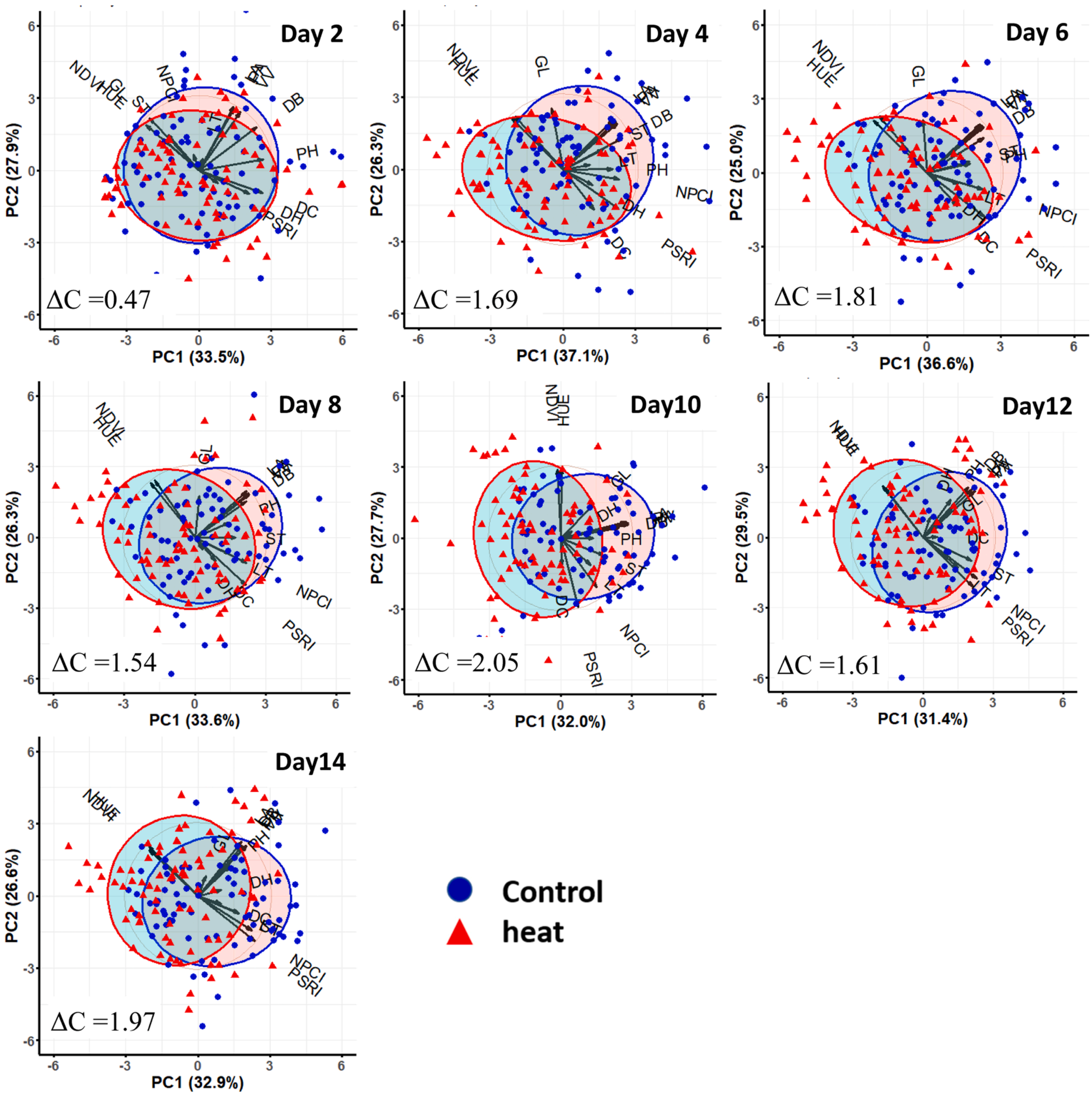

Fig. 4. PCA of morphological and spectral traits of pepper genotypes influenced by heat treatment. Values in parentheses indicate the percentage of variance explained by the corresponding PC. Ellipses represent the 95 % confidence interval for each class, viz. control and heat treatment ($n = 80$). ΔC indicates the Euclidean distance between the centroids of both groups. The morphological traits: LA (3D leaf area), PA (projected leaf area), DB (digital biomass), PH (plant height), DC (canopy light penetration depth), DH (convex hull area), VV (voxel volume average), The spectral traits: GL (GLI), HUE, LT (lightness), NDVI, NPCI, PSRI, ST (saturation).

(GPC003350)' exhibited severe heat sensitivity with scores declining to ~1 by day 14, while Pep01 (GPC035430), 'Pep09 (GPC003040)', and several other genotypes maintained scores above 4. Among the 20 genotypes studied, only 8 genotypes ('Pep01 (GPC035430)', 'Pep02 (GPC042150)', 'Pep04 (GPC003010)', 'Pep06 (GPC003350)', 'Pep08 (GPC005480)', 'Pep10 (GPC014630)', 'Pep11 (GPC007080)', and 'Pep16 (GPC121620)') produced flowers during the experimental period. Heat treatment significantly influenced flowering dynamics in these genotypes. 'Pep04 (GPC003010)' and 'Pep10 (GPC014630)' showed accelerated flowering under heat stress (60 % and 50 % respectively) compared to minimal flowering in controls. 'Pep11 (GPC007080)' achieved full flowering under both conditions, though heat treatment hastened the process. In contrast, 'Pep01 (GPC035430)', 'Pep08 (GPC005480)', and 'Pep16 (GPC121620)' displayed limited flowering response regardless of treatment.

Heat stress also affected flower morphology significantly. Control plants of 'Pep02 (GPC042150)' and 'Pep11 (GPC007080)' produced normal, fully developed white flowers. However, heat-treated plants exhibited abnormal flower development, characterized by incomplete petal formation and irregular flower structure (Fig. S12).

4. Discussion

Phenotyping has historically been a major bottleneck in plant

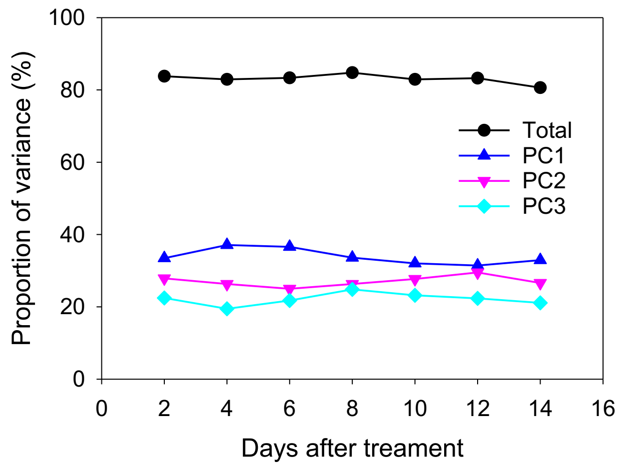

Fig. 5. Time series of PCA explained variance ratio during heat treatment.

breeding programs, primarily due to its labor-intensive nature and the challenge of simultaneously measuring multiple traits (Yang et al., 2020). Conventional methods of assessing stress tolerance often rely on destructive measurements and subjective visual scoring, which limit the ability to track dynamic responses in individual plants over time (Lee et al., 2024). High-throughput phenotyping systems effectively address these limitations by enabling automated, non-destructive measurements of multiple traits with high temporal resolution and precision (Lee et al., 2018; Fumia et al., 2023; Poorter et al., 2023; Agarwal et al., 2024).

Controlled condition phenotyping provide significant advantages over field-based approaches, particularly for stress tolerance studies. While outdoor phenotyping presents challenges in controlling environmental variables, controlled condition facilities enable precise control of temperature, light and humidity, which is crucial for studying specific stress responses (Yang et al., 2020). Under field conditions, heat stress is frequently accompanied by drought stress, as plants increase transpiration to regulate leaf temperature (Zandalinas et al., 2018). Additionally, fluctuating light intensity, soil heterogeneity, pest pressure, and varying nutrient availability can all impact plant responses, making it difficult to isolate heat stress effects. To address this challenge, our study ensured optimal irrigation and nutrient management throughout the experiment, with daily watering and controlled fertilization regimes. Additionally, the controlled environment enabled us to maintain consistent temperature and humidity levels throughout the experiment, which is difficult to achieve under field conditions (Poorter et al., 2016).

Our study demonstrated the effectiveness of the TraitFinder system for evaluating heat stress responses in pepper seedlings, offering several advantage over traditional phenotyping approaches. The system enables the simultaneous measurement of both morphological and physiological traits, including leaf area, digital biomass, and spectral indices, while minimizing human error and labor requirement. The reliability of these non-destructive measurements was validated through strong correlations with traditional destructive measurements, with 3D leaf area showing positive correlations with fresh weight ($r = 0.66$) and dry weight ($r = 0.48$). Furthermore, the non-destructive nature of these measurements allows for continuous monitoring of individual plants, revealing temporal patterns in stress response that would be impossible to detect with conventional destructive methods. This ability to track dynamic responses in the same plants throughout the experiment provides valuable insights into the temporal progression of heat stress responses.

The key innovation of our study is the application of PC-space distance calculations to quantifying heat stress responses. Unlike traditional approaches that evaluate individual trait responses separately, this approach provides a comprehensive metric for assessing overall stress adaptation patterns. The Euclidean distance between the control and heat-treated groups in the PC space effectively captured the magnitude of stress responses, enabling the objective identification of heat-tolerant genotypes. This approach builds upon recent multivariate stress analysis methods (Biermann et al., 2022), while introducing a more nuanced quantification of stress response patterns.

Across our 20 chili pepper genotypes, we observed a clear association between PC distance magnitude and phenotypic stability under heat stress. Genotypes exhibiting smaller PC distances, such as 'Pep15 (GPC001740)', 'Pep17 (GPC121710)', 'Pep12 (GPC018160)', and 'Pep3 (GPC017800)', maintained growth patterns similar to control conditions, suggesting efficient homeostatic mechanisms (Hasanuzzaman et al., 2013). This finding aligns with recent research on phenotypic stability under stress conditions (Abdelghany et al., 2024), providing a quantitative framework for identifying stable genotypes under heat stress.

Our PC-distance based approach reveals important parallels with previous temperature response classifications in *Capsicum* species. Similar to the Type III genotypes identified by Jang et al. (2008), which maintained stable relative growth rates across temperature regimes, our

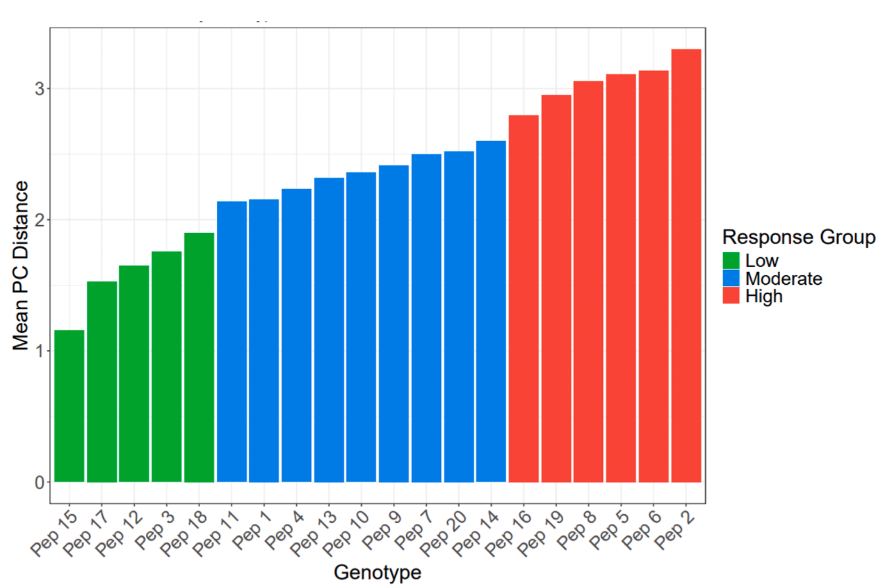

Fig. 6. Mean PC distance by genotype.

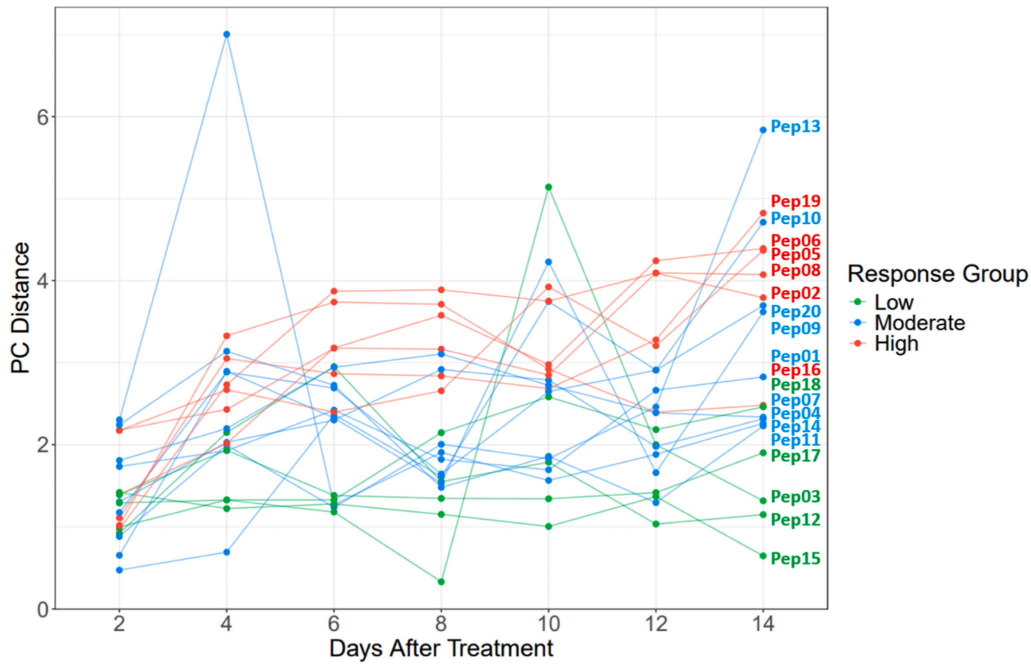

Fig. 7. PC distance changes over time by response group.

genotypes with minimal PC distances (such as 'Pep15 (GPC001740)', 'Pep17 (GPC121710)', 'Pep12 (GPC018160)', and 'Pep3 (GPC017800)') demonstrated substantial growth stability under heat stress. This physiological homeostasis across temperature conditions appears to be a key adaptive mechanism for heat tolerance. Just as Jang et al. found that Type III genotypes maintained consistent shoot-to-root ratios and exhibited minimal impact on root development across temperature ranges, our low-distance genotypes preserved their multidimensional growth patterns despite heat stress exposure.

This thermal stability is particularly valuable for breeding programs, as it indicates robust physiological buffering mechanisms that maintain essential growth functions under stress. Such genotypes likely possess superior respiratory homeostasis, as described by Kurimoto et al. (2004), enabling them to maintain similar metabolic rates regardless of temperature fluctuations. The identification of these temperature-stable genotypes through PC-distance measurements provides an efficient screening approach for heat tolerance breeding programs, as it captures the multidimensional growth stability that characterizes the most resilient genotypes.

The Euclidean distance measurements revealed distinct heat response categories among the genotypes. Those with minimal distances between control and heat-treated groups ($\leq$1.2) demonstrated remarkable stability in their growth patterns despite temperature stress. Intermediate-distance genotypes (1.2–2.5) showed moderate alterations in their growth parameters but maintained overall productivity. In contrast, large-distance genotypes (>2.5) exhibited substantial shifts in their growth characteristics under heat stress, indicating significant physiological adjustments or potential stress susceptibility.

Our multi-dimensional PCA approach revealed that the first three principal components consistently explained approximately 83 % of the total variance across all time points, with PC1 accounting for 33.9 %, PC2 for 27.1 %, and PC3 for 22.2 %. This high percentage indicates that these components effectively capture the major patterns in the data, validating our use of these dimensions for distance calculations.

Our observations on reproductive development under heat stress revealed interesting patterns of flowering responses among genotypes. Notably, 'Pep04 (GPC003010)', 'Pep10 (GPC014630)', and 'Pep11 (GPC007080)' exhibited heat-induced flowering acceleration, with 'Pep04 (GPC003010)' showing about 60 % flowering in heat treatments versus minimal flowering in controls. While 'Pep11 (GPC007080)' achieved full flowering under both conditions, heat treatment accelerated the process. In contrast, 'Pep01 (GPC035430)', 'Pep08 (GPC005480)', and 'Pep 16 (GPC007080)' showed limited flowering (<10 %) regardless of treatment, and 'Pep06 (GPC003350)' exhibited low flowering rates (~10 %) despite the growth reduction by heat stress.

However, these heat-induced flowers frequently displayed morphological abnormalities and failed to set fruit, ultimately leading to chlorosis and abscission. This morphological alteration suggests that heat stress not only affects flowering time but also disrupts normal flower development processes. These findings align with those of previous studies on the effects of heat stress on the reproductive development of *Capsicum* species (Erickson and Markhart, 2002; Kim et al., 2023). The occurrence of flowering only in control conditions for some genotypes (e.g., 'Pep16 (GPC121620)') further emphasizes the complex interaction between temperature and reproductive development.

The differential sensitivity between vegetative growth and reproductive development presents crucial implications for breeding programs. While some genotypes maintained stable vegetative growth under heat stress, reproductive success was consistently compromised, suggesting independent genetic control mechanisms for these traits (Pagamas and Nawata, 2008). This dichotomy between vegetative stability and reproductive sensitivity highlights the need for comprehensive breeding strategies that address both aspects of heat tolerance.

A limitation of our study is the inherent difficulty of indoor phenotyping in fully replicating the complex field conditions. The gap between controlled environment studies and field performance remains a significant challenge (Araus et al., 2018), particularly when translating seedling-stage heat tolerance to reproductive success under field conditions. In fact, most of the heat tolerant entries identified by Fumia et al. (2023) from field-based phenotyping were identified as being in the high response group when evaluated at the seedling stage in controlled conditions. Among the low response group, only 'Pep03 (GPC017800)' was also found to be highly tolerant in the field screening (Fumia et al., 2023). Similarly, among the high response group, only 'Pep16 (GPC121620)' and 'Pep19 (GPC123750)' were also found to be highly sensitive to heat stress in the field-based screen (Fumia et al., 2023). This issue is especially relevant given our observations of compromised reproductive development under heat stress.

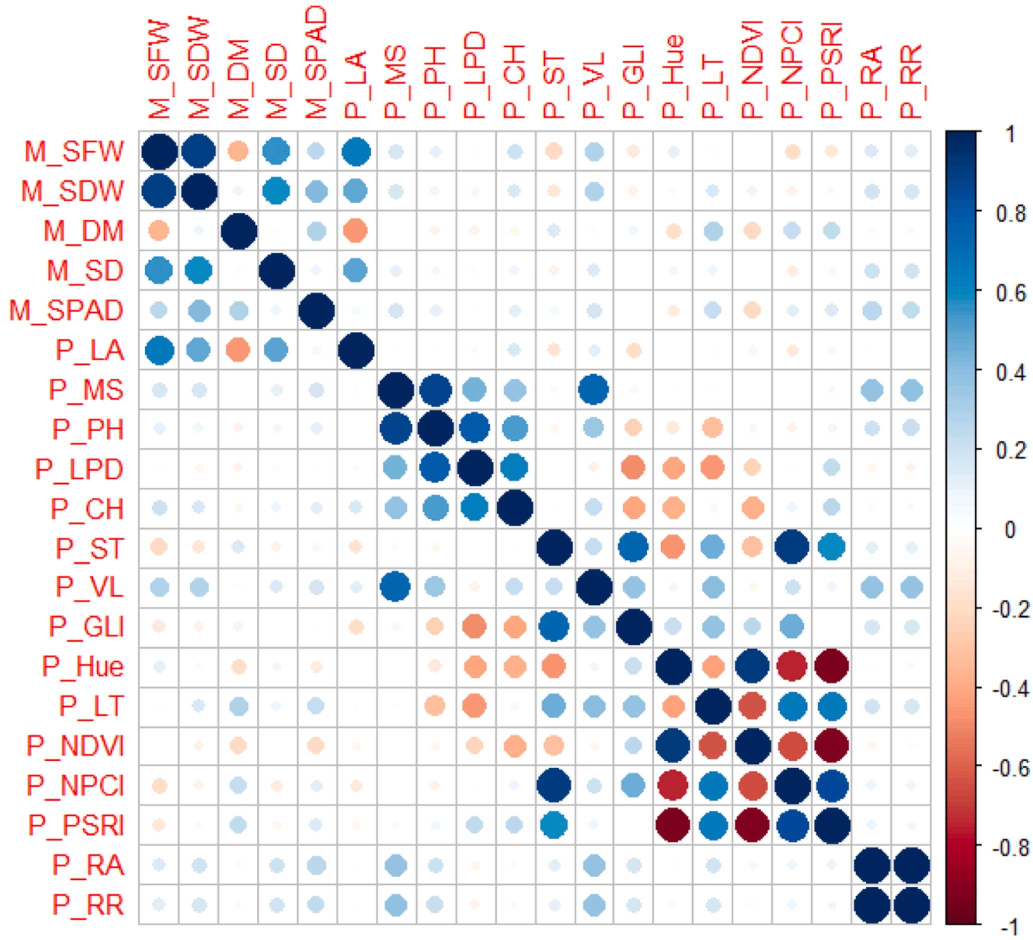

Fig. 8. Pairwise Pearson correlation of all the phenotypic parameters collected at 14 days after heat treatment. Traits beginning with "M" in the upper left represent the actual measurements taken after heat treatment termination, including fresh shoot weight (M_SFW), dry shoot weight (M_SDW), dry matter (M_DM), stem diameter (M_SD), and M_SPAD values. Below these, traits beginning with "P" indicate characteristics measured using the Traitfinder system and RGB camera (and Image J) on day 14 of heat treatment. The abbreviations for each trait are defined as follows: P_LA is 3D leaf area, P_MS is digital biomass, P_PH is plant height average, P_LPD is canopy light penetration depth, P _CH is convex hull area, P_ST is saturation average, P_VL is voxel volume total, P_GLI is GLI average, P_Hue is Hue average, P_LT is lightness average, P_LT is lightness average, P_NDVI is NDVI average, P_NPCI is NPCI average, P_PSRI is PSRI average, P_RA is root areas, and P_RR is root ratio.

Future research should focus on bridging the gap between indoor and field phenotyping through several approaches. While our controlled environment study successfully identified heat-tolerant genotypes based on vegetative traits, developing integrated screening protocols that effectively link controlled environment and field performance is crucial. Specifically, protocols that can predict field reproductive success from early-stage controlled environment screening would be valuable, give our observation that vegetative heat tolerance did not necessarily correspond to reproductive heat tolerance. These protocols should incorporate both developmental stage-specific traits and environmental interactions that influence the expression of heat tolerance. First, sequential screening approaches that start with controlled environment evaluation of early vigor and vegetative heat tolerance, followed by field validation of reproductive success, could help optimize the selection process. Second, long-term studies that track genotypes from the seedling to reproductive stages under varying environmental conditions will help validate early-stage phenotyping results. Additionally, incorporating physiological markers and molecular tools could deepen our understanding of heat tolerance mechanisms across developmental stages (Ohama et al., 2017; Lee et al., 2022), particularly in relation to the connection between vegetative and reproductive heat tolerance.

The development of mathematical models linking controlled condition phenotyping data with field performance could help identifying key developmental stages and environmental conditions that influence the expression of heat tolerance. These models should specifically account for the complex interactions between temperature and reproductive development, as observed in this study.

5. Conclusion

This study demonstrated the efficacy of advanced 3D multispectral phenotyping under controlled environments for evaluating heat stress responses in pepper (*Capsicum* spp.) genotypes. The integration of continuous, non-destructive measurements of morphological and spectral traits facilitated the development of a novel screening methodology based on principal component analysis and Euclidean distance calculations, which quantifies heat stress adaptation patterns with enhanced precision and objectivity.

Temporal dynamics analysis revealed characteristic stress response patterns with maximum separation between control and heat-treated plants occurring at day 10, establishing an optimal timepoint for phenotypic evaluation. Significant variation in heat response patterns among pepper genotypes indicated substantial genetic diversity in adaptation mechanisms, providing valuable resources for heat tolerance breeding programs.

The multivariate PC-distance approach provides a comprehensive alternative to conventional single-trait evaluations by simultaneously capturing both morphological and physiological responses to heat stress.

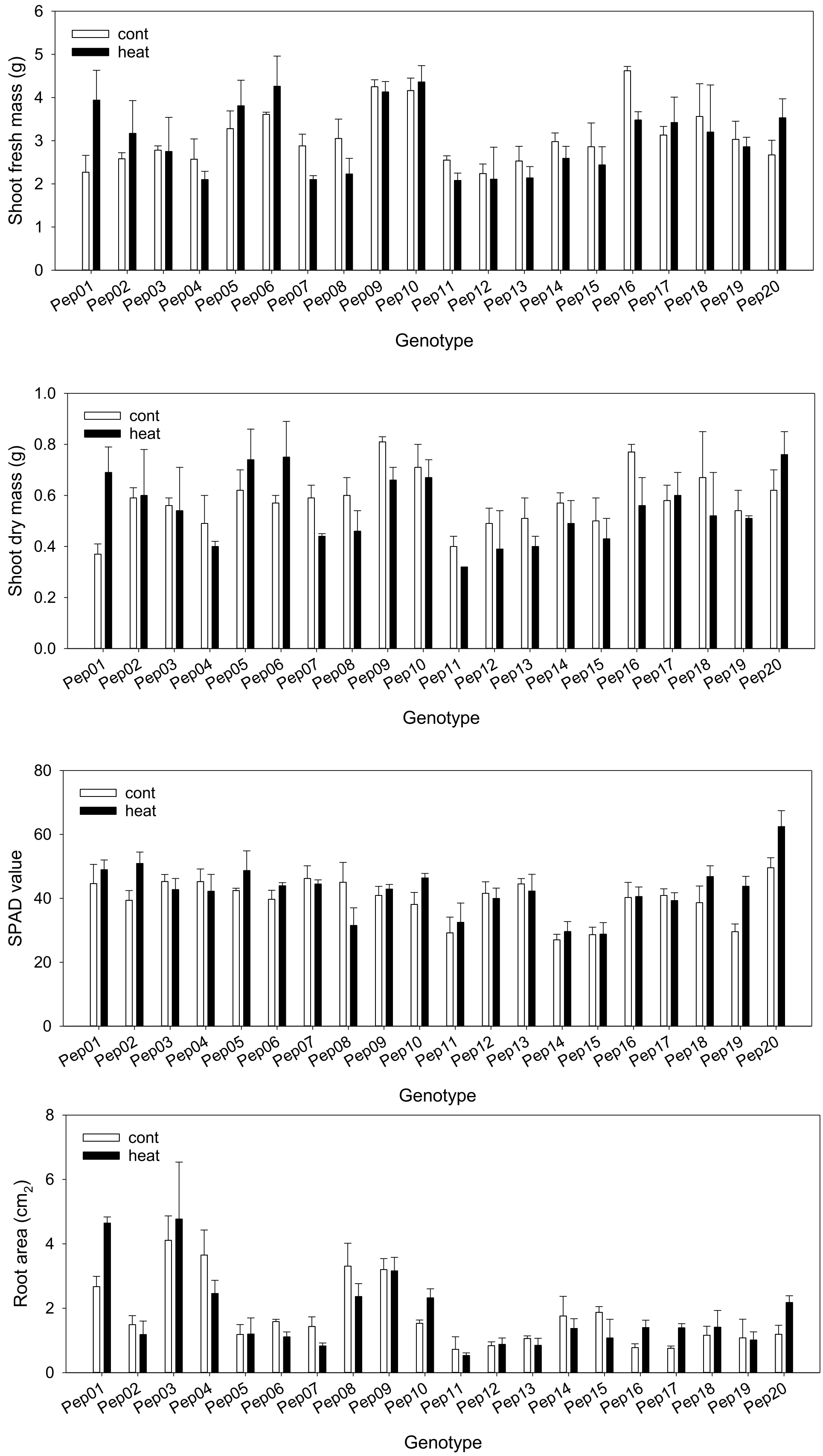

Fig. 9. Shoot fresh and dry mass, SPAD value, and root area of pepper genotypes after 14-day heat treatment. Values have been expresses as mean ± SD.

This integrated methodology enables efficient identification of genotypes with stable performance across multiple parameters, establishing a robust selection criterion for climate resilience breeding.

Strong correlations between non-destructive phenotypic measurements and traditional destructive assessments validated the reliability of this phenotyping system for germplasm evaluation. The precise environmental control achieved in growth chambers allowed for the assessment of heat stress responses in isolation from confounding factors, a significant advantage over field-based screening approaches.

This research provides a methodological foundation for breeding strategies that integrate high-throughput phenotyping for developing climate-resilient pepper varieties. Future investigations should focus on characterizing the genetic basis of stable genotypes with minimal PC distances for heat stress responses, elucidating the genetic architecture underlying stable heat response patterns, and validating the relationship between seedling-stage resilience and reproductive-stage performance under field conditions.

CRediT authorship contribution statement

Yoonah Jang: Writing – original draft, Methodology, Conceptualization. **Roland Schafleitner:** Writing – review & editing, Supervision, Funding acquisition. **Derek W. Barchenger:** Writing – review & editing, Resources. **Ya-ping Lin:** Writing – review & editing, Data curation. **Junho Lee:** Visualization, Software.

Declaration of competing 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2025.114136.

Data availability

Data will be made available on request.

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