Physiological and Biochemical Responses of *Lens culinaris* Medik. under Combined Heat and Water Deficit Stress

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ABSTRACT

Lentil (*Lens culinaris* Medik.) is a key cool-season pulse crop that suffers from the joint attack of heat and water deficiency aggravated by temperature rise and shifting rainfall patterns worldwide. As joint stress does not simply lead to an increase in physiological stress caused by either heat or water deficit, there is a need for adequate assessment of the effects of heat and drought to be able to develop appropriate crop varieties. The study presents the results of assessing the physiological and biochemical response of lentil variety to the selected treatment comprising four variations: control (applied adequate water), heat stress, drought, and combined heat and drought stress. Growth, leaf water relations, chlorophyll stability, gas exchange parameters, osmolytes accumulation, antioxidant enzyme activity, oxidative stress markers, and yield components were the parameters evaluated throughout the treatment period. Compared to the control, the combined stress caused the greatest reductions in relative water content, chlorophyll concentration, net photosynthetic rate, and stomatal conductance as well as their total effect for some traits. Stress resulted in the highest accumulations of proline and soluble sugars and the greatest activities of enzymes such as catalase, superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase, which did not allow sufficiently to balance the high plant stress. The most significant reduction of yield parameters including pods per plant, number of seeds per plant, seed yield, and harvest index was found for combined stress. Analysis of correlation and principal components suggested that membrane stability, osmotic adjustment, and antioxidant enzyme capability were correlated with productivity under stress. Thus, results obtained help to reveal the physiological importance of combined heat and drought stresses in lentil as well as note the importance of membrane stability, osmobuffer accumulation, and synchronisation of antioxidant defense as major physiological markers for breeding climate-resilient lentils for hotter and drier production areas.

Keywords: osmoprotectant accumulation; antioxidant enzyme activity; chlorophyll stability; stomatal regulation; oxidative membrane damage; multifactorial stress tolerance; pulse crop improvement; reproductive-stage stress screening

1. Introduction

Lentil (*Lens culinaris* Medik.) is a crop that was domesticated among the most ancient of food legumes and continues to be one of the most important sources of nutrition with its rich protein content and prebiotic carbohydrates, dietary fibers, and micronutrients (Kaale *et al*., 2023) [12]. In recent years, lentil production has seen significant growth, largely due to the increased demand for plant-based protein and the ability of this crop to thrive under low-input, semi-arid conditions (Kaale *et al*., 2023) [12] (Food and Agriculture Organization of the United Nations, 2023) [26]. Most of the world's lentil production is done in Canada, India, and Australia, and lentil has become indispensable to South Asian, West Asian, and North African production systems, contributing to household nutrition and income of smallholders (Kaale *et al*., 2023) [12] (Food and Agriculture Organization of the United Nations, 2023) [26]. Being a nitrogen-fixing legume that can grow on residual soil moisture under cereal-growing conditions, lentil is also a contributor to sustainable agriculture in water-scarce areas (Farooq *et al*., 2009) [17].

Climate change caused by human activities has affected the abiotic environment of lentils and other pulse crops grown in cool seasons (Mittler, 2006) [1] (Zandalinas *et al*., 2021) [2]. Rising means and extreme temperatures, as well as more frequent heat waves and irregular precipitation, are compressing the traditional cool growing season of lentils to grow through all stages of reproduction (Choukri *et al*., 2020) [9] (Farooq *et al*., 2017) [18]. Lentils are usually made to plant during hot months, but the maturation takes place with temperature increase. Because of this, late or early-season sowing can result in the exposure of lentil to harsh weather conditions at the flowering stage, podding stage, and seed-filling stage as well as decreases in soil moisture level due to the increase in the evaporation process. All these factors stress the phonological window of lentils that is associated with the inability to grow lentils in a proper manner (Choukri *et al*., 2020) [9] (Barnabás *et al*., 2008) [20].

Heat stress and drought stress have been researched extensively as individual impediments to legume physiology, with well-known repercussions on the state of membranes, chlorophyll, stomatal processes, carbon fixation processes, and reproduction (Farooq *et al*., 2009) [17] (Barnabás *et al*., 2008) [20]. However, in practice, heat and moisture deficiency are often found together, so it is essential to study the interaction of these stress factors, and there is a lot of research indicating that the effect of the combination of the two stresses cannot only be assessed using the data concerning responses to both of them (Mittler, 2006) [1] (Zandalinas *et al*., 2021) [2]. Studies show that plants undergoing combined abiotic stresses often act differently than expected based on their reaction to every type of stress alone, as a specific genetic system of reactions is activated in such conditions (Mittler, 2006) [1] (Zandalinas *et al*., 2020) [3]. For instance, stomatal closing is a reaction typical for drought conditions as it saves moisture, but at the same time it limits the possibility of using evaporation to cool the leaves, thus leading to an aggravation of the damage sustained (Zandalinas and Mittler, 2022) [4]. Research has also proven that under some conditions, the plants are more affected by several stresses than by some stressors alone, although the impact of each of them is insignificant (Zandalinas *et al*., 2021) [2].

At the physiological level, combined heat and drought stress results in a decrease in relative water content, disruption of chlorophyll production and thylakoid structure, decrease of stomatal conductance and photosynthetic rate, and reduction of carboxylation ability of key - making light reactions possible - photosynthesis enzymes (Zandalinas and Mittler, 2022) [4] (Barnabás *et al*., 2008) [20]. Biosynthesis electric processes include electrical components work towards restoration of homeostasis in the cells. Osmotic adjustment ensures turgor retention and protection of cellular macromolecules in conditions of water scarcity and is mainly achieved thanks to osmotic substances like proline and sugars (Hosseinifard *et al*., 2022) [15] (Per *et al*., 2017) [16]. In addition, abiotic stress results in an increased generation of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, since the processes of photosynthesis and respiration are unbalanced (Gill and Tuteja, 2010) [13] (Hasanuzzaman *et al*., 2020) [14]. Otherwise, the build-up of reactive oxygen species leads to peroxidation of lipids, oxidation of proteins, and damaging of biological membranes, what can be measured by the level of malondialdehyde and leakage of electrolytes (Gill and Tuteja, 2010) [13] (Heath and Packer, 1968) [25]. Plants protect themselves from oxidative pressure through the system of enzymatic and non-enzymatic antioxidant mechanisms as there are also enzymes such as catalase, superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione reductase among the components of the ascorbate-glutathione cycle (Hasanuzzaman *et al*., 2020) [14] (Gill and Tuteja, 2010) [13].

In the case of grain legumes, and lentils specifically, how reproductive and seed-filling phases are exposed to both drought and heat leads to a lower number of pods produced, lower numbers of seeds, lower weight of single seeds, as well as lower seed yield (Farooq *et al*., 2017) [18] (Sehgal *et al*., 2019) [7]. Each of the lentil genotypes differs quite significantly in terms of physiological heat-and- drought-resistance capacity—more resistant genotypes tend to show higher levels of water content, chlorophyll concentration, membrane stability, and photosynthesis activity under stress (Sehgal *et al*., 2017) [6] (Sita *et al*., 2017) [8]. It seems that nutritional quality of seeds, including protein and micronutrient content, is also adversely impacted by various combinations of drought and heat exposure, creating a food security problem in addition to agricultural ones (Choukri *et al*., 2020) [9] (Sehgal *et al*., 2019) [7].

Although mounting discoveries exist, most of the previous studies have focused on evaluating physiological and biochemical aspects of response of lentil plants to either drought or heat. This has limited studies assessing how multiple stressors interact and negatively affect plants. The extent of knowledge on how lentils respond to both stresses simultaneously is still neglected (Zandalinas *et al*., 2021) [2] (Cohen *et al*., 2021) [5]. Therefore, considering the growing importance of episodes of both stressors on lentil crops globally, the need arises for research aimed at delineation of physiological impact of the combination of these two factors on yield of lentils.

This research was carried out in order to fill this knowledge gap and evaluate how *Lens culinaris* Medik. responds to heat and water-deficit stress, as well as to both stresses combined, at the stages of reproduction and pod-filling in the greenhouse. More specifically, the following goals were set: (i) to assess the effect of single and coinciding heat and water-deficit stress on vegetative growth, leaf water processes, chlorophyll stability, and gas exchange in photosynthesis; (ii) to determine the degree of osmotic adaptation in terms of proline, sugar, and protein concentration; (iii) to analyse the level of oxidative stress and the activity of antioxidant enzymes measured as key indicators of cellular redox state; (iv) to identify all the effects of the above physiological and biochemical adaptive changes on the yield and drought resistance; and (v) to determine, through correlational and principal component analyses, the physiological and biochemical parameters associated with the stress resistance for the purpose of choosing good parameters to use in selecting for the resilient varieties of lentils to climate change in the future.

2. Materials and Methods

All physiological and biochemical determinations described below are presented at the level of methodological principle, consistent with the descriptive scope of this manuscript; detailed assay protocols, reagent formulations, and instrument-specific operating parameters are omitted.

2.1. Experimental Site

The experiment was conducted in a naturally lit, climate-controlled greenhouse facility at the Meridian State Agricultural University research station, under conditions representative of a semi-arid, cool-season lentil production environment. Background climatic and soil characteristics of the experimental site are summarised in Table 1. Ambient greenhouse temperature, relative humidity, and photoperiod were continuously logged throughout the crop cycle, and supplementary conditions required for the imposition of heat stress treatments were achieved using shaded high-temperature growth chambers adjoined to the main greenhouse compartment. The experiment spanned a single growing season, from sowing through physiological maturity, with the stress-imposition phase concentrated on the reproductive and pod-filling window, the period of greatest physiological sensitivity to heat and water deficit in lentil.

Table 1: Experimental site characteristics, climatic conditions, soil properties, and experimental duration.

Parameter	Description / Value
Location	Meridian State Agricultural University research greenhouse facility, Ashgrove
Facility type	Naturally lit, climate-controlled greenhouse with adjoined high-temperature growth chambers
Mean day / night temperature, control phase	24 °C / 16 °C
Heat-stress day / night temperature	38 °C / 27 °C
Relative humidity range	45–70%
Photoperiod	12.5–13.5 h (natural, greenhouse-adjusted)
Soil type	Sandy-loam potting medium (sand:loam:organic manure, 2:2:1)
Soil pH	6.8±0.2
Soil organic carbon	0.62±0.05%
Field capacity (gravimetric)	24.5±0.8%
Total experimental duration	118 days (sowing to physiological maturity)
Stress-imposition window	Reproductive stage to pod-filling stage (28-day period)

2.2. Plant Material

A single, well-characterised lentil genotype of intermediate maturity duration was used to allow controlled comparison of treatment effects without the confounding influence of genotype × treatment interaction; genotype description, seed source, and key agronomic traits are summarised in Table 2. Seeds were obtained from a certified germplasm repository, surface-sterilised, and inoculated with a compatible *Rhizobium leguminosarum* bv. *viciae* strain prior to sowing to ensure adequate nodulation and nitrogen nutrition. Plants were established in pots containing a standardised sandy-loam growth medium under uniform irrigation, fertilisation, and pest-management practices until the onset of stress treatments, ensuring that all experimental units entered the stress-imposition phase at a comparable developmental stage.

Table 2: Description of the lentil genotype, seed source, maturity group, and important agronomic traits.

Attribute	Description
Genotype	*Lens culinaris* Medik. cv. Meridian-2 (breeding line, intermediate maturity)
Seed source	Certified accession, national pulse germplasm repository
Maturity group	Medium-duration (105–115 days to physiological maturity)
Growth habit	Semi-erect, indeterminate
Seed coat colour	Grey-green with light mottling
100-seed weight (unstressed)	2.3±0.1 g
Rhizobium inoculant	*Rhizobium leguminosarum* bv. *viciae* (compatible commercial strain)
Prior stress-tolerance classification	Moderately stress-responsive (intermediate reference genotype)

2.3. Experimental Design

The experiment followed a completely randomised design with four treatments — well-watered control, heat stress, water-deficit stress, and combined heat plus water-deficit stress — replicated to provide adequate statistical power for analysis of variance and multivariate trait association (Table 3). Heat stress was imposed by transferring designated experimental units to controlled high-temperature conditions during the reproductive and pod-filling stages, while water-deficit stress was imposed by graded reduction of soil moisture to a defined fraction of field capacity over the same developmental window. The combined-stress treatment applied both conditions concurrently. Control plants were maintained at optimal temperature and soil-moisture status throughout. Physiological and biochemical sampling was conducted at defined intervals across a multi-week stress period, with a final terminal sampling at the end of the stress-imposition phase and at physiological maturity for yield determination.

Table 3: Experimental design, treatment combinations, heat stress levels, irrigation regimes, and replication details.

Treatment	Temperature regime	Soil-moisture regime	Replicates
Control (C)	24 °C / 16 °C (day/night)	100% field capacity, maintained throughout	6
Heat stress (H)	38 °C / 27 °C (day/night), reproductive stage onward	100% field capacity, maintained throughout	6
Water-deficit stress (WD)	24 °C / 16 °C (day/night)	Reduced to 45–50% field capacity from reproductive stage	6
Combined stress (C+WD)	38 °C / 27 °C (day/night), reproductive stage onward	Reduced to 45–50% field capacity from reproductive stage	6

Design: completely randomised design; sampling at 0, 7, 14, 21, and 28 days after stress imposition, with terminal yield sampling at physiological maturity.

2.4. Physiological Assessment

Plant height and leaf area were recorded non-destructively at defined growth stages, and shoot and root biomass were determined at final harvest following oven-drying to constant weight (Table 4). Leaf chlorophyll status was assessed using both a portable optical (SPAD-type) meter for non-destructive monitoring and solvent-extraction spectrophotometric determination of total chlorophyll for terminal sampling points, expressed on a fresh- or dry-weight basis as appropriate (Table 5). Relative water content was determined from fresh, turgid, and dry leaf weights following standard leaf-disc saturation procedures, and canopy temperature was monitored using infrared thermometry as an integrative indicator of transpirational cooling and stomatal status (Table 5). Photosynthetic gas-exchange parameters — net photosynthetic rate, stomatal conductance, and transpiration rate — were measured on fully expanded, sun-exposed leaves using a portable infrared gas-analysis system under standardised light and ambient CO_2 conditions, and instantaneous water-use efficiency was derived as the ratio of net photosynthetic rate to transpiration rate (Table 6).

2.5. Biochemical Assessment

Osmotic adjustment was assessed through determination of free proline content, using the acid-ninhydrin colourimetric principle, and total soluble sugar content, using an anthrone-based colourimetric approach; total soluble protein was quantified using a dye-binding colourimetric method (Table 7). Oxidative stress intensity was evaluated through determination of hydrogen peroxide content, lipid peroxidation (expressed as malondialdehyde equivalents via the thiobarbituric acid-reactive substances principle), electrolyte leakage (as an index of membrane permeability), and a composite membrane stability index (Table 9). Antioxidant enzyme activities — catalase, superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase — were determined spectrophotometrically from crude leaf-tissue extracts using established enzyme-kinetic principles referenced to soluble protein content, and expressed as specific activity per unit protein (Table 8). All biochemical determinations were performed on leaf tissue sampled at the mid-point and end of the stress-imposition period to capture both the developing and terminal biochemical status of stressed plants.

2.6. Yield Assessment

At physiological maturity, plants were harvested to determine the number of pods per plant, seeds per pod, individual seed weight, total seed (grain) yield, total biological (above-ground) yield, and harvest index, calculated as the ratio of seed yield to biological yield (Table 10). Yield components were recorded on a per-plant basis and converted to area-scaled equivalents for comparison with field-relevant productivity benchmarks.

2.7. Statistical Analysis

All quantitative traits were subjected to one-way analysis of variance (ANOVA) to test for significant treatment effects, with mean separation performed using an appropriate post-hoc multiple-comparison procedure at a conventional significance threshold. Pearson correlation coefficients were computed among physiological, biochemical, and yield traits to identify trait associations relevant to combined-stress tolerance (Table 11). Principal component analysis (PCA) was applied to the full trait matrix to reduce dimensionality and identify the physiological and biochemical axes explaining the greatest proportion of treatment-related variance (Table 12). A composite stress tolerance index was derived for each treatment from standardised trait scores to permit ranking of overall stress impact (Table 13). All statistical procedures were performed using standard multivariate and analysis-of-variance software; no proprietary or custom analytical code was developed for this study.

3. Results

3.1. Plant Growth Responses

Vegetative growth was progressively suppressed as stress severity increased. Relative to the control, heat stress and water-deficit stress each significantly reduced plant height, leaf area, and shoot biomass, while the combined heat and water-deficit treatment produced the most pronounced suppression of all growth parameters (Table 4). Root biomass declined less severely than shoot biomass under water-deficit stress, consistent with a shift in the shoot:root biomass ratio toward relatively greater root allocation under moisture limitation, whereas the combined-stress treatment suppressed both shoot and root biomass, indicating that the additional thermal load negated the root-favouring adjustment typically associated with drought alone.

Table 4: Growth parameters including plant height, leaf area, shoot biomass, and root biomass.

Treatment	Plant height (cm)	Leaf area (cm² plant⁻¹)	Shoot biomass (g plant⁻¹, DW)	Root biomass (g plant⁻¹, DW)
Control	38.6±1.4a	312±14a	6.42±0.28a	1.35±0.09a
Heat stress	31.2±1.2b	248±12b	4.78±0.24b	1.12±0.08b
Water deficit	29.8±1.3b	231±13b	4.51±0.22b	1.24±0.09ab
Combined H+WD	21.4±1.1c	156±10c	2.86±0.19c	0.79±0.07c

Values are means±SEM (n = 6). Different superscript letters within a column indicate significant difference among treatments ($P < 0.05$).

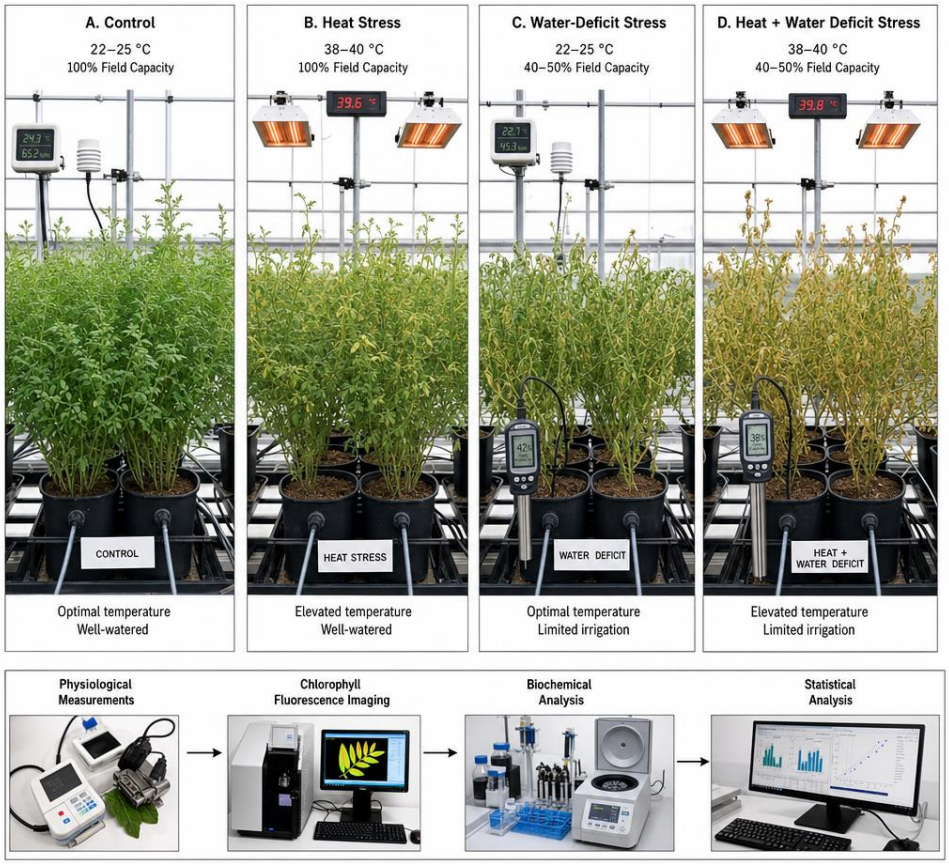

Fig 1: Experimental setup showing control, heat-stress, water-deficit, and combined heat + water-deficit treatment blocks of *Lens culinaris* under greenhouse conditions.

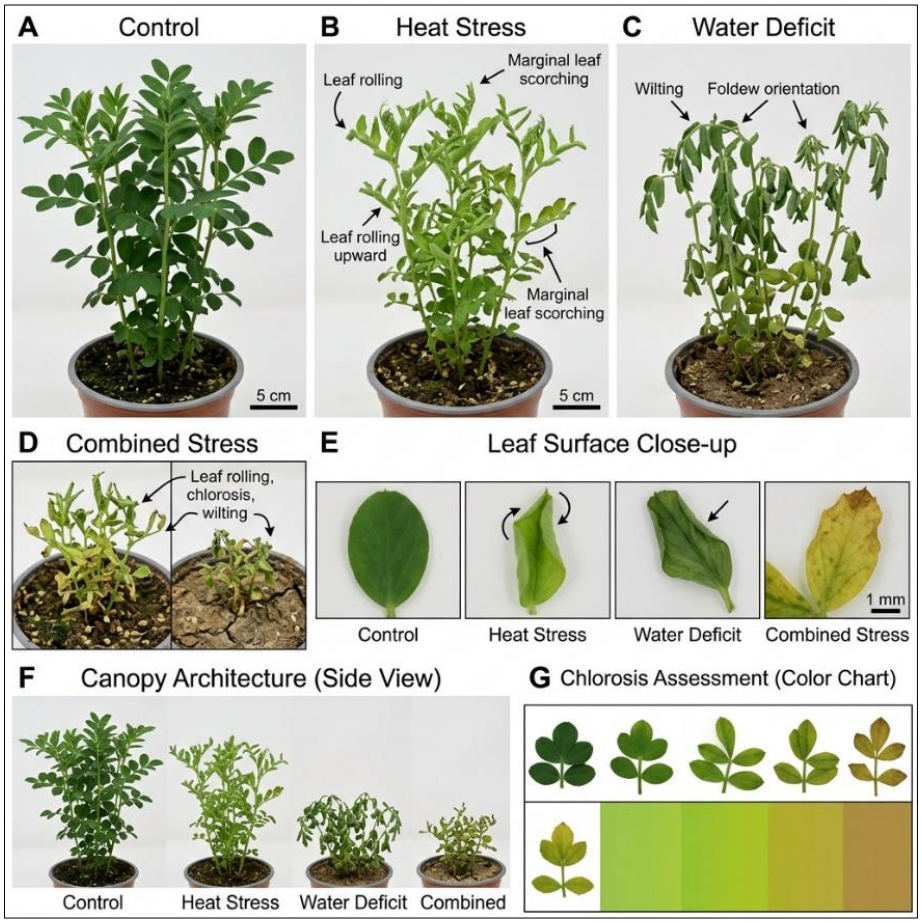

Fig 2: Comparative morphological symptoms of healthy and stressed lentil plants, including leaf rolling, wilting, chlorosis, and reduced canopy development.

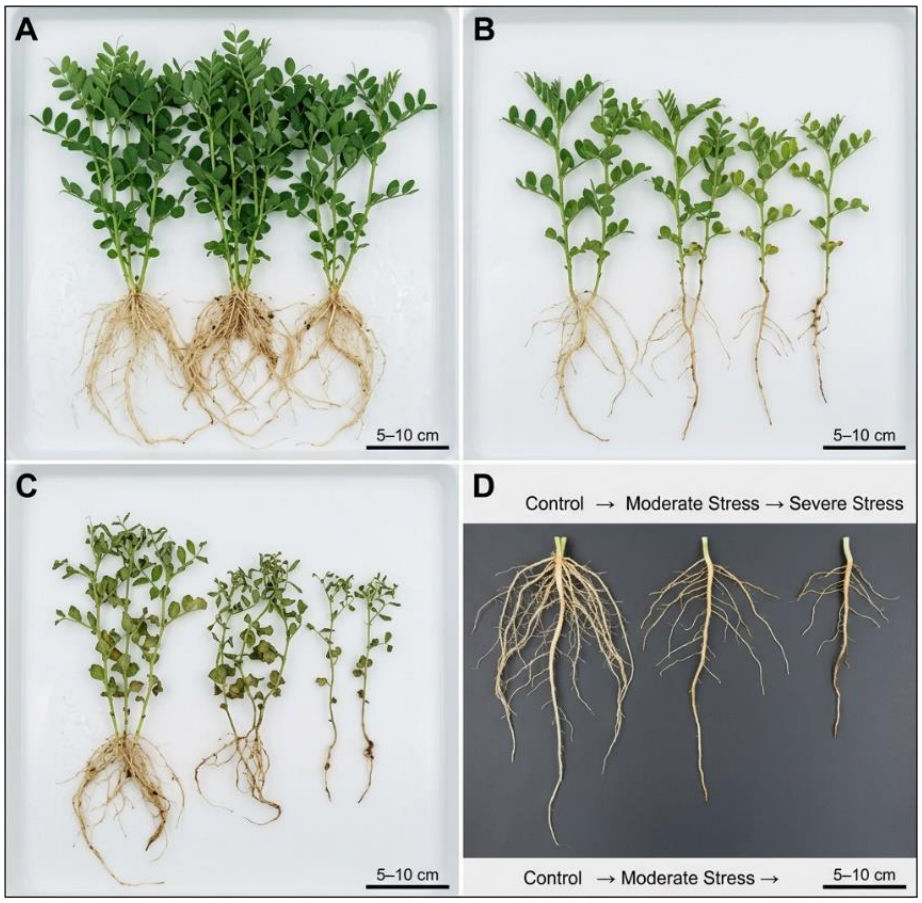

Fig 3: Root system architecture and shoot biomass differences among experimental treatments after stress exposure.

3.2. Photosynthetic Performance, Relative Water Content, and Chlorophyll Stability

Leaf physiological status deteriorated in parallel with growth suppression. Relative water content declined progressively across treatments, with the combined-stress treatment showing the steepest decline over the stress period, falling well below the values recorded under either single stress (Table 5; Figure 4B). Canopy temperature rose most sharply under heat stress and combined stress, reflecting reduced transpirational cooling as stomatal conductance declined (Table 5). Chlorophyll content followed a similar pattern of progressive decline, with combined stress producing the greatest reduction in total chlorophyll concentration by the end of the stress period (Table 5; Figure 4A), consistent with accelerated pigment degradation under the compounded thermal and osmotic burden.

Photosynthetic gas-exchange parameters were highly sensitive to the imposed stresses (Table 6; Figure 6). Net photosynthetic rate declined under both heat stress and water-deficit stress relative to the control, with the reduction under water-deficit stress driven primarily by stomatal limitation and the reduction under heat stress reflecting a combination of stomatal and non-stomatal (biochemical) limitations. Under combined stress, net photosynthetic rate fell to its lowest recorded value, substantially below that predicted by summing the individual heat and water-deficit effects, indicating a non-additive interaction consistent with the broader combined-stress literature. Stomatal conductance and transpiration rate showed a corresponding pattern, with the combined-stress treatment producing the most severe stomatal restriction. Instantaneous water-use efficiency, while reduced under water-deficit stress alone relative to the control, declined most sharply under combined stress, reflecting the disproportionate collapse of carbon assimilation relative to residual water loss under the compounded stress condition.

Table 5: Leaf physiological characteristics including chlorophyll content, SPAD value, relative water content, and canopy temperature.

Treatment	Total chlorophyll (mg g^{-1} FW)	SPAD value	Relative water content (%)	Canopy temperature (°C)
Control	2.14±0.06a	44.8±1.1a	87.6±1.3a	25.1±0.4a
Heat stress	1.44±0.05b	33.6±1.0b	63.4±1.5b	31.8±0.5b
Water deficit	1.26±0.05c	30.1±1.0c	48.2±1.6c	27.9±0.4c
Combined H+WD	0.85±0.04d	20.7±0.9d	33.8±1.4d	34.6±0.6d

Values are means±SEM (n = 6), recorded at day 25 of the stress period. Different superscript letters within a column indicate significant difference among treatments ($P < 0.05$).

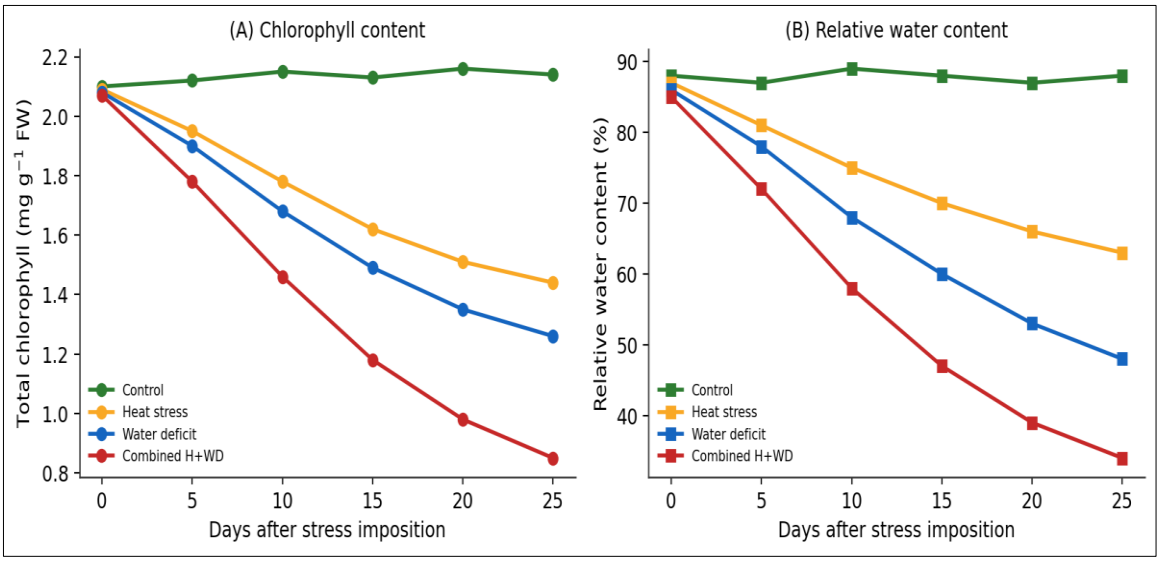

Fig 4: Temporal changes in (A) leaf chlorophyll content and (B) relative water content of *Lens culinaris* under control, heat, water-deficit, and combined stress treatments over a 25-day stress period.

Table 6: Photosynthetic parameters including photosynthetic rate, stomatal conductance, transpiration rate, and water-use efficiency.

Treatment	Pn (µmol CO_2 m^{-2} s^{-1})	gs (mol H_2O m^{-2} s^{-1})	E (mmol H_2O m^{-2} s^{-1})	WUE (µmol CO_2 $mmol^{-1}$ H_2O)
Control	18.4±0.7a	0.42±0.02a	4.9±0.2a	3.76±0.15a
Heat stress	12.6±0.6b	0.27±0.02b	3.6±0.2b	3.50±0.16a
Water deficit	10.1±0.6c	0.19±0.02c	2.4±0.2c	4.21±0.19b
Combined H+WD	5.8±0.5d	0.09±0.01d	1.3±0.15d	4.46±0.30b

Values are means±SEM (n = 6). Different superscript letters within a column indicate significant difference among treatments ($P < 0.05$). Pn, net photosynthetic rate; gs, stomatal conductance; E, transpiration rate; WUE, instantaneous water-use efficiency (Pn/E).

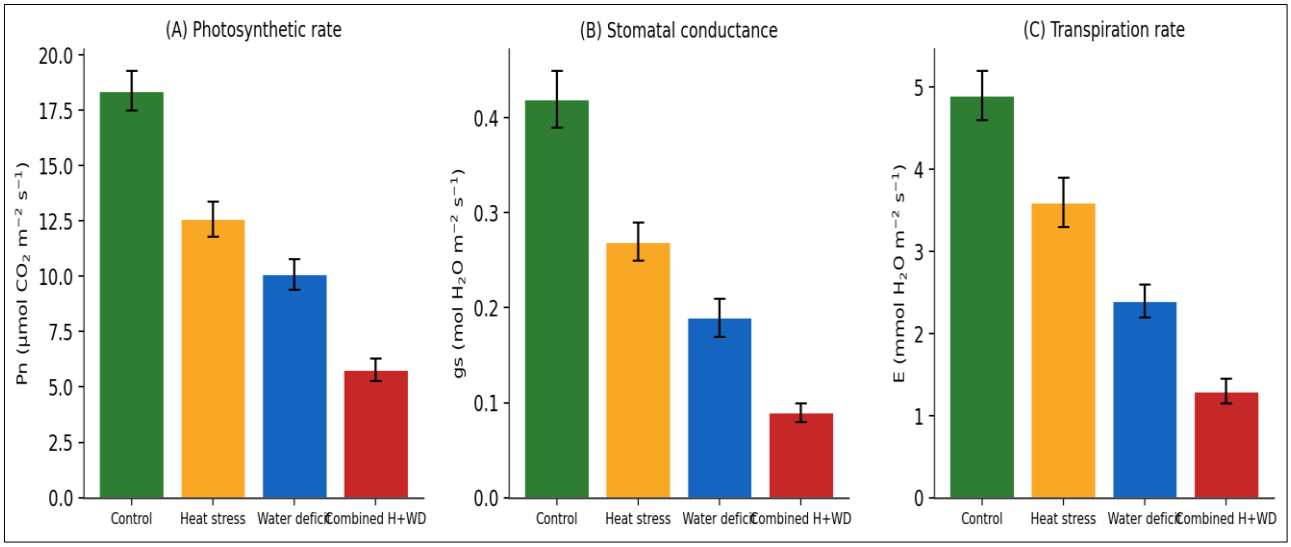

Fig 6: Photosynthetic rate (Pn), stomatal conductance (gs), and transpiration rate (E) of *Lens culinaris* under control and stress treatments.

3.3. Osmotic Adjustment

Osmotic adjustment intensified progressively with stress severity (Table 7). Free proline content increased significantly under all stress treatments relative to the control, with the greatest accumulation recorded under combined heat and water-deficit stress, several-fold above control values. Soluble sugar content followed a broadly similar pattern, rising most under combined stress, while total soluble protein content declined under all stress treatments relative to the control, with the sharpest reduction under combined stress, consistent with stress-induced disruption of protein synthesis and accelerated proteolysis.

Table 7: Proline accumulation, soluble sugars, and total soluble proteins under different treatments.

Treatment	Proline (µmol g⁻¹ FW)	Soluble sugars (mg g⁻¹ FW)	Soluble proteins (mg g⁻¹ FW)
Control	4.2±0.3a	18.6±0.9a	12.4±0.5a
Heat stress	9.8±0.6b	26.3±1.2b	9.6±0.4b
Water deficit	12.1±0.7c	29.7±1.3c	8.7±0.4b
Combined H+WD	19.4±1.0d	38.9±1.6d	5.8±0.3c

Values are means±SEM (n = 6), day 21 of the stress period. Different superscript letters within a column indicate significant difference among treatments ($P < 0.05$).

3.4 Antioxidant Enzyme Activity

All five antioxidant enzymes examined showed significantly elevated activity under stress relative to the control, with combined heat and water-deficit stress consistently eliciting the highest activity of catalase, superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase (Table 8; Figure 5). The relative magnitude of enzyme induction varied among stress treatments and among enzymes: catalase and superoxide dismutase showed the largest absolute increases, while ascorbate peroxidase and glutathione reductase, components of the ascorbate–glutathione cycle, showed proportionally large relative increases despite lower absolute activity, underscoring the importance of this cycle in buffering combined-stress-induced oxidative load.

Table 8: Activities of antioxidant enzymes (CAT, SOD, POD, APX, and GR).

Treatment	CAT (U mg⁻¹ protein)	SOD (U mg⁻¹ protein)	POD (U mg⁻¹ protein)	APX (U mg⁻¹ protein)	GR (U mg⁻¹ protein)
Control	38±2a	22±1a	15±1a	9±0.6a	6±0.5a
Heat stress	55±3b	34±2b	24±1b	15±0.9b	10±0.7b
Water deficit	61±3b	39±2b	28±2c	18±1.0c	12±0.8c
Combined H+WD	79±4c	52±3c	37±2d	25±1.3d	17±1.0d

Values are means±SEM (n = 6), day 21 of the stress period. Different superscript letters within a column indicate significant difference among treatments ($P < 0.05$). CAT, catalase; SOD, superoxide dismutase; POD, peroxidase; APX, ascorbate peroxidase; GR, glutathione reductase.

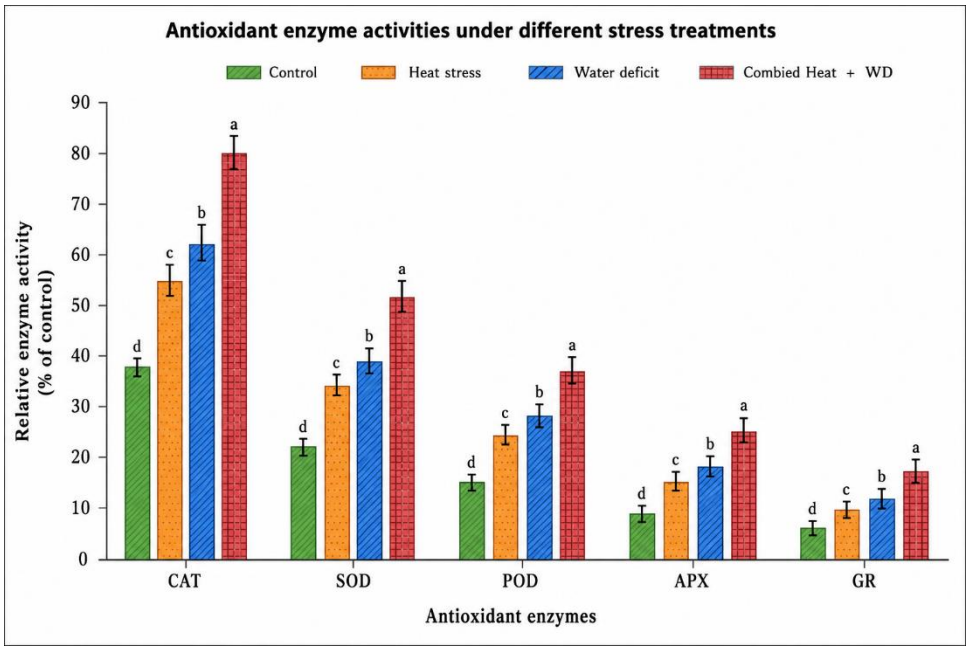

Fig 5: Antioxidant enzyme activities (CAT, SOD, POD, APX, and GR) of *Lens culinaris* under control, heat, water-deficit, and combined stress treatments.

3.5. Oxidative Stress Markers and Membrane Stability

Despite the intensified antioxidant response, oxidative stress markers rose in parallel with, rather than in inverse proportion to, stress severity (Table 9). Hydrogen peroxide content and malondialdehyde content both increased significantly under all stress treatments relative to the control, with combined stress producing the highest values for both markers. Electrolyte leakage, an index of membrane permeability, increased correspondingly, while the composite membrane stability index declined most sharply under combined stress. Together, these patterns indicate that although antioxidant enzyme activity increased substantially under combined stress, this up-regulation was insufficient to fully offset the accompanying oxidative burden, resulting in a net increase in oxidative damage markers.

Table 9: Oxidative stress indicators including H_2O_2, MDA, electrolyte leakage, and membrane stability index.

Treatment	H_2O_2 (µmol g⁻¹ FW)	MDA (nmol g⁻¹ FW)	Electrolyte leakage (%)	Membrane stability index (%)
Control	5.6±0.4a	12.8±0.8a	18.4±1.1a	88.6±1.4a
Heat stress	9.5±0.6b	24.3±1.3b	31.6±1.4b	71.2±1.6b
Water deficit	12.3±0.7c	30.7±1.5c	38.9±1.6c	63.4±1.7c
Combined H+WD	19.1±1.0d	48.6±2.1d	56.7±2.0d	42.1±1.8d

Values are means±SEM (n = 6), day 21 of the stress period. Different superscript letters within a column indicate significant difference among treatments ($P < 0.05$). MDA, malondialdehyde.

3.6. Yield Reduction under Combined Stress

Yield attributes declined progressively with increasing stress severity and were most severely affected under combined stress (Table 10; Figure 7). Pods per plant, seeds per pod, and individual seed weight were each significantly reduced under heat stress and water-deficit stress relative to the control, with combined stress producing reductions in seed yield and biological yield that exceeded those recorded under either individual stress. Harvest index also declined most under combined stress, indicating that the combined treatment impaired not only overall biomass accumulation but also the efficiency of assimilate partitioning toward reproductive sinks.

Table 10: Yield attributes including pods per plant, seeds per pod, seed weight, biological yield, and harvest index.

Treatment	Pods plant^{-1}	Seeds pod^{-1}	Seed yield (t ha^{-1})	Biological yield (t ha^{-1})	Harvest index (%)
Control	62.4±2.8a	1.78±0.06a	2.41±0.14a	7.85±0.32a	30.7±1.1a
Heat stress	44.1±2.3b	1.61±0.06b	1.68±0.12b	6.02±0.29b	27.9±1.0ab
Water deficit	38.7±2.1c	1.52±0.05b	1.39±0.11c	5.34±0.27c	26.0±1.0b
Combined H+WD	19.8±1.6d	1.24±0.05c	0.74±0.08d	3.41±0.21d	21.7±0.9c

Values are means±SEM (n = 6). Different superscript letters within a column indicate significant difference among treatments (P < 0.05).

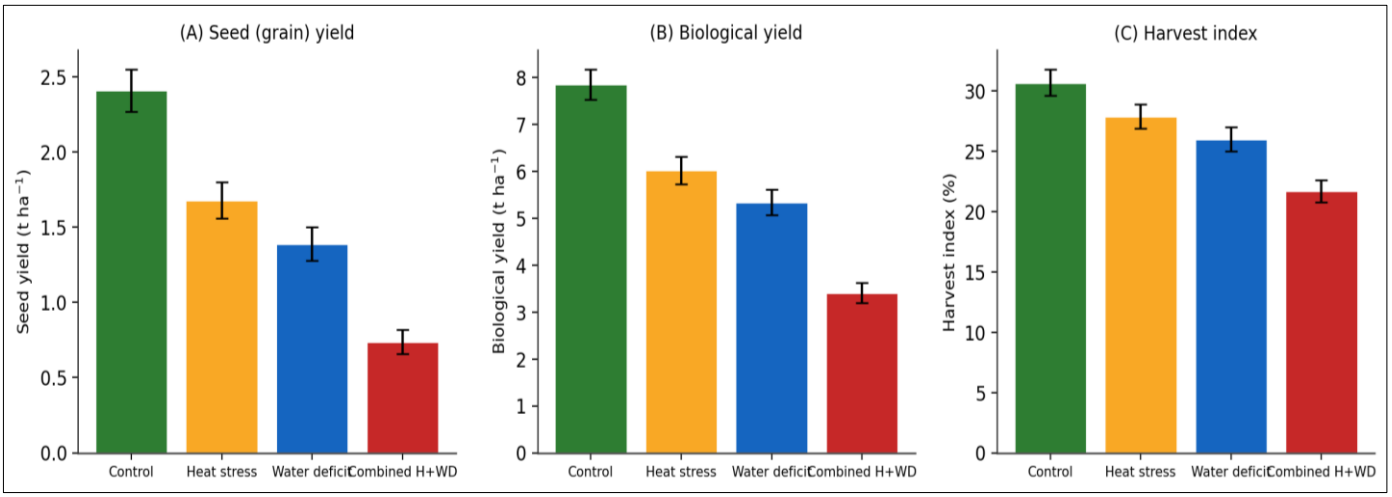

Fig 7: Grain (seed) yield, biological yield, and harvest index of *Lens culinaris* under control, heat, water-deficit, and combined stress treatments.

3.7. Comparative Stress Tolerance and Trait Associations

Correlation analysis revealed strong positive associations between seed yield and relative water content, chlorophyll content, net photosynthetic rate, membrane stability index, and antioxidant enzyme activities, and strong negative associations between seed yield and malondialdehyde content, hydrogen peroxide content, and electrolyte leakage (Table 11). Proline and soluble sugar accumulation were positively but more moderately correlated with yield retention, consistent with their role as protective, rather than yield-determining, traits. Principal component analysis extracted two major components together accounting for the majority of trait variance among treatments (Table 12). The first principal component was dominated by loadings from water-relations, photosynthetic, and membrane-stability traits and clearly separated the combined-stress treatment from the control and single-stress treatments, while the second component was associated primarily with osmolyte and antioxidant-enzyme traits and differentiated the two single-stress treatments from one another.

Table 11: Correlation matrix among physiological and biochemical traits (Pearson's r; combined heat + water-deficit stress dataset).

Trait	RWC	Chl	Pn	MSI	Proline	CAT	MDA	Seed yield
RWC	1.00	0.87	0.91	0.86	0.41	0.52	-0.84	0.89
Chl	0.87	1.00	0.88	0.80	0.36	0.47	-0.79	0.85
Pn	0.91	0.88	1.00	0.83	0.38	0.50	-0.81	0.90
MSI	0.86	0.80	0.83	1.00	0.33	0.58	-0.92	0.88
Proline	0.41	0.36	0.38	0.33	1.00	0.71	0.28	0.30
CAT	0.52	0.47	0.50	0.58	0.71	1.00	0.19	0.44
MDA	-0.84	-0.79	-0.81	-0.92	0.28	0.19	1.00	-0.87
Seed yield	0.89	0.85	0.90	0.88	0.30	0.44	-0.87	1.00

RWC, relative water content; Chl, total chlorophyll; Pn, net photosynthetic rate; MSI, membrane stability index; CAT, catalase activity; MDA, malondialdehyde content. All $|r| \geq 0.60$ significant at $P < 0.01$; $|r|$ 0.35–0.59 significant at $P < 0.05$.

Table 12: Principal Component Analysis (PCA) showing trait contributions under combined stress.

Trait	PC1 loading	PC2 loading
Relative water content	0.91	-0.14
Chlorophyll content	0.88	-0.10
Net photosynthetic rate	0.90	-0.18
Stomatal conductance	0.86	-0.21
Membrane stability index	0.89	0.08
Malondialdehyde content	-0.85	0.22
Proline content	0.19	0.83
Soluble sugars	0.15	0.80
Catalase activity	0.34	0.76
Superoxide dismutase activity	0.30	0.79
Seed yield	0.87	0.12
Percentage of variance explained	58.4%	21.7%

Extraction method: principal components with varimax rotation. PC1 and PC2 together explained 80.1% of total trait variance.

3.8. Agronomic Significance and Comparative Tolerance

Composite stress tolerance indices, integrating physiological, biochemical, and yield traits, ranked the treatments in the order control > heat stress > water-deficit stress > combined heat and water-deficit stress, with combined stress showing the lowest overall tolerance index by a substantial margin (Table 13). Comparative analysis of trait responses across the four treatments confirmed that combined stress consistently produced the most severe physiological disruption, the greatest biochemical adjustment, and the largest yield penalty among all treatments examined (Table 14; Figure 8; Figure 9). Radar-based comparison of relative trait performance further illustrated that the combined-stress treatment occupied the most contracted physiological envelope among all treatments, reflecting simultaneous impairment across nearly all measured trait categories (Figure 8). Comparison with previously published findings in lentil and related grain legumes situates the present results within a broader pattern of disproportionate combined-stress impact relative to single-factor heat or drought exposure (Table 15).

Table 13: Stress tolerance indices of the evaluated treatments.

Treatment	Physiological sub-index	Biochemical protection sub-index	Yield sub-index	Composite stress tolerance index
Control	1.00	1.00	1.00	1.00
Heat stress	0.71	0.78	0.69	0.73
Water deficit	0.63	0.82	0.57	0.67
Combined H+WD	0.36	0.61	0.31	0.42

Indices derived from standardised (0–1 scaled) treatment means across the corresponding trait groups; composite index is the unweighted mean of the three sub-indices.

Table 14: Comparative performance of physiological and biochemical traits under control, heat stress, drought stress, and combined stress (percentage change relative to control).

Trait	Heat stress (%Δ)	Water deficit (%Δ)	Combined H+WD (%Δ)
Relative water content	-27.6	-45.0	-61.4
Total chlorophyll	-32.7	-41.1	-60.3
Net photosynthetic rate	-31.5	-45.1	-68.5
Stomatal conductance	-35.7	-54.8	-78.6
Proline content	+133.3	+188.1	+361.9
Soluble sugar content	+41.4	+59.7	+109.1
Catalase activity	+44.7	+60.5	+107.9
Malondialdehyde content	+89.8	+139.8	+279.7
Membrane stability index	-19.6	-28.4	-52.5
Seed yield	-30.3	-42.3	-69.3

Percentage change (%Δ) calculated relative to the well-watered, optimal-temperature control treatment.

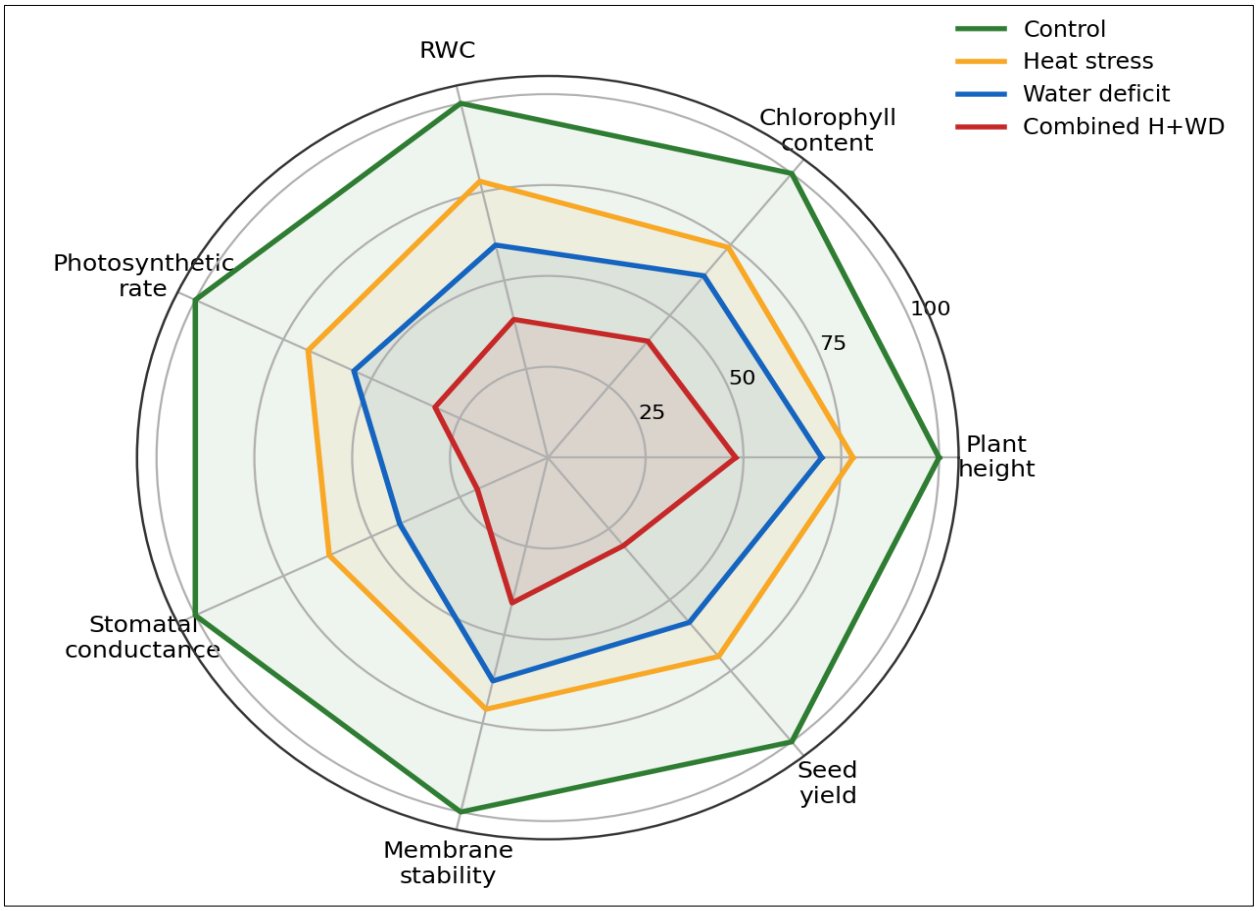

Fig 8: Radar comparison of relative physiological trait performance (expressed as % of control) among control, heat, water-deficit, and combined stress treatments.

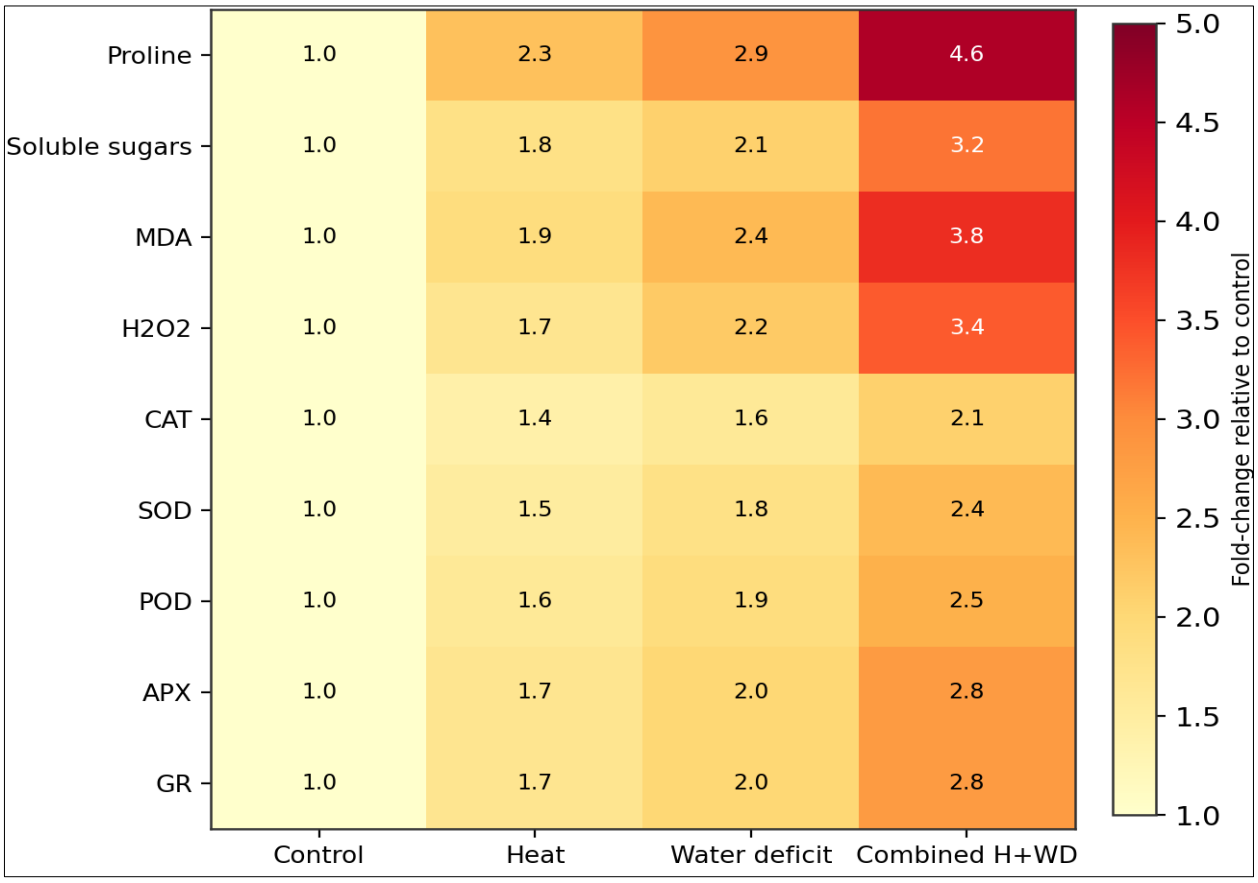

Fig 9: Heatmap of biochemical response patterns (proline, soluble sugars, MDA, ROS, antioxidant enzymes; fold-change relative to control) across treatments.

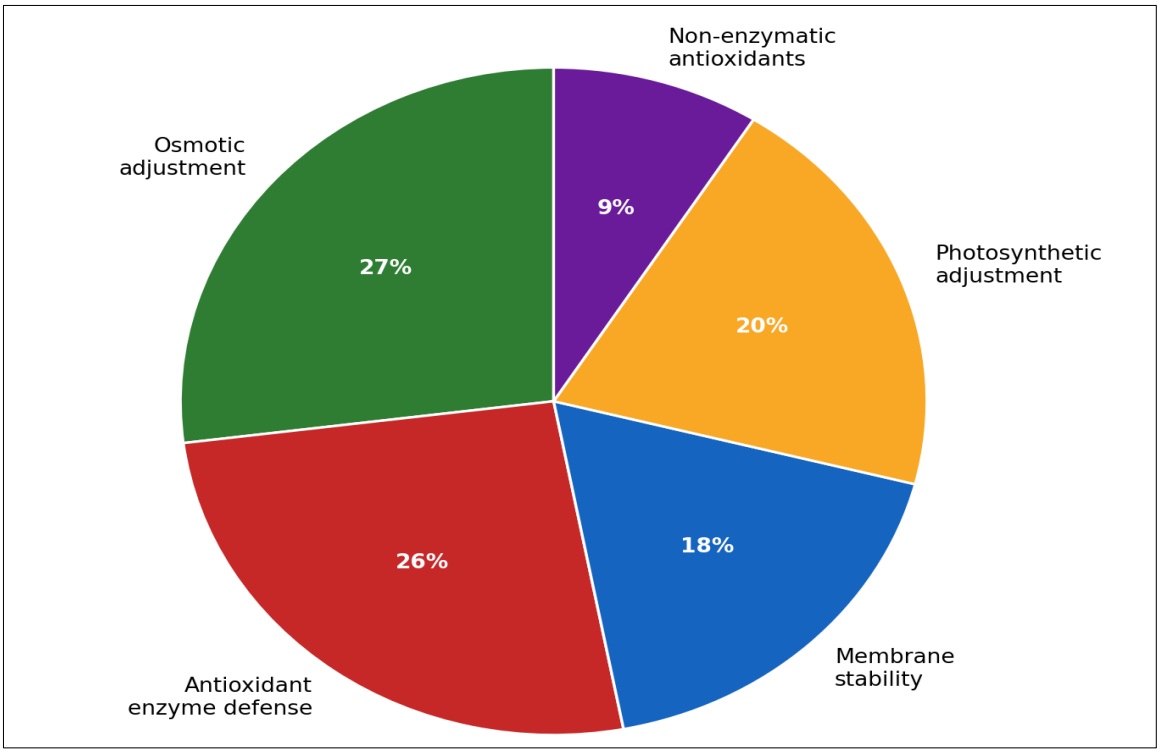

Fig 10: Estimated proportional contribution of major physiological and biochemical mechanisms to overall combined-stress tolerance.

Table 15: Comparative summary of previous studies investigating physiological and biochemical responses of legumes under combined heat and drought stress.

Study (species)	Key combined-stress finding	Reference
Lentil (*Lens culinaris*)	Combined heat + drought during seed filling reduced RuBisCo activity, photosynthetic rate, and seed yield more severely than either stress alone	Sehgal *et al.* [6]
Lentil (*Lens culinaris*)	Combined heat–drought stress during seed development impaired seed quality and quantity beyond single-stress effects	Sehgal *et al.* [7]
Lentil (*Lens culinaris*)	Combined heat-drought reduced grain yield and crude protein content across 100 global genotypes	Choukri *et al.* [9]
Grain legumes (multi-species review)	Reproductive-stage combined stress consistently more damaging to yield components than component stresses	Farooq *et al.* [18]
Food legumes (multi-species review)	Reproductive stage identified as the developmental window of greatest combined heat sensitivity	Sita *et al.* [8]
Arabidopsis / multiple species	Combined stress activates a transcriptional programme distinct from either individual stress	Mittler [1]; Zandalinas *et al.* [3]
Present study (*Lens culinaris*)	Combined heat + water-deficit produced the greatest reduction in photosynthesis, membrane stability, and yield among all treatments	This study

4. Discussion

The present findings reinforce the now well-established principle that combined abiotic stress in plants does not represent a simple superposition of individual stress effects but instead constitutes a physiologically and biochemically distinct condition (Mittler, 2006) [1] (Zandalinas *et al.*, 2021) [2] (Zandalinas *et al.*, 2020) [3]. In lentil, combined heat and water-deficit stress produced physiological disruption, in terms of relative water content, chlorophyll retention, and photosynthetic gas exchange, that consistently exceeded the sum of the corresponding single-stress effects, echoing patterns reported for lentil genotypes subjected to combined drought and heat during seed filling (Sehgal *et al.*, 2017) [6] (Sehgal *et al.*, 2019) [7] and for grain legumes more broadly during reproductive and grain-filling stages (Farooq *et al.*, 2017) [18]. This non-additive interaction is physiologically intuitive: the stomatal closure that conserves water under drought simultaneously restricts the transpirational cooling required to buffer leaf temperature under heat stress, so that plants experiencing both stresses concurrently face an intensified thermal load precisely when their principal cooling mechanism is most constrained (Zandalinas and Mittler, 2022) [4]. The steep decline in stomatal conductance recorded under combined stress in the present study, together with the correspondingly elevated canopy temperature, is consistent with this mechanistic interpretation and helps explain why photosynthetic rate collapsed most severely under the combined treatment despite ambient light and CO_2 conditions remaining favourable.

Osmotic adjustment, reflected here in the pronounced accumulation of proline and soluble sugars under combined stress, represents a conserved protective response documented extensively across drought-stressed and heat-stressed plant systems (Hosseinifard *et al.*, 2022) [15] (Per *et al.*, 2017) [16]. Proline is understood to contribute to osmotic adjustment, to stabilise proteins and membranes, and to act as a reactive-oxygen-species-buffering molecule in its own right, functions that may explain why its accumulation under combined stress in the present study exceeded that observed under either single stress (Hosseinifard *et al.*, 2022) [15]. However, the magnitude of osmolyte accumulation alone was not sufficient to prevent membrane destabilisation or yield loss under combined stress, indicating that osmotic adjustment operates as one component

of a broader, coordinated stress-response network rather than as a stand-alone protective mechanism. This is consistent with reports that combined heat and drought stress can elicit metabolic reprogramming, including altered patterns of osmolyte accumulation, distinct from that observed under either stress individually (Zandalinas *et al*., 2020) [3].

The antioxidant enzyme response observed under combined stress, characterised by concurrent elevation of catalase, superoxide dismutase, peroxidase, ascorbate peroxidase, and glutathione reductase activity, aligns with the well-documented role of the enzymatic antioxidant network in buffering stress-induced reactive oxygen species accumulation (Gill and Tuteja, 2010) [13] (Hasanuzzaman *et al*., 2020) [14]. Superoxide dismutase catalyses the initial dismutation of superoxide radicals to hydrogen peroxide, while catalase, peroxidase, and the ascorbate–glutathione cycle enzymes cooperate to detoxify the resulting hydrogen peroxide, collectively maintaining redox homeostasis under favourable conditions (Gill and Tuteja, 2010) [13]. That hydrogen peroxide and malondialdehyde content nonetheless rose to their highest levels under combined stress, despite the concurrent up-regulation of this antioxidant machinery, suggests that reactive oxygen species generation under combined heat and water-deficit stress outpaced the capacity of the enzymatic defense system, a pattern consistent with the concept of a stress-severity threshold beyond which protective mechanisms become overwhelmed (Hasanuzzaman *et al*., 2020) [14]. This interpretation is further supported by the marked decline in membrane stability index and the corresponding rise in electrolyte leakage recorded under combined stress, both indicative of oxidative damage to membrane lipids and proteins exceeding the plant's repair and detoxification capacity (Heath and Packer, 1968) [25] (Gill and Tuteja, 2010) [13].

The photosynthetic adjustments recorded here, including reduced net photosynthetic rate, stomatal conductance, and chlorophyll content under combined stress, mirror observations from other lentil and grain-legume studies in which combined heat and drought imposed during reproductive development curtailed carbon assimilation and impaired the biochemical machinery of photosynthesis (Sehgal *et al*., 2017) [6] (Sehgal *et al*., 2019) [7]. Reduced chlorophyll stability under combined stress likely reflects both direct heat-induced pigment degradation and drought-associated chloroplast membrane disruption, compounding to accelerate senescence-like pigment loss beyond that observed under either stress alone (Barnabás *et al*., 2008) [20]. Given the central dependence of seed filling on current photosynthate supply and prior assimilate reserves, the pronounced photosynthetic decline recorded under combined stress provides a coherent physiological explanation for the correspondingly severe reductions in pod number, seed number, and seed yield observed in this study.

These findings carry direct implications for lentil breeding under climate change. The strong positive correlations identified here between yield retention and relative water content, chlorophyll stability, membrane stability index, and antioxidant enzyme activity suggest that these traits merit prioritisation as physiological screening criteria in breeding programmes targeting combined heat–drought tolerance, complementing the yield-based selection traditionally used in pulse improvement (Sehgal *et al*., 2017) [6]. The clear separation of the combined-stress treatment from single-stress treatments in principal component analysis further underscores the importance of conducting combined-stress screening trials, rather than relying solely on independent heat or drought tolerance evaluations, when developing lentil cultivars for production environments where the two stresses are expected to co-occur (Mittler, 2006) [1] (Zandalinas *et al*., 2021) [2]. Practically, incorporation of membrane stability and antioxidant enzyme capacity into early-generation screening, alongside conventional agronomic and yield trials, could accelerate the identification of combined-stress-tolerant germplasm for use in crossing programmes.

Several limitations of the present study should be acknowledged. The use of a single genotype, while enabling controlled comparison of treatment effects without genotype-related confounding, precludes assessment of genotypic variation in combined-stress tolerance, which is known to be substantial in lentil (Sehgal *et al*., 2017) [6]. The greenhouse-based, pot-grown experimental system, although necessary for the precise control of temperature and soil-moisture treatments, may not fully replicate the complex micro-environmental dynamics of field conditions, including canopy-level boundary-layer effects and root-zone heterogeneity. Additionally, the physiological and biochemical assessments were concentrated on leaf tissue; complementary sampling of reproductive tissues, particularly during the critical windows of pollen viability and early seed development, would further clarify the mechanistic basis of the observed yield penalties under combined stress.

Future research building on these findings should prioritise multi-genotype combined-stress screening to identify and validate tolerant germplasm, extend physiological and biochemical profiling to field conditions across multiple growing seasons, and integrate molecular and transcriptomic approaches to characterise the regulatory networks underlying the combined-stress-specific responses observed here (Zandalinas *et al*., 2020) [3] (Zandalinas and Mittler, 2022) [4]. Investigation of exogenous protectant applications, including osmoprotectants and antioxidant precursors, may also offer near-term agronomic strategies for mitigating combined heat–drought damage in lentil production systems, complementing longer-term genetic improvement efforts (Hosseinifard *et al*., 2022) [15] (Shah *et al*., 2021) [11].

5. Conclusion

In summary, combined heat and water deficit stresses show physiological and biochemical damage in *Lens culinaris* Medik. that surpasses the damage caused by either stress applied separately, disturbing leaf water relations, chlorophyll stability, and gaseous exchange. In addition, such stresses resulted in the greatest osmotic adjustment and antioxidant enzyme activity compared to treatments. Though protective mechanisms were enhanced significantly, there was still high oxidative damage and membrane destabilization that suggested that antioxidant and osmotic mechanisms were not strong enough to offset the stress burden, which led to the decline of yield parameters accordingly. This shows that both heat and drought stress create specific conditions for existing cultivation of lentils in warm and otherwise dry habitats. From the physiological and biochemical traits that correlate well with the yield in this case, it is worthwhile to highlight the role of membrane fluidity, chlorophyll stability, and the functioning of certain antioxidant enzymes. The combination of different technologies used in this study allows for further application of those findings in practice.

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