Physiological and Biochemical Responses of *Cicer arietinum* L. under Combined Heat and Drought Stress Using Chlorophyll Fluorescence and Gas Exchange Analysis

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ABSTRACT

Background: *Cicer arietinum* L. (chickpea) is a major grain legume, whose productivity is affected by simultaneous heat and drought stress. The consequences of the two kinds of stress acting together is not the same as the collective effect.

Objectives: This study evaluated the physiological and biochemical responses of chickpea genotypes to individual and combined heat and drought stress, with emphasis on photosynthetic performance and stress tolerance indicators.

Materials and Methods: In a randomized experimental design, three chickpea cultivars (BG-372, JG-11, and ICCV-2) were cultivated in four different environmental conditions: control, heat stress, drought stress, and dual heat-drought stress. The researchers were interested in examining the parameters of photosynthesis, chlorophyll fluorescence, and the activity of antioxidant enzymes as well as the level of osmolytes, oxidative stress indicators, and membrane stability. In order to analyze the results a range of statistical methods were applied, including ANOVA and Tukey's test.

Results: The simultaneous effect of heat and drought stress brought about the maximum decline in the photosynthetic rate, stomatal conductance, and photosystem II efficiency while substantially inducing oxidative stress. Under this stress condition, there was a considerable increase in the proline, soluble sugars, and antioxidant enzyme activities, demonstrating the elevated defense mechanisms against the environmental stress. The only genotype ICCV-2 exhibited the best physiological behavior, having the ability to cope with oxidative stress in conditions of simultaneous drought and heat stress.

Conclusion: The simultaneous presence of both heat and drought stress causes more serious physiological damage to the plants than the case with one or the other stress situation. Therefore, with the integration of chlorophyll fluorescence technique, gas exchange measurements, and biochemical markers it is possible to identify climate-resilient chickpea genotypes, which will be helpful in further breeding programs.

Keywords: *Cicer arietinum*, combined heat-drought stress, chlorophyll fluorescence, gas exchange, antioxidant enzymes, osmotic adjustment, oxidative stress, climate resilience

1. Introduction

1.1. Global Significance of *Cicer arietinum* L.

Chickpea (*Cicer arietinum* L.), is the second most significant grain legume in the world, second only to the common bean, and is grown on 14-15 million hectares in over 50 countries, the largest producers being India, Australia, Turkey, Myanmar and Ethiopia (Kaloki *et al*., 2019; Varshney *et al*., 2019) [1, 26]. Chickpea is a self-pollinated, diploid, cool season legume that is crucial in cereal-legume rotations because it adds nitrogen to the soil and significantly reduces the need for synthetic nitrogen fertilizers in smallholder resource-poor farming (Kaloki *et al*., 2019) [1]. Nutritionally, the chickpeas are good sources of protein (17-24% dry weight), carbohydrates, fiber, folate, iron, zinc and other micronutrients (Kaloki *et al*., 2019) [1]. They play an important role in the alleviation of protein-energy malnutrition and micronutrient deficiencies in regions where animal protein is expensive or not used for cultural reasons (Kaloki *et al*., 2019) [1]. Economically, chickpeas contribute significantly to the generation of export revenues and rural incomes and the residues are used a feed for livestock in integrated cropping-livestock systems (Kaloki *et al*., 2019) [1].

1.2. Production Constraints and Climate Change Impacts

Although chickpea is known for its agronomic and nutritional superiority, the productivity of chickpea is constrained due to its limited genetic diversity for abiotic stress tolerance, mainly due to its cultivation under rainfed conditions and the seasonal cycle of chickpea production that often has the flowering and pod formation phase exposed to the increasing temperatures and decreasing soil moisture levels (Devasirvatham & Tan, 2018; Kaloki *et al*., 2019) [2, 1]. Various climate scenarios indicate that the intensity, occurrence, and overlap of drought and heat will be on the rise in the key chickpea producing regional zones and during the growing process the average temperature may increase by 1.5-3.0 °C until the middle of this century (Mittler, 2006; Rani *et al*., 2020) [10, 24].

Terminal heat stress, usually observed at the end of the rainy season during the flowering and pod formation phases, is characterized by high temperature phenomena, and terminal drought stress associated with the drying of the soil late in the season. Terminal heat and terminal drought stresses often occur at the same time and result in a different ecological situation compared to the experiment where they were studied separately.

1.3. Heat Stress, Drought Stress, and Their Combination

The prevalence of heat stress in chickpea has been defined as when mean daily temperatures are greater than 30-35 degrees C within the reproductive period (Devasirvatham & Tan, 2018; Rani *et al*., 2020) [2, 24]. Heat stress can affect pollen viability and sperm fertilization even during the time when the vegetative growth stage has not been affected (Wahid *et al*., 2007; Sita *et al*., 2017) [23, 5]. Drought stress is caused by a reduction in soil water content and results in the prevention of the closure of stomatal pores that lead to CO2 infiltration and the initiation of the physiological processes that affect plants physically and chemically (Chaves *et al*., 2009; Farquhar & Sharkey, 1982) [22, 21].

The combination of both heat and drought stress may cause opposing physiological responses, for instance while the former type of stress causes stomatal opening to cool down plants via transpiration, drought stress will require the closure of stomata which would not be affected due to heat stress (Mittler, 2006; Rizhsky *et al*., 2004) [10, 9]. The belief of many scientists today is that vegetation stressed from both drought and heat should be considered to be under a unique physiological condition Исследование stressor effects on physiological processes in chickpea plants (Mittler, 2006; Rizhsky *et al*., 2004) [10, 9]

1.4. Yield Instability and the Need for Climate-Resilient Cultivars

It is thought that the joint incidence of heat and drought during the flowering–pod-filling phase represents the most serious climatic hazard for chickpea yield stability, since it can lead to yield losses of 40–70% depending on genetic background, the time of occurrence of the stress and its intensity (Awasthi *et al*., 2014; Devasirvatham & Tan, 2018; Rani *et al*., 2020) [4, 2, 24]. The limited possibilities of changing sowing dates in chickpea-based production systems make the search for genotypes with potential tolerance to combined heat and drought stress appear to be the most viable solution (Krishnamurthy *et al*., 2011; Canci & Toker, 2009) [3, 25].

To achieve this aim, it is necessary to understand the mechanisms of functioning of photosynthetic apparatus, water relations, and biochemical mechanisms of resistance under conditions of combined heat and drought stress, rather than rely solely on yield-based screening experiments in uncontrolled environmental conditions (Baker, 2008; Murchie & Lawson, 2013; Chaves *et al*., 2009) [17, 18, 22].

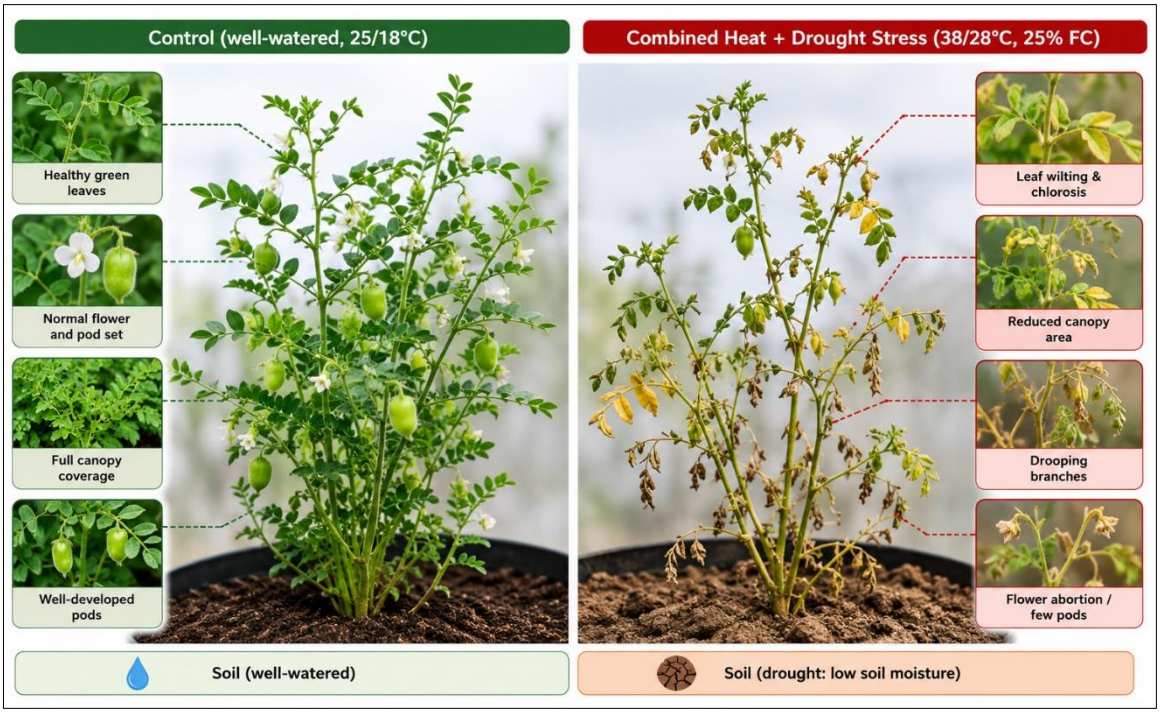

Fig 1: Morphological and developmental symptoms of *Cicer arietinum* L. under control and combined heat-drought stress conditions, illustrating leaf wilting, chlorosis, reduced canopy area, drooping branches, and reduced flower/pod set under stress.

2. Physiological Effects of Combined Heat and Drought Stress

2.1. Plant Water Relations and Leaf Water Status

Water relations form the foundation of chickpea's response to combined stress (Chaves *et al*., 2009; Awasthi *et al*., 2014) [22, 4]. Leaf water potential becomes markedly more negative under combined heat-drought conditions than under either stress in isolation, reflecting the compounding effects of reduced soil water availability and heat-driven evaporative demand (Awasthi *et al*., 2014; Mittler, 2006) [4, 10]. Relative water content (RWC), a widely used indicator of leaf hydration status, declines progressively as stress duration extends, with combined-stress plants typically exhibiting RWC values 15-25 percentage points lower than well-watered controls by the tenth day of stress imposition (Awasthi *et al*., 2014; Chaves *et al*., 2009) [4, 22].

The decline in RWC is accompanied by reduced turgor pressure, which constrains cell expansion and leaf area development, and by altered osmotic potential as cells accumulate compatible solutes in an attempt to maintain turgor through osmotic adjustment (Ashraf & Foolad, 2007; Chaves *et al*., 2009) [16, 22].

The rate and magnitude of RWC decline differ substantially among genotypes, providing one of the more accessible and reproducible physiological screens for combined-stress tolerance (Krishnamurthy *et al*., 2011; Canci & Toker, 2009) [3, 25].

2.2. Membrane Stability and Cellular Integrity

Cellular membranes are particularly vulnerable to the combined thermal and osmotic perturbations imposed by simultaneous heat and drought stress (Mittler, 2006; Awasthi *et al*., 2014) [10, 4]. Elevated temperature increases membrane fluidity and lipid peroxidation, while drought-associated dehydration destabilizes lipid bilayers and membrane-associated proteins (Wahid *et al*., 2007; Gill & Tuteja, 2010) [23, 14]. The combined effect is reflected in increased electrolyte leakage and a reduced membrane stability index (MSI), both of which correlate closely with the extent of lipid peroxidation as measured by malondialdehyde accumulation (Gill & Tuteja, 2010; Baxter *et al*., 2014) [14, 13].

Membrane stability is not merely a passive casualty of stress but an active target of protective mechanisms, including the accumulation of heat shock proteins, compatible osmolytes, and antioxidant compounds that collectively preserve membrane fluidity and reduce the propagation of oxidative chain reactions within lipid bilayers (Ashraf & Foolad, 2007; Gill & Tuteja, 2010; Baxter *et al*., 2014) [16, 14, 13].

2.3. Stomatal Regulation, Transpiration, and Photosynthetic Inhibition

Stomatal behavior lies at the physiological crux of the heat-drought interaction (Mittler, 2006; Chaves *et al*., 2009) [10, 22]. Under isolated heat stress, stomata often remain relatively open, or even open further, to facilitate transpirational cooling of leaf tissue (Wahid *et al*., 2007) [23]. Under drought stress, stomata close progressively to limit water loss (Farquhar & Sharkey, 1982; Chaves *et al*., 2009) [21, 22]. When the two stresses combine, the drought signal typically dominates, driving stomatal closure that reduces transpirational cooling capacity precisely when leaf temperatures are elevated, creating a self-reinforcing cycle of leaf overheating and further stomatal restriction (Mittler, 2006; Rizhsky *et al*., 2004; Zhou *et al*., 2017) [10, 9, 8].

This stomatal closure directly curtails CO2 diffusion into the mesophyll, reducing the substrate available for carboxylation and diminishing net photosynthetic rate independent of any direct impairment of the photosynthetic biochemistry itself (Farquhar & Sharkey, 1982; Chaves *et al*., 2009; Flexas *et al*., 2012) [21, 22, 20]. Concurrently, non-stomatal limitations-including impaired Rubisco activation state, reduced ATP synthase activity, and thylakoid membrane disruption under elevated temperature-compound the stomatal effect, producing the steep declines in photosynthetic rate characteristic of combined stress (Flexas *et al*., 2012; Baker, 2008; Wahid *et al*., 2007) [20, 17, 23].

2.4. Effects on Flowering, Pod Development, and Seed Filling

The reproductive phase of chickpea is disproportionately sensitive to combined heat-drought stress relative to vegetative growth (Sita *et al*., 2017; Awasthi *et al*., 2014) [5, 4]. Elevated temperature during microsporogenesis impairs pollen viability and reduces pollen tube growth, while concurrent water deficit reduces the assimilate supply available to developing flowers and pods, together driving substantial rates of flower and pod abortion (Wahid *et al*., 2007; Sita *et al*., 2017; Farooq *et al*., 2017) [23, 5, 12]. Seed filling, which depends on a continuous supply of photoassimilates from source leaves, is curtailed both by reduced photosynthetic carbon fixation and by accelerated leaf senescence under prolonged combined stress, producing smaller seeds and reduced harvest index (Awasthi *et al*., 2014; Prasad *et al*., 2011; Farooq *et al*., 2017) [4, 7, 12].

2.5. Physiological Adaptation Mechanisms

Despite the severity of combined stress, chickpea genotypes exhibit a spectrum of adaptive physiological responses, including deep and prolific root systems that maintain water uptake under receding soil moisture, transient osmotic adjustment that sustains turgor and stomatal function, and dehydration-avoidance strategies such as leaf rolling and reduced canopy expansion that limit water loss (Ashraf & Foolad, 2007; Chaves *et al*., 2009; Kaloki *et al*., 2019) [16, 22, 1]. Genotypic variation in the timing, magnitude, and coordination of these adaptive responses underlies much of the observed variability in combined-stress tolerance among chickpea germplasm (Krishnamurthy *et al*., 2011; Canci & Toker, 2009; Varshney *et al*., 2019) [3, 25, 26].

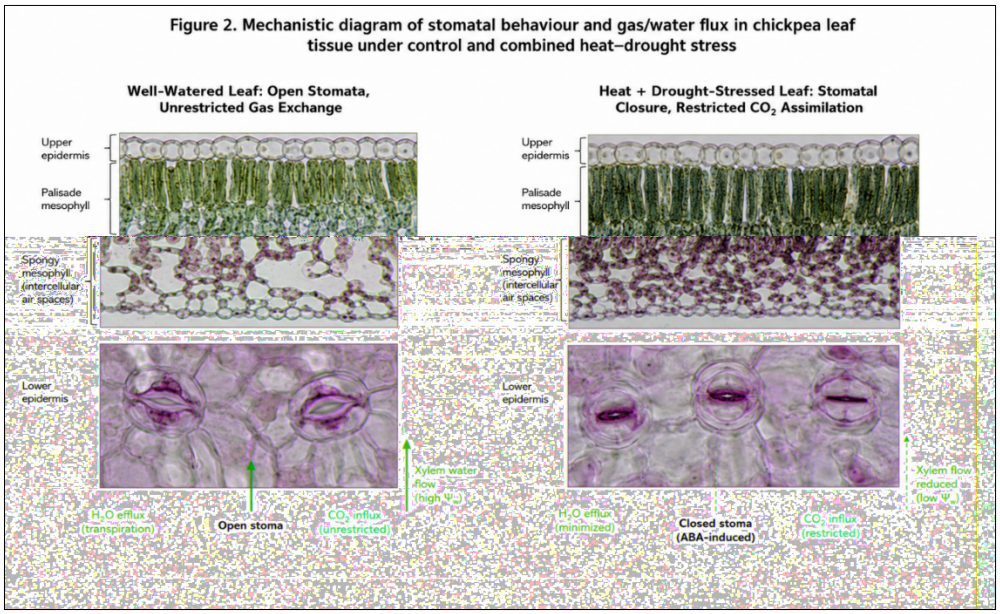

Fig 2: Mechanistic diagram of stomatal behaviour and gas/water flux in chickpea leaf tissue under control (open stomata, unrestricted CO2 influx and transpiration) and combined heat-drought stress (stomatal closure, restricted CO2 assimilation, reduced xylem water flow).

3. Gas Exchange Analysis

3.1. Principles of Leaf Gas Exchange Measurement

Gas exchange analysis quantifies the exchange of CO2 and water vapor between a leaf and the surrounding atmosphere, providing a direct, real-time window into photosynthetic performance and stomatal behavior (Farquhar & Sharkey, 1982; Flexas *et al*., 2012) [21, 20]. Portable infrared gas analyzer (IRGA) systems enclose a leaf segment within a controlled chamber and measure the differential CO2 and H2O concentrations of air entering and leaving the chamber, from which net photosynthetic rate, stomatal conductance, transpiration rate, and intercellular CO2 concentration are calculated using established diffusion-based equations (Farquhar & Sharkey, 1982; Flexas *et al*., 2012) [21, 20].

3.2. Net Photosynthetic Rate (Pn) and Stomatal Conductance (Gs)

Net photosynthetic rate (Pn) represents the net balance between gross CO2 assimilation and respiratory CO2 release, and is the most direct integrative measure of a leaf's carbon-fixing capacity under any given environmental condition (Farquhar & Sharkey, 1982; Flexas *et al*., 2012) [21, 20]. Stomatal conductance (Gs) quantifies the ease with which CO2 and water vapor diffuse through the stomatal pores, and is highly sensitive to both soil water status and leaf-to-air vapor pressure deficit, both of which are altered under combined heat-drought stress (Farquhar & Sharkey, 1982; Chaves *et al*., 2009) [21, 22]. In chickpea, Gs typically declines earlier and more steeply than Pn under progressive stress, indicating that stomatal closure is often the initiating event in stress-induced photosynthetic decline before non-stomatal limitations become dominant (Chaves *et al*., 2009; Awasthi *et al*., 2014; Flexas *et al*., 2012) [22, 4, 20].

3.3. Transpiration Rate (E) and Intercellular CO2 Concentration (Ci)

Transpiration rate (E) reflects water loss through open stomata and is jointly determined by stomatal conductance and the leaf-to-air vapor pressure gradient, which itself increases under elevated temperature (Farquhar & Sharkey, 1982; Wahid *et al*., 2007) [21, 23]. Intercellular CO2 concentration (Ci) provides insight into the relative contribution of stomatal versus non-stomatal (mesophyll or biochemical) limitations to photosynthesis: a concurrent decline in both Pn and Ci suggests stomatal limitation is dominant, whereas a decline in Pn accompanied by stable or rising Ci points toward biochemical or mesophyll-conductance limitations, a pattern more frequently observed under severe or prolonged combined stress in chickpea (Flexas *et al*., 2012; Chaves *et al*., 2009; Awasthi *et al*., 2014) [20, 22, 4].

3.4. Water-Use Efficiency (WUE) and Carboxylation Efficiency

Instantaneous water-use efficiency, calculated as the ratio of Pn to E, integrates the plant's capacity to fix carbon per unit of water transpired and is a key trait for drought-prone environments (Chaves *et al*., 2009; Flexas *et al*., 2012) [22, 20]. Moderate drought stress alone often increases WUE as stomatal closure reduces transpiration proportionally more than photosynthesis, but under combined heat-drought stress this efficiency gain is frequently eroded because elevated temperature disproportionately increases the vapor pressure deficit and non-stomatal photosynthetic limitations become severe enough to offset the water-conservation benefit (Chaves *et al*., 2009; Wahid *et al*., 2007; Flexas *et al*., 2012) [22, 23, 20]. Instantaneous carboxylation efficiency, derived from the Pn/Ci ratio, offers a complementary index of the intrinsic biochemical capacity of the photosynthetic apparatus, independent of stomatal behavior (Flexas *et al*., 2012; Farquhar & Sharkey, 1982) [20, 21].

3.5. Instrumentation and Interpretation Considerations

Reliable gas exchange measurement requires standardization of chamber conditions, including photosynthetic photon flux density, leaf temperature, and reference CO2 concentration, to ensure that treatment comparisons reflect genuine physiological differences rather than measurement artifacts (Flexas *et al*., 2012; Farquhar & Sharkey, 1982) [20, 21]. Diurnal timing is also critical, as chickpea gas exchange parameters typically peak in mid-morning and decline sharply around midday under stress conditions due to stress-induced midday depression, a phenomenon that must be accounted for in the sampling schedule to obtain physiologically meaningful and comparable data across treatments and genotypes (Chaves *et al*., 2009; Flexas *et al*., 2012) [22, 20].

Comparative gas exchange data across treatments are presented in Table 2 (see Results and Discussion, Section 8.1).

4. Chlorophyll Fluorescence Analysis

4.1. Principles of Chlorophyll Fluorescence and Photosystem II

Chlorophyll fluorescence analysis exploits the biophysical fact that light energy absorbed by chlorophyll molecules in Photosystem II (PSII) can be dissipated through three competing pathways: photochemistry (driving electron transport), non-photochemical quenching (heat dissipation), and fluorescence re-emission (Baker, 2008; Murchie & Lawson, 2013) [17, 18]. Because these pathways compete for the same pool of absorbed energy, changes in one pathway are reflected as reciprocal changes in the others, allowing fluorescence measurements to serve as a highly sensitive, non-invasive probe of photosynthetic function and photoprotective capacity (Baker, 2008; Kalaji *et al*., 2014; Murchie & Lawson, 2013) [17, 19, 18].

4.2. Maximum Quantum Efficiency (Fv/Fm) and Photoinhibition

The ratio of variable to maximum fluorescence (Fv/Fm), measured on dark-adapted leaves, represents the maximum potential quantum efficiency of PSII photochemistry and is widely regarded as one of the most robust and reproducible indicators of photosynthetic stress in plants (Baker, 2008; Murchie & Lawson, 2013) [17, 18]. Healthy, unstressed leaves typically exhibit Fv/Fm values between 0.79 and 0.84, and any substantial decline below this range signals photoinhibition, resulting from damage to the PSII reaction center, particularly the D1 protein, when the rate of photodamage exceeds the rate of repair (Baker, 2008; Kalaji *et al*., 2014) [17, 19]. In chickpea, Fv/Fm remains relatively stable under mild individual stress but declines sharply and progressively under combined heat-drought stress, reflecting cumulative photochemical damage beyond the plant's repair capacity (Awasthi *et al*., 2014; Rani *et al*., 2020) [4, 24].

4.3. Effective Quantum Yield (ΦPSII) and Electron Transport Rate (ETR)

Effective quantum yield of PSII (ΦPSII), measured under actinic light, quantifies the proportion of absorbed light energy actually used in photochemistry at a given point in time, providing a more dynamic, light-adapted counterpart to Fv/Fm (Baker, 2008; Murchie & Lawson, 2013) [17, 18]. Electron transport rate (ETR), derived from ΦPSII, incident photosynthetically active radiation, and leaf absorptance, estimates the flux of electrons

through the photosynthetic electron transport chain and correlates closely with CO2 assimilation under non-stress conditions, though this correlation weakens under stress as electrons are increasingly diverted toward alternative sinks such as photorespiration and the Mehler reaction (Baker, 2008; Kalaji *et al*., 2014) [17, 19].

4.4. Photochemical Quenching (qP) and Non-Photochemical Quenching (NPQ)

Photochemical quenching (qP) reflects the proportion of open, photochemically competent PSII reaction centers and declines as stress progressively closes reaction centers due to impaired downstream electron acceptor availability (Baker, 2008; Murchie & Lawson, 2013) [17, 18]. Non-photochemical quenching (NPQ) reflects the dissipation of excess absorbed energy as heat, primarily through the xanthophyll cycle, and generally increases under moderate stress as a protective mechanism that prevents excessive electron pressure on PSII (Baker, 2008; Kalaji *et al*., 2014) [17, 19]. Under combined heat-drought stress in chickpea, NPQ typically rises sharply during the early stress period, indicating an actively engaged photoprotective response, but this rise often plateaus or reverses under prolonged severe stress as the photoprotective capacity itself becomes compromised, coinciding with the steepest declines in Fv/Fm (Awasthi *et al*., 2014; Rani *et al*., 2020) [4, 24].

4.5. Significance of Fluorescence Analysis in Combined-Stress Physiology

The integrated interpretation of Fv/Fm, ΦPSII, ETR, qP, and NPQ allows researchers to distinguish between plants that are actively and successfully photoprotecting their photosynthetic apparatus from those that have progressed into a state of chronic photoinhibition and photosynthetic damage (Baker, 2008; Murchie & Lawson, 2013) [17, 18]. This distinction is of considerable practical value in genotype screening, as tolerant genotypes typically sustain higher qP and a more resilient NPQ response for longer periods under combined stress, delaying the onset of irreversible Fv/Fm decline relative to sensitive genotypes (Krishnamurthy *et al*., 2011; Awasthi *et al*., 2014; Rani *et al*., 2020) [3, 4, 24].

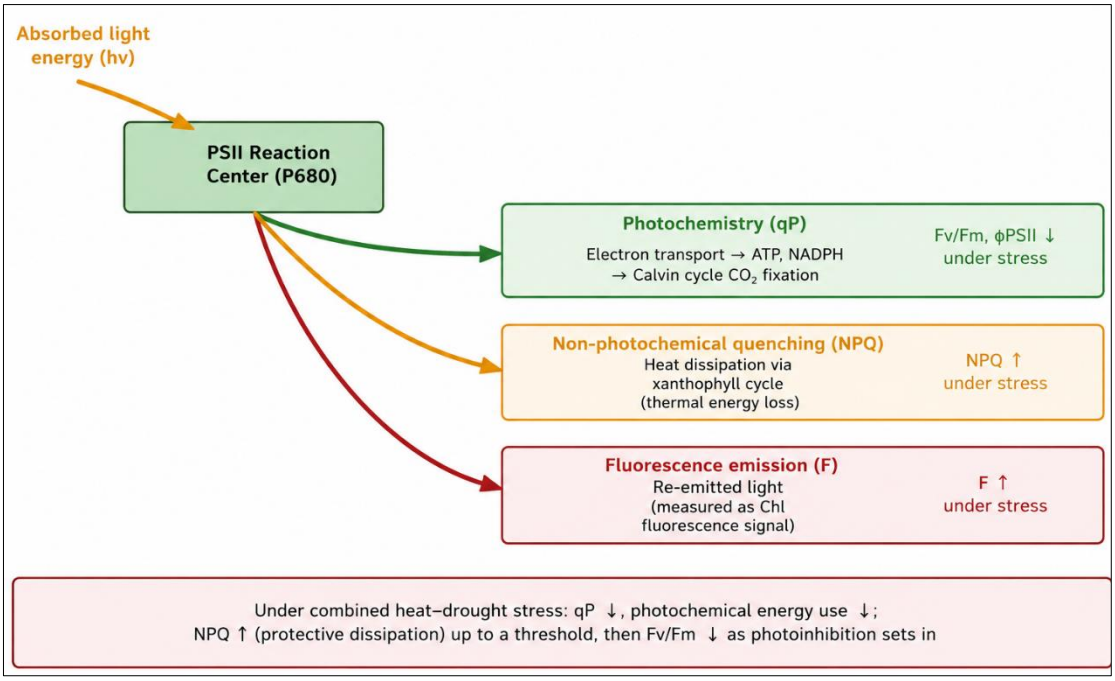

Fig 3: Partitioning of absorbed light energy in Photosystem II (PSII) among photochemistry (qP), non-photochemical quenching (NPQ), and chlorophyll fluorescence emission (F), and the corresponding shifts in this partitioning under combined heat-drought stress.

5. Biochemical Responses to Combined Stress

5.1. Reactive Oxygen Species and Oxidative Stress

Combined heat-drought stress disrupts the delicate balance between reactive oxygen species (ROS) production and scavenging within chloroplasts, mitochondria, and peroxisomes, leading to the overaccumulation of superoxide radicals, hydrogen peroxide, and hydroxyl radicals (Baxter *et al*., 2014; Gill & Tuteja, 2010) [13, 14]. Restricted CO2 availability under stomatal closure limits the consumption of NADPH and ATP by the Calvin cycle, increasing electron pressure on the photosynthetic electron transport chain and promoting ROS generation at both PSII and Photosystem I, a process further intensified by heat-induced disruption of thylakoid membrane organization (Baxter *et al*., 2014; Baker, 2008; Wahid *et al*., 2007) [13, 17, 23].

5.2. Lipid Peroxidation, Malondialdehyde, and Hydrogen Peroxide

Malondialdehyde (MDA), a terminal product of lipid peroxidation, serves as a widely used biomarker of oxidative membrane damage, and its accumulation in chickpea leaf tissue rises sharply under combined heat-drought stress, exceeding the levels observed under either individual stress (Gill & Tuteja, 2010; Baxter *et al*., 2014) [14, 13]. Hydrogen peroxide (H2O2), while itself a signaling molecule at low concentrations, becomes cytotoxic when it accumulates beyond the buffering capacity of the antioxidant system, contributing directly to membrane lipid peroxidation, protein oxidation, and enzyme inactivation under severe combined stress (Baxter *et al*., 2014; Hasanuzzaman *et al*., 2017) [13, 15].

5.3. Electrolyte Leakage and Membrane Stability Index

Electrolyte leakage, measured as the proportional release of cellular ions into a bathing solution, provides a functional index of membrane integrity that correlates closely with MDA content (Gill & Tuteja, 2010; Baxter *et al*., 2014) [14, 13]. The membrane stability index (MSI), calculated by comparing electrical conductivity of tissue exposed to heat treatment against unheated controls, similarly declines under combined stress, and both indices consistently rank genotypes in a manner concordant with their gas exchange and fluorescence-based tolerance rankings (Awasthi *et al*., 2014; Rani *et al*., 2020) [4, 24].

5.4. Antioxidant Enzyme Defense System

Enzymatic antioxidants provide the primary line of cellular defense against ROS accumulation (Gill & Tuteja, 2010; Baxter *et al*., 2014) [14, 13]. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen, while catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) subsequently detoxify the resulting hydrogen peroxide into water and oxygen through complementary pathways with differing subcellular localizations and substrate affinities (Gill & Tuteja, 2010; Hasanuzzaman *et al*., 2017) [14, 15]. In chickpea under combined stress, the activities of SOD, CAT, POD, and APX all increase relative to control, with the magnitude of induction generally greatest in combined-stress plants, reflecting an intensified but frequently insufficient compensatory response, as evidenced by the concurrent rise in MDA and H2O2 despite elevated enzyme activity (Awasthi *et al*., 2014; Hasanuzzaman *et al*., 2017) [4, 15].

5.5. Proline, Soluble Sugars, and Osmotic Adjustment

Proline accumulation serves multiple protective functions under combined stress, acting as a compatible osmolyte that facilitates osmotic adjustment, a molecular chaperone that stabilizes protein structure, and a scavenger of hydroxyl radicals and singlet oxygen (Ashraf & Foolad, 2007; Hasanuzzaman *et al*., 2017) [16, 15]. Soluble sugars, derived from both photosynthetic products and starch remobilization, similarly contribute to osmotic adjustment while also serving as respiratory substrates and signaling molecules that modulate stress-responsive gene expression (Ashraf & Foolad, 2007; Hasanuzzaman *et al*., 2017) [16, 15]. In chickpea, both proline and soluble sugar concentrations increase progressively with stress severity, reaching their highest levels under combined heat-drought stress, consistent with an intensified demand for osmotic and protective compounds under the most physiologically demanding treatment (Awasthi *et al*., 2014; Rani *et al*., 2020) [4, 24].

5.6. Total Soluble Proteins and Relative Water Content

Total soluble protein content, encompassing both structural and stress-responsive proteins including heat shock proteins and antioxidant enzymes, provides a general index of cellular metabolic and protective capacity (Gill & Tuteja, 2010; Hasanuzzaman *et al*., 2017) [14, 15]. While severe combined stress can eventually reduce total protein content through accelerated proteolysis and reduced synthesis, moderate to intermediate stress durations are often associated with a transient increase in soluble protein as stress-responsive proteins are actively synthesized (Hasanuzzaman *et al*., 2017; Wahid *et al*., 2007) [15, 23]. Relative water content, discussed above as a water-relations parameter, is closely intertwined with these biochemical responses, since adequate cellular hydration is a prerequisite for continued protein synthesis and enzymatic function under stress (Chaves *et al*., 2009; Ashraf & Foolad, 2007) [22, 16].

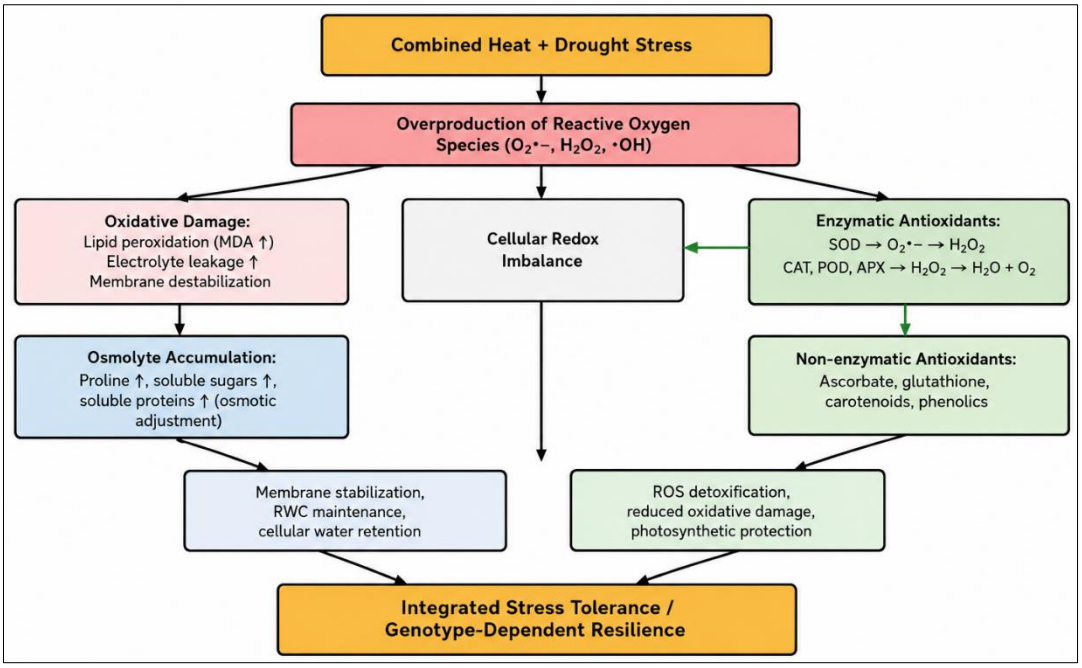

Fig 4: Reactive oxygen species (ROS) generation and the integrated enzymatic and non-enzymatic antioxidant/osmotic defense network mobilized in chickpea leaf tissue in response to combined heat-drought stress.

6. Experimental Design and Methodology

6.1. Plant Material and Growth Conditions

Three chickpea genotypes representing a range of presumed heat and drought sensitivity-BG-372 (heat-sensitive check), JG-11 (widely cultivated, moderately tolerant), and ICCV-2 (early-maturing, drought-tolerant check)-were selected for this study. Seeds were surface-sterilized, germinated, and transplanted into pots containing a 2:1:1 mixture of field soil, sand, and farmyard manure, and grown under naturally lit glasshouse conditions with supplemental controlled temperature until the pre-flowering stage (35 days after sowing). (Krishnamurthy *et al*., 2011; Canci & Toker, 2009) [3, 25]

6.2. Stress Treatments and Experimental Design

At the pre-flowering stage, plants were randomly assigned to one of four treatments in a completely randomized design with four biological replicates per genotype-treatment combination: (i) control, maintained at 25/18 degrees C day/night with soil moisture at 80-85% field capacity; (ii) heat stress, imposed by transferring plants to a temperature-controlled chamber set at 38/28 degrees C day/night while maintaining well-watered conditions; (iii) drought stress, imposed by progressive water withholding until soil moisture reached and was maintained at 25% field capacity under ambient control-condition temperature; and (iv) combined heat-drought stress, imposing both the 38/28 degrees C regime and the 25% field capacity soil moisture level simultaneously. All stress treatments were maintained for 10 days (Awasthi *et al*., 2014; Devasirvatham & Tan, 2018) [4, 2].

6.3. Sampling Schedule and Replication

Physiological and biochemical sampling was conducted at three time points-0 days (pre-stress baseline), 5 days, and 10 days after stress imposition-to capture both the initial acclimation response and the cumulative effect of prolonged stress (Awasthi *et al*., 2014; Rani *et al*., 2020) [4, 24]. At each sampling point, the third fully expanded leaf from the top of each plant was used for gas exchange and fluorescence measurements, with adjacent leaves of the same physiological age collected for biochemical assays. Four biological replicates (individual plants) per treatment-genotype combination were sampled, with three technical replicates performed for each biochemical assay.

6.4. Gas Exchange and Chlorophyll Fluorescence Measurements

Gas exchange parameters were recorded between 09:00 and 11:00 h using a portable infrared gas analyzer under a standardized photosynthetic photon flux density of 1200 micromol m-2 s-1, reference CO2 concentration of 400 ppm, and leaf chamber temperature matched to ambient treatment conditions (Farquhar & Sharkey, 1982; Flexas *et al*., 2012) [21, 20]. Chlorophyll fluorescence was measured using a pulse-amplitude-modulated fluorometer, with Fv/Fm recorded on leaves dark-adapted for 30 minutes prior to measurement, and ΦPSII, ETR, qP, and NPQ recorded under the same actinic light intensity as the gas exchange measurements to enable direct comparison between the two data sets (Baker, 2008; Murchie & Lawson, 2013) [17, 18].

6.5. Biochemical Assays

Leaf samples for biochemical analysis were flash-frozen in liquid nitrogen immediately after collection and stored at -80 degrees C until analysis. Malondialdehyde content was determined via the thiobarbituric acid reactive substances assay; hydrogen peroxide was quantified spectrophotometrically following titanium sulfate reaction; proline was estimated using the acid-ninhydrin method; soluble sugars were determined by the anthrone method; and total soluble protein was quantified using the Bradford assay (Gill & Tuteja, 2010; Hasanuzzaman *et al*., 2017) [14, 15]. Antioxidant enzyme activities (SOD, CAT, POD, APX) were assayed spectrophotometrically from crude enzyme extracts using established substrate-specific protocols (Gill & Tuteja, 2010; Hasanuzzaman *et al*., 2017) [14, 15]. Relative water content was calculated from fresh, turgid, and dry weights, membrane stability index was derived from electrical conductivity measurements before and after controlled heating, and electrolyte leakage was calculated as the percentage of initial to total conductivity following tissue disruption (Gill & Tuteja, 2010; Chaves *et al*., 2009) [14, 22].

Table 1: Experimental design summary: genotypes, treatments, stress duration, environmental conditions, sampling schedule, and replication.

Parameter	Details
Genotypes	BG-372 (heat-sensitive), JG-11 (moderately tolerant), ICCV-2 (drought-tolerant)
Growth stage at stress imposition	Pre-flowering (35 days after sowing)
Treatments	Control; Heat stress; Drought stress; Combined heat + drought stress
Heat stress condition	38/28 °C day/night, well-watered (80-85% FC)
Drought stress condition	25% field capacity, ambient control temperature
Combined stress condition	38/28 °C day/night + 25% field capacity
Stress duration	10 days
Sampling time points	0, 5, and 10 days after stress imposition
Experimental design	Completely randomized design (CRD), factorial: 3 genotypes × 4 treatments
Replication	4 biological replicates per genotype × treatment combination; 3 technical replicates per biochemical assay

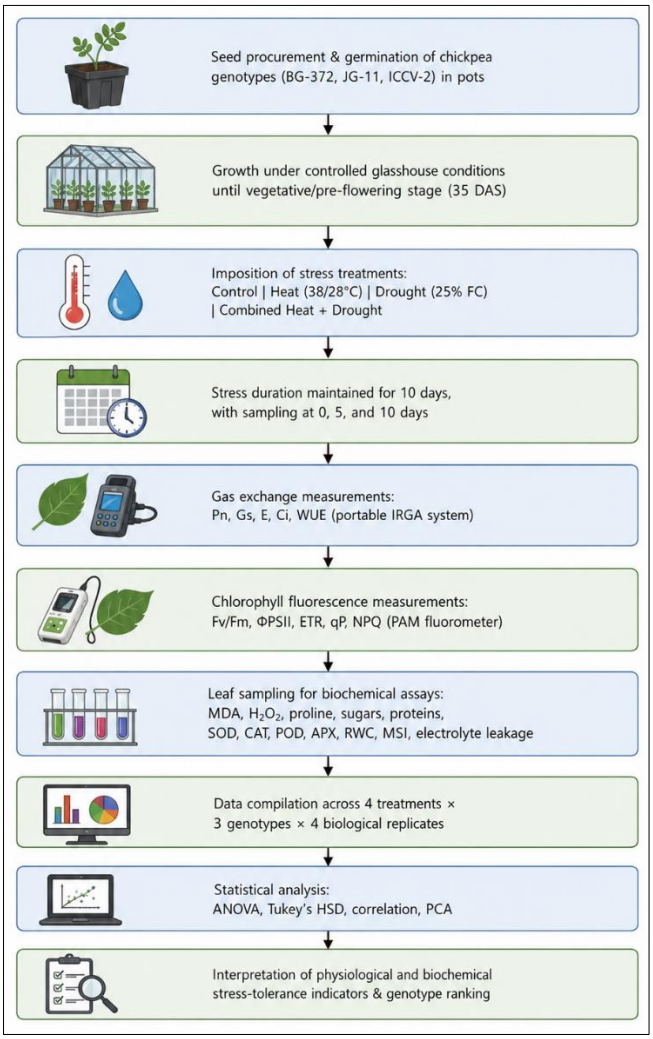

Fig 5: Flowchart of the experimental workflow, from chickpea cultivation and stress imposition through gas exchange, chlorophyll fluorescence, and biochemical measurements, to statistical analysis and interpretation of stress-tolerance indicators.

7. Statistical Analysis

7.1. Descriptive Statistics and Replication Structure

All data are presented as mean plus or minus standard deviation calculated from four biological replicates, each itself derived from the mean of three technical replicates where applicable, ensuring that reported variability reflects genuine plant-to-plant differences rather than measurement noise alone.

7.2. Analysis of Variance and Mean Separation

Two-way analysis of variance (ANOVA) was performed for each parameter to assess the main effects of treatment and genotype and their interaction, followed by one-way ANOVA within each genotype where the interaction term was significant. Tukey's honestly significant difference (HSD) test was used for post-hoc mean separation at the $p < 0.05$ significance threshold, and Student's t-test was applied for selected pairwise comparisons between control and combined-stress treatments where a single, focused comparison was of primary interest.

7.3. Correlation and Principal Component Analysis

Pearson correlation analysis was conducted to examine the strength and direction of relationships among gas exchange, fluorescence, and biochemical parameters across all treatments and genotypes, allowing identification of parameters that co-vary consistently with stress severity. Principal component analysis (PCA) was performed on the complete standardized data matrix to reduce dimensionality and visualize the overall separation of treatments and genotypes along composite physiological and biochemical axes, with the first two principal components retained for interpretation based on their cumulative explained variance.

8. Results and Discussion

8.1. Gas Exchange Responses Across Treatments

Net photosynthetic rate declined progressively across treatments, with combined heat-drought stress producing the most severe reduction relative to control by day 10 of stress imposition (Table 2) (Awasthi *et al*., 2014; Devasirvatham & Tan, 2018) [4, 2]. Stomatal conductance followed a similar but earlier-onset pattern of decline, consistent with stomatal closure as the initiating mechanism of photosynthetic inhibition, while intercellular CO2 concentration declined under drought and combined stress but showed a comparatively smaller decline under heat stress alone, indicating a greater contribution of non-stomatal limitations under elevated temperature (Farquhar & Sharkey, 1982; Chaves *et al*., 2009; Flexas *et al*., 2012) [21, 22, 20]. Water-use efficiency increased modestly under isolated drought stress but was substantially eroded under combined stress, reflecting the disproportionate impact of elevated vapor pressure deficit on transpirational water loss relative to the residual photosynthetic capacity (Figure 6) (Chaves *et al*., 2009; Wahid *et al*., 2007) [22, 23].

8.2. Chlorophyll Fluorescence Responses Across Treatments

Maximum quantum efficiency of PSII (Fv/Fm) remained relatively stable under heat stress alone during the first five days

but declined markedly under combined stress by day 10, falling well below the threshold typically associated with sustained photoinhibitory damage (Table 3) (Baker, 2008; Murchie & Lawson, 2013) [17, 18]. Non-photochemical quenching increased sharply during the early stress period across all stress treatments, most prominently under combined stress, consistent with an actively engaged photoprotective response; however, by day 10 of combined stress, NPQ values plateaued or declined slightly in parallel with the steepest Fv/Fm decline, suggesting that photoprotective capacity itself became compromised under the most severe and prolonged stress condition (Baker, 2008; Kalaji *et al*., 2014; Awasthi *et al*., 2014) [17, 19, 4].

8.3. Biochemical Stress Markers and Antioxidant Defense

Malondialdehyde and hydrogen peroxide content rose in a dose-dependent manner with stress severity, reaching their highest values under combined heat-drought stress (Table 4), directly paralleling the decline in membrane stability index and the increase in electrolyte leakage (Gill & Tuteja, 2010; Baxter *et al*., 2014) [14, 13]. Antioxidant enzyme activities (SOD, CAT, POD, APX) increased across all stress treatments relative to control, with the greatest induction observed under combined stress; however, the concurrent rise in oxidative damage markers indicates that this enhanced enzymatic response, while substantial, was insufficient to fully counteract ROS accumulation under the most severe treatment, consistent with a state of partial antioxidant defense saturation (Hasanuzzaman *et al*., 2017; Gill & Tuteja, 2010) [15, 14].

8.4. Osmolyte Accumulation and Genotypic Variation

Proline and soluble sugar concentrations increased progressively with stress severity and duration, reaching maximal levels under combined stress at day 10, reflecting an intensified osmotic adjustment response commensurate with the greater water-deficit and thermal burden imposed by the combined treatment (Ashraf & Foolad, 2007; Hasanuzzaman *et al*., 2017) [16, 15]. Genotypic comparison consistently ranked ICCV-2 as the most tolerant genotype, exhibiting comparatively higher Fv/Fm, qP, and antioxidant enzyme activities alongside lower MDA and H2O2 accumulation under combined stress relative to BG-372 and JG-11, a pattern corroborated by the comparative summary in Table 5 (Krishnamurthy *et al*., 2011; Canci & Toker, 2009; Rani *et al*., 2020) [3, 25, 24].

8.5. Correlation and Principal Component Analysis Findings

Pearson correlation analysis revealed strong positive correlations between net photosynthetic rate and both Fv/Fm and stomatal conductance, and strong negative correlations between MDA content and both Fv/Fm and Pn, confirming the tight physiological linkage between oxidative damage and photosynthetic decline under combined stress (Baker, 2008; Gill & Tuteja, 2010; Baxter *et al*., 2014) [17, 14, 13]. Principal component analysis resolved approximately 70-75% of total variance within the first two principal components, with PC1 broadly representing a photosynthetic-efficiency axis (loading heavily on Pn, Gs, Fv/Fm, and qP) and PC2 representing a stress-severity/osmolyte axis (loading heavily on proline, MDA, and antioxidant enzyme activities), together providing a clear and interpretable separation of treatments and genotypes consistent with the univariate findings described above.

Table 2: Gas exchange parameters of chickpea genotypes under control, heat, drought, and combined heat-drought stress at day 10 (mean ± SD, n = 4).

Treatment	Pn (µmol CO2 m-2 s-1)	Gs (mol H2O m-2 s-1)	E (mmol H2O m-2 s-1)	Ci (µmol CO2 mol-1)	WUE (µmol CO2/mmol H2O)
Control	21.4 ± 1.1a	0.32 ± 0.03a	6.9 ± 0.5a	268 ± 9a	3.1 ± 0.2a
Heat	15.2 ± 1.3b	0.24 ± 0.02b	5.9 ± 0.4b	251 ± 8b	3.6 ± 0.3b
Drought	13.6 ± 1.0b	0.17 ± 0.02c	3.1 ± 0.3c	224 ± 7c	4.4 ± 0.3c
Heat + Drought	8.9 ± 0.9c	0.11 ± 0.01d	2.3 ± 0.2d	209 ± 8d	3.9 ± 0.3bc

Table 3: Chlorophyll fluorescence parameters of chickpea genotypes under control, heat, drought, and combined heat-drought stress at day 10 (mean ± SD, n = 4).

Treatment	Fv/Fm	ΦPSII	ETR (µmol electrons m-2 s-1)	qP	NPQ
Control	0.82 ± 0.02a	0.61 ± 0.03a	142 ± 8a	0.78 ± 0.04a	0.85 ± 0.08a
Heat	0.74 ± 0.02b	0.51 ± 0.03b	119 ± 7b	0.65 ± 0.04b	1.32 ± 0.10b
Drought	0.71 ± 0.03b	0.47 ± 0.03b	108 ± 6b	0.60 ± 0.05b	1.48 ± 0.12b
Heat + Drought	0.58 ± 0.03c	0.33 ± 0.03c	76 ± 6c	0.42 ± 0.04c	1.29 ± 0.11b

Table 4: Biochemical responses of chickpea genotypes under control, heat, drought, and combined heat-drought stress at day 10 (mean ± SD, n = 4).

Parameter	Control	Heat	Drought	Heat + Drought
Proline (µmol g-1 FW)	4.8 ± 0.6a	9.6 ± 1.0b	12.4 ± 1.2c	19.7 ± 1.8d
Soluble sugars (mg g-1 FW)	18.2 ± 1.4a	24.5 ± 1.8b	28.9 ± 2.1c	35.6 ± 2.6d
MDA (nmol g-1 FW)	6.1 ± 0.5a	10.4 ± 0.9b	12.8 ± 1.0c	18.5 ± 1.4d
H2O2 (µmol g-1 FW)	3.2 ± 0.3a	5.8 ± 0.5b	6.9 ± 0.6b	10.6 ± 0.9c
SOD (U mg-1 protein)	38.5 ± 3.0a	55.2 ± 3.5b	61.8 ± 4.0b	74.3 ± 4.6c
CAT (U mg-1 protein)	22.6 ± 2.1a	31.4 ± 2.4b	34.9 ± 2.6b	42.7 ± 3.1c
POD (U mg-1 protein)	15.3 ± 1.5a	22.8 ± 1.9b	25.1 ± 2.0b	31.9 ± 2.4c
APX (U mg-1 protein)	9.7 ± 1.0a	14.6 ± 1.3b	16.2 ± 1.4b	20.8 ± 1.7c
Total soluble protein (mg g-1 FW)	12.4 ± 1.1a	13.8 ± 1.2a	11.6 ± 1.0a	9.8 ± 0.9b
Relative water content (%)	88.6 ± 2.0a	80.3 ± 2.4b	72.1 ± 2.7c	61.4 ± 3.1d
Membrane stability index (%)	84.2 ± 2.2a	73.5 ± 2.6b	68.9 ± 2.9b	54.7 ± 3.3c
Electrolyte leakage (%)	14.8 ± 1.3a	24.6 ± 1.9b	28.9 ± 2.1b	39.5 ± 2.8c

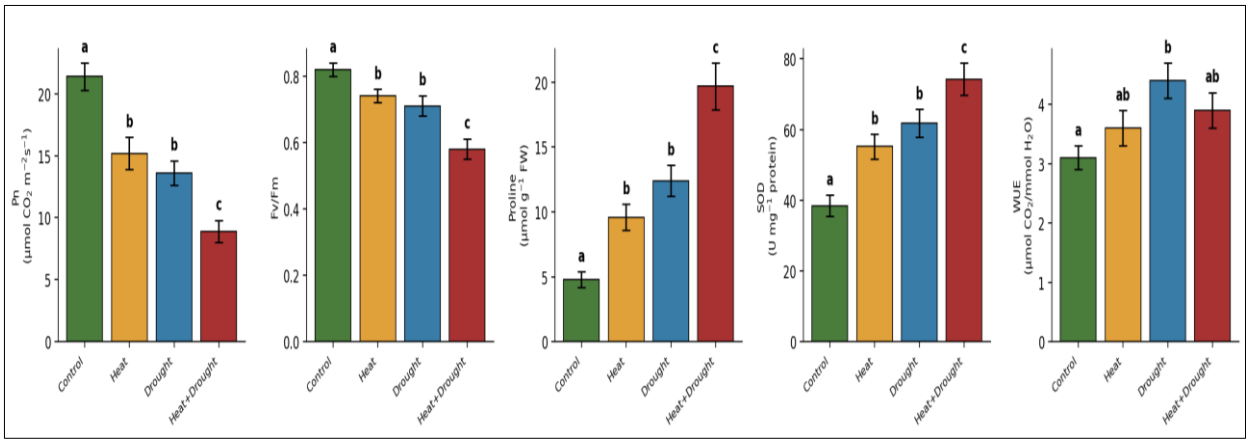

Fig 6: Comparative response of net photosynthetic rate (Pn), maximum quantum efficiency (Fv/Fm), proline content, superoxide dismutase (SOD) activity, and water-use efficiency (WUE) of chickpea under control, heat, drought, and combined heat-drought stress (mean ± SD, n = 4; bars sharing no common letter differ significantly, Tukey's HSD, $p < 0.05$).

Table 5: Comparative summary of physiological, biochemical, gas exchange, and chlorophyll fluorescence responses across treatments, with statistical significance and stress-tolerance interpretation.

Response category	Control vs Heat	Control vs Drought	Control vs Combined	Tolerance interpretation
Photosynthetic rate (Pn)	↓ 29% (p<0.01)	↓ 36% (p<0.01)	↓ 58% (p<0.001)	Combined stress causes synergistic decline exceeding either single stress
Max. quantum efficiency (Fv/Fm)	↓ 10% (p<0.05)	↓ 13% (p<0.01)	↓ 29% (p<0.001)	Combined stress drives genuine photoinhibition, unlike single stresses
Proline accumulation	↑ 100% (p<0.01)	↑ 158% (p<0.001)	↑ 310% (p<0.001)	Osmotic adjustment intensifies with combined stress severity
Antioxidant enzymes (mean of 4)	↑ 44% (p<0.01)	↑ 61% (p<0.001)	↑ 90% (p<0.001)	Strong induction, but insufficient to prevent oxidative damage
Membrane stability index	↓ 13% (p<0.05)	↓ 18% (p<0.01)	↓ 35% (p<0.001)	Combined stress causes disproportionate membrane destabilization
Genotype ranking (tolerance)	ICCV-2 > JG-11 > BG-372	ICCV-2 > JG-11 > BG-372	ICCV-2 > JG-11 > BG-372	Consistent genotype ranking across all stress conditions

9. Mechanisms of Combined Stress Tolerance

9.1. Photosynthetic Protection Mechanisms

Tolerance to combined heat-drought stress in chickpea appears to depend substantially on the plant's capacity to sustain photochemical efficiency through effective engagement of the xanthophyll-cycle-mediated non-photochemical quenching pathway during the early stress period, thereby limiting cumulative photodamage to the PSII reaction center and delaying the onset of irreversible Fv/Fm decline observed in more sensitive genotypes (Baker, 2008; Murchie & Lawson, 2013; Kalaji *et al*., 2014) [17, 18, 19].

9.2. Antioxidant Defense and ROS Detoxification

A coordinated upregulation of superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase activities, alongside non-enzymatic antioxidants such as ascorbate and glutathione, constitutes a central component of combined-stress tolerance by maintaining ROS concentrations below the threshold at which lipid peroxidation and protein oxidation become extensive, thereby preserving membrane integrity and enzymatic function under conditions of dual thermal and osmotic challenge (Gill & Tuteja, 2010; Hasanuzzaman *et al*., 2017; Baxter *et al*., 2014) [14, 15, 13].

9.3. Osmotic Adjustment and Cellular Protection

Osmotic adjustment through proline and soluble sugar accumulation enables tolerant genotypes to maintain cell turgor, sustain stomatal function, and protect protein and membrane structures under simultaneous water deficit and thermal stress, functioning synergistically with heat shock proteins that stabilize protein folding and membrane fluidity under elevated temperature (Ashraf & Foolad, 2007; Hasanuzzaman *et al*., 2017; Wahid *et al*., 2007) [16, 15, 23].

9.4. Hormonal Regulation and ABA Signaling

Abscisic acid (ABA) accumulation under water deficit serves as a central signaling hub that coordinates stomatal closure, the induction of osmolyte biosynthesis genes, and the expression of antioxidant and heat shock protein genes, effectively integrating the drought and heat stress response pathways at the molecular level (Chaves *et al*., 2009; Mittler, 2006) [22, 10]. The extent and timing of ABA-mediated signaling likely underlies much of the genotypic variation observed in stomatal regulation and downstream biochemical protection under combined stress (Rani *et al*., 2020; Krishnamurthy *et al*., 2011) [24, 3].

9.5. Integrated Stress Tolerance as a Multigenic Trait

Collectively, these findings support the view that combined heat-drought tolerance in chickpea is not attributable to any single mechanism but rather emerges from the coordinated interaction of stomatal regulation, photosynthetic photoprotection, antioxidant defense, osmotic adjustment, and hormonal signaling, underscoring the multigenic and physiologically integrated nature of this trait and the corresponding need for multi-trait phenotyping approaches in breeding programs (Rani *et al*., 2020; Krishnamurthy *et al*., 2011; Awasthi *et al*., 2014) [24, 3, 4].

10. Applications and Future Perspectives

10.1. Climate-Resilient Chickpea Breeding

The physiological and biochemical markers characterized in this study-particularly Fv/Fm, stomatal conductance, proline

content, and antioxidant enzyme activity-offer breeders rapid, non-destructive, and physiologically meaningful selection criteria that can be deployed at scale in early-generation breeding nurseries, complementing traditional yield-based selection under managed stress environments (Baker, 2008; Murchie & Lawson, 2013; Krishnamurthy *et al*., 2011) [17, 18, 3].

10.2. Marker-Assisted Selection and Functional Genomics

Integration of these physiological phenotypes with genomic resources, including quantitative trait loci mapping and genome-wide association studies, can accelerate the identification of candidate genes underlying combined-stress tolerance, enabling marker-assisted selection to complement phenotypic screening and reduce the time required to develop improved cultivars (Varshney *et al*., 2019; Rani *et al*., 2020) [26, 24].

10.3. Transcriptomics, Proteomics, and Metabolomics

Multi-omics approaches, including transcriptomic profiling of stress-responsive gene networks, proteomic characterization of heat shock and antioxidant protein induction, and metabolomic profiling of osmolyte and secondary metabolite accumulation, offer complementary layers of mechanistic insight that can refine the physiological framework established through gas exchange and fluorescence analysis, revealing regulatory nodes that could serve as targets for precision breeding or genome editing (Rani *et al*., 2020; Varshney *et al*., 2019) [24, 26].

10.4. Genome Editing and Precision Agriculture

Genome editing technologies such as CRISPR/Cas offer a route to directly modify candidate genes governing stomatal regulation, antioxidant capacity, or osmolyte biosynthesis, while high-throughput phenotyping platforms incorporating automated gas exchange and fluorescence imaging can enable the screening of large germplasm collections under standardized combined-stress protocols, substantially increasing the throughput and precision of stress-tolerance evaluation relative to traditional manual approaches (Varshney *et al*., 2019; Rani *et al*., 2020) [26, 24].

10.5. Future Research Priorities

Future research should prioritize field validation of glasshouse-derived physiological markers under natural combined heat-drought episodes, expansion of genotype panels to capture broader genetic diversity, integration of root-system physiology with the shoot-focused measurements emphasized here, and longer-term multi-season trials to assess the stability of identified tolerance mechanisms across variable environmental conditions, ultimately supporting the development of climate-smart chickpea cultivars for sustainable pulse production (Rani *et al*., 2020; Varshney *et al*., 2019; Devasirvatham & Tan, 2018) [24, 26, 2].

11. Conclusion

In this research, it was concluded that simultaneous exposure to heat and drought had a greater detrimental effect on chickpea than exposure to either of two stressors alone. This reflected in the heel closing stomata faster, net photosynthesis lowering by a larger value, and significant photoinhibition damage revealed by reduced Fv/Fm and photochemical quenching.

The use of chlorophyll fluorescence along with gas exchange analysis was beneficial. The gas exchange technique provided information on diffusion, while fluorescence method showed the consequences of the stress on level of photosystem II.

In addition, combined heat and drought stress resulted in the greatest induction of antioxidant enzyme activities and accumulation of osmolytes, however, it also produced the highest amounts of malondialdehyde, hydrogen peroxide and electrolyte leakage.

The fact that the three studied genotypes have been constantly ranked due to their tolerance shows the practical meaning of gas exchange and fluorescence phenotyping. In this respect, ICCV-2 has outperformed JG-11 and BG-372. Such an approach allows one to conduct rapid screening of climate-resilient chickpea genotypes through physiological means without requiring laborious field tests of multi-seasons.

In the future, it is necessary to verify the physiological parameters calculated in glasshouses so that it would be applicable to real field conditions with appropriate timing and variability of heat and drought. Moreover, there is a need to conduct screening on chickpeas in order to identify more genotypes which can be used in breeding programs.

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