



Unraveling the physiological and molecular mechanisms regulating grain yield under combined drought and heat stress in wheat (*Triticum aestivum*)

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ABSTRACT

An experiment was conducted during winter (*rabi*) seasons of 2020–21 and 2021–22 at the research farm of ICAR-Indian Agricultural Research Institute, New Delhi to assess the impacts of heat and drought stresses, both individually and combined, on wheat (*Triticum aestivum* L.) plants during the reproductive stage. Four wheat genotypes (C306, HD2967, Raj3765 and WL711) were subjected to heat stress (H), drought stress (D) and combined heat and drought stress (HD) conditions at the anthesis stage. The research investigated various physiological, biochemical and grain yield parameters, as well as the relative expression of genes involved in the proline and abscisic acid (ABA) metabolic pathways. Among the tested genotypes, Raj3765 exhibited decreased ABA levels and increased proline accumulation during the anthesis stage under both individual and combined stress conditions. Notably, Raj3765 also displayed higher grain yield compared to the other 3 genotypes under all stress conditions, indicating that elevated proline levels and reduced ABA levels likely contributed to its resilience. Furthermore, the study revealed that the combination of heat and drought stresses had a more severe detrimental effect on wheat plants compared to individual stress treatments. These findings underscore the significance of comprehensively studying combined stress conditions, as they can result in substantial yield losses in wheat crop development and productivity.

Keywords: Abiotic stress tolerance, Combined stress, Drought, Free radicals, Heat, Oxidative damage

Wheat (*Triticum aestivum* L.) is a globally significant crop and a major calorie source (Abdelrahman *et al.* 2020). India, as the second-largest wheat producer, faces the challenge of meeting food demand with limited land (CGIAR 2020). India contributes around 14% to global wheat production (FAO 2021). Heat waves and drought stress threaten wheat production, causing potential yield losses of up to 86% and 69% respectively (Bhunia *et al.* 2020, Valipour *et al.* 2021). High temperatures can lead to pollen abortion and shorter crop cycles, while heat and drought stress induce oxidative damage (Baidya *et al.* 2023). However, the specific impacts of combined heat and drought stress on wheat are not well understood. Although research on the physiological and biochemical responses to simultaneous heat and drought stress in wheat is limited, gaining insights into these mechanisms is essential for effective management and adaptation strategies (Qaseem *et al.* 2019, Mansoor *et al.* 2022). The objectives of the current study were to evaluate the performance of different

wheat genotypes under simultaneous heat and drought stress and to explore the physiological and biochemical mechanisms involved in stress tolerance during the reproductive stages. By understanding the mechanisms of combined stress tolerance, this research aimed to identify wheat genotypes with improved performance under heat and drought conditions.

MATERIALS AND METHODS

Experimental material, design and treatments: The experiment was conducted at the research farm of ICAR-Indian Agricultural Research Institute, New Delhi, to study the response of 4 wheat genotypes (C306, HD2967, Raj3765 and WL711) to drought and heat stress during the winter (*rabi*) seasons of 2020–21 and 2021–22. The genotypes were subjected to different treatments including timely sowing with irrigation (control), without irrigation (drought stress), induced heat stress in controlled chambers at National Innovations in Climate Resilient Agriculture (NICRA), ICAR-IARI, New Delhi and combined heat and drought stress. Each genotype had 9 seeds sown in a pot on 19th and 20th November, 2020 and 2021, respectively and after thinning, 4 plants were retained until heading. The samples for physio-biochemical and molecular analysis

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were collected at the anthesis stage and the total grain yield (TGY) was calculated at the time of harvesting. Treatment application began at heading and continued until maturity. The experiment followed standard agronomic practices and recorded temperature (°C) and precipitation (mm) data during the wheat development period (Supplementary Fig 1).

Soil moisture measurements: Soil moisture content (SMC) was determined at the anthesis stage using a gravimetric technique (Black 1965). Soil samples were collected at a depth of 20 cm from the middle of the earthen pots using a tube auger. The SMC values were measured as 24.4% under control conditions, 18.99% during drought stress, 22.05% during heat stress and 12.14% during combined heat and drought stress.

Plant physiological traits: Relative water content (RWC) and membrane stability index (MSI) were measured in the flag leaf at the anthesis stage following the methods recommended by Barrs and Weatherly (1962) and Premachandra *et al.* (1990), respectively. The total chlorophyll content (mg/g DW) in the flag leaf was estimated using the method described by Hiscox and Israelstam (1979). The photosynthetic rate (Pn) and associated parameters of the flag leaf were measured using a portable Infrared Gas Analyzer (IRGA), LI-6400XT Model (Li-COR Ltd., Lincoln, Nebraska, USA), based on the method given by Long *et al.* (1996). Total leaf area (cm²/plant) was measured using a leaf area meter at the anthesis stage under all 4 stress conditions.

Biochemical traits: Oxidative stress, indicated by lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS), was estimated in the flag leaves of control and stressed samples using the methods described by Heath and Packer (1968) and Alexieva *et al.* (2001), respectively. Proline content and total antioxidant capacity (TAC) was determined by FRAP assay, and abscisic acid (ABA) content were measured in the flag leaves of control and stressed samples at the anthesis stage following the methods given by Bates *et al.* (1973), Benzie and Szeto (1999) and Zeevaart *et al.* (1999), respectively. The enzymatic activity of total catalase (CAT), peroxidase (POX) and glutathione reductase (GR) was studied in the flag leaves of control and stressed samples based on the methods described by Aebi *et al.* (1984), Castillo *et al.* (1984) and Smith *et al.* (1988), respectively.

Yield components: The total grain yield (g/plant) was recorded for all 4 genotypes under the 4 different stress conditions.

Total RNA isolation and cDNA synthesis: Plant material weighing 100 mg was collected and total RNA was manually extracted from flag leaf tissues using TRIzol® reagent from Invitrogen, USA. The cDNA Synthesis Superscript® III First-Strand Synthesis Kit from Invitrogen, USA, was used to synthesize complementary DNA (cDNA) from the isolated RNA. The resulting cDNA can be stored at -20°C for further use as a template in Real-Time quantitative PCR (qPCR) procedures.

Primer designing and real-time quantitative PCR:

Real-time quantitative PCR primers were designed using OligoAnalyzer 3.1 (Supplementary Fig 2). The designed primers were used in Real-Time quantitative PCR reactions, which were analyzed using an Applied Biosystems Step One Plus™ real-time detection system.

Statistical analysis: The experimental data collected from the field study were analyzed using statistical methods in the "R" software. The average mean and standard error (SE) were calculated based on at least 3 biological replicates. Analysis of variance (ANOVA) techniques appropriate for a completely randomized design (CRD) were applied to evaluate the significance of the treatment effects. Pairwise comparisons were conducted using the DMRT function from the agricolae package. Graphs depicting the results were created using the ggplot2 package.

RESULTS AND DISCUSSIONS

Combination stress led to higher changes in physiological and biochemical traits: Significant variations were observed in physio-biochemical parameters including RWC, MSI, Pn, proline, TAC, H₂O₂ levels, MDA and ABA content among the wheat genotypes under different stress conditions. Drought stress primarily affected RWC, while heat stress caused a decline in MSI. Irrigation was used to mitigate the impact of heat stress. Previous studies have reported similar findings (Farooq *et al.* 2019). WL711 had the highest reduction in leaf area (76.79%), followed by HD2967 (67.82%), C306 (63.80%) and Raj3765 (51.24%). Drought stress caused a greater decrease in leaf area compared to heat stress. The previous study also reported that drought stress reduces leaf number, size and longevity, resulting in decreased photosynthesis (Shao *et al.* 2008). It was found that combined stress has a more detrimental impact on plant physiological traits and yield compared to individual stresses (Rollins *et al.* 2013).

Chlorophyll concentration and membrane stability declined under independent and combined stress, leading to a decrease in photosynthesis rate. Combined stress resulted in a more significant loss of these traits compared to individual stresses (Kumar *et al.* 2022). Leaf chlorophyll content was greatly reduced under combined stress, with WL711 showing the highest percentage loss (79.05%). Chlorophyll concentration in all genotypes was significantly impacted by individual and combined heat and drought conditions. Heat stress caused greater damage compared to drought stress alone.

WL711 showed higher membrane permeability and increased lipid peroxidation, indicated by elevated malondialdehyde levels (Farooq *et al.* 2019). All genotypes exhibited increased ABA content under stress conditions, with the highest accumulation observed under combined stress, while no significant difference was observed under heat or drought stress alone. In response to reactive oxygen species (ROS) damage, osmoregulatory molecules like proline, TAC and enzymatic antioxidants accumulated under all stressors (Mansoor *et al.* 2022). TAC was significantly higher under all stress conditions and with the combined

stress showing the greatest increase across all wheat genotypes. Raj3765 exhibited the highest spike in TAC, followed by C306, HD2967 and WL711 under combined stress, indicating the cumulative impact of drought and heat stress on enhancing antioxidant capacity.

Effect of individual and combined stress on enzymatic antioxidant activities: Our study found that wheat genotypes exhibited increased activity of enzymatic antioxidants under different stress conditions, with the highest accumulation observed under combined drought and heat stress. Among the genotypes, Raj3765 consistently showed the highest activity of CAT, GR and POX at the anthesis stage, indicating its superior ability to cope with oxidative stress. This suggests that genotypes with higher enzymatic antioxidant activity, such as Raj3765, possess better tolerance and resilience to stressful conditions, supporting their growth and development.

Effect of individual and combined stress on pollen viability percentage: We found that all stress conditions significantly reduced pollen viability compared to control conditions, with the greatest decrease observed under combined stress. Specifically, the percentages of viable pollen under combined stress ranged from 25.43 to 51.04% across the genotypes. The high temperature had a more pronounced negative effect on pollen viability compared to drought stress alone (Bheemanahalli *et al.* 2019). These findings highlight the detrimental effects of both combined stress and individual heat stress on pollen viability in wheat genotypes. The results are consistent with the previous reports (Baidya *et al.* 2023).

Tolerant genotypes accumulate more proline under combined stress: Our study revealed that proline levels increased in all tested conditions, with the highest accumulation observed under combined stress, followed by drought stress during anthesis in wheat genotypes. Raj3765, known for its tolerance, exhibited the highest proline accumulation, while WL711 showed lower accumulation, indicating its greater sensitivity to heat stress. These findings emphasize

the significance of proline accumulation as an adaptive response to stress, with tolerant genotypes like Raj3765 showing higher proline levels to cope with combined stress. Conversely, sensitive genotypes like WL711 may face challenges in effectively accumulating proline under heat stress.

In our study, we found that under combined stress, the expression of proline biosynthesis genes, *TaP5CS1* (Fig 1A), *TaP5CS2* (Fig 1B) and *TaP5CR* (Fig 1C), was significantly higher in the Raj3765 compared to individual stress conditions. Additionally, all genotypes showed increased expression of these genes under drought, heat, and combined stress compared to control conditions. These results are consistent with earlier research demonstrating that proline accumulation can be attributed to increased biosynthesis and decreased degradation under heat and drought stress (Silva *et al.* 2008).

Abscisic acid production was lower in Raj3765 as compared to WL711: In our study, WL711 demonstrated the highest percentage change in ABA concentration under

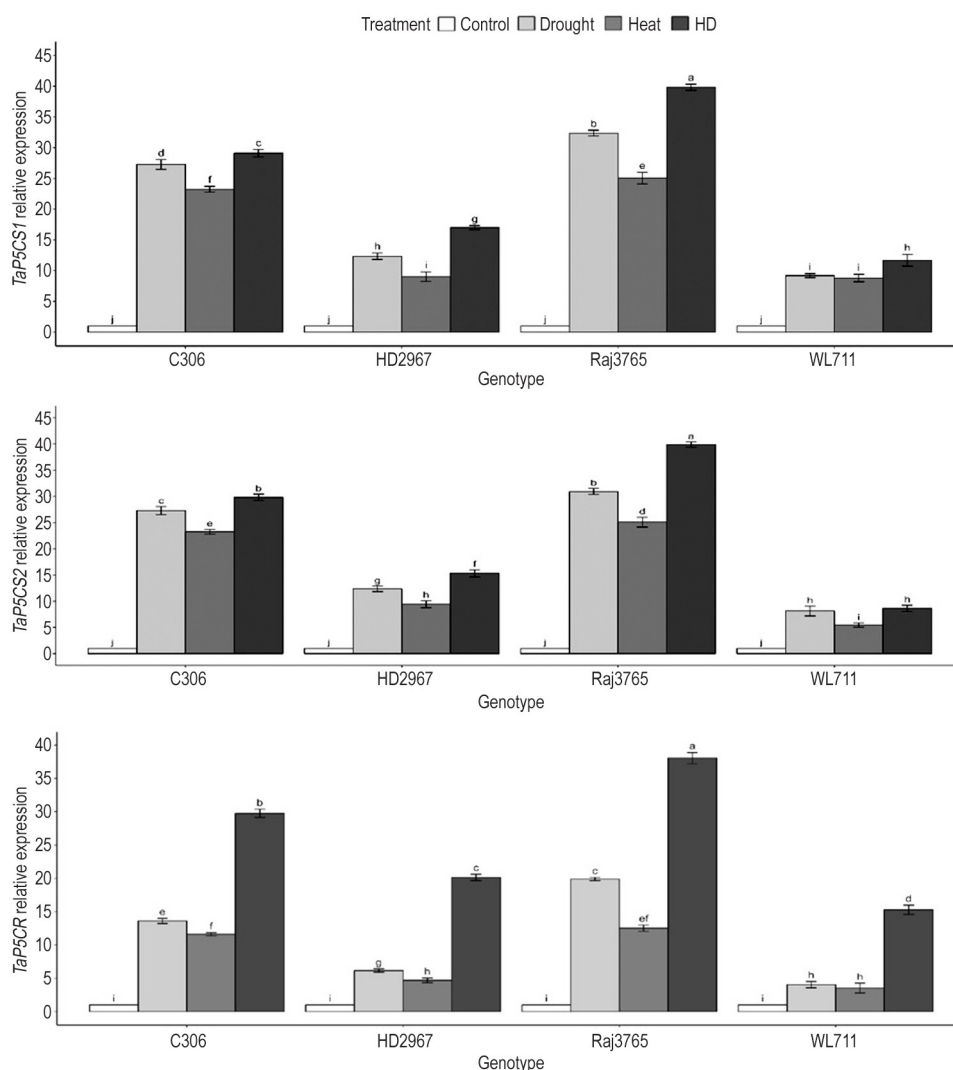


Fig 1 Relative expression of *TaP5CS1* (A), *TaP5CS2* (B) and *TaP5CR* (C) in all 4 contrasting wheat genotypes under control, drought, heat and combined stress conditions.

drought, heat and combined stress, followed by HD2967, C306 and Raj3765. Increased ABA levels have been associated with leaf yellowing and senescence in previous studies (Kumar *et al.* 2022). Our findings suggest that Raj3765 exhibits delayed senescence compared to WL711, likely due to lower ABA accumulation in the flag leaves at anthesis. This supports the notion that differential ABA accumulation contributes to the varying stress responses and senescence patterns observed among the wheat genotypes.

Tolerant genotypes under combined stress exhibit mildly decreased *TaNCED3* and *TaAAO3* gene expression: The expression of two important genes involved in ABA production, *TaNCED3* (Fig 2A) and *TaAAO3* (Fig 2B), significantly increased in all wheat genotypes in all stress conditions. However, Raj3765 exhibited reduced expression of *TaNCED3* and *TaAAO3* compared to the other genotypes under stress conditions (Fig 2). These findings are consistent with previous research highlighting the activation of ABA production-related genes under drought stress (Seiler *et al.* 2011). Other studies conducted on various plant species, such as maize, Arabidopsis, avocado, tomato, cowpea and bean, have also reported increased expression of *NCED3* under drought stress (Ali *et al.* 2020). Additionally, Zdunek-Zastocka (2008) observed elevated *AAO3* expression in pea leaves during salt stress.

Raj3765, tolerant as compared to other tested wheat genotypes, exhibited higher chlorophyll content under stress conditions by suppressing the expression of ABA biosynthetic genes. Specifically, the expression of *TaABA8'OH1* (Fig 2C), involved in ABA breakdown, was decreased in the sensitive genotype WL711 under all stresses. In contrast, all genotypes, including Raj3765, showed increased *TaABA8'OH1* expression under individual and combined stress compared to control conditions. These findings align with previous research showing that impaired ABA production leads to altered senescence in *Arabidopsis* mutants. The upregulation of *TaABA8'OH1* in response to stress has also been observed in barley, suggesting a compensatory mechanism for elevated ABA

levels. Overall, Raj3765's ability to maintain lower ABA levels through *TaABA8'OH1* upregulation may contribute to its stress tolerance, while WL711 exhibits higher ABA levels, potentially contributing to its sensitivity (Seiler *et al.* 2011).

Effect of individual and combined stress on total grain yield: Combined stress has a greater detrimental effect on wheat yield compared to independent stress, thus causing significant yield losses. Under drought stress, yield reductions ranged from 17.7 to 32.24%, while the combination of drought and heat stress resulted in more severe losses ranging from 42.13 to 61.06%. Heat stress alone led to yield losses ranging from 29.98 to 46.55%. Among the studied wheat genotypes, WL711 experienced the highest reduction in yield, while Raj3765 exhibited the least reduction in yield.

Correlation between physio-biochemical traits and total grain yield under combined stress: Our study identified significant positive correlations between various physiological and biochemical traits during the anthesis stage of wheat. Traits such as total chlorophyll content,

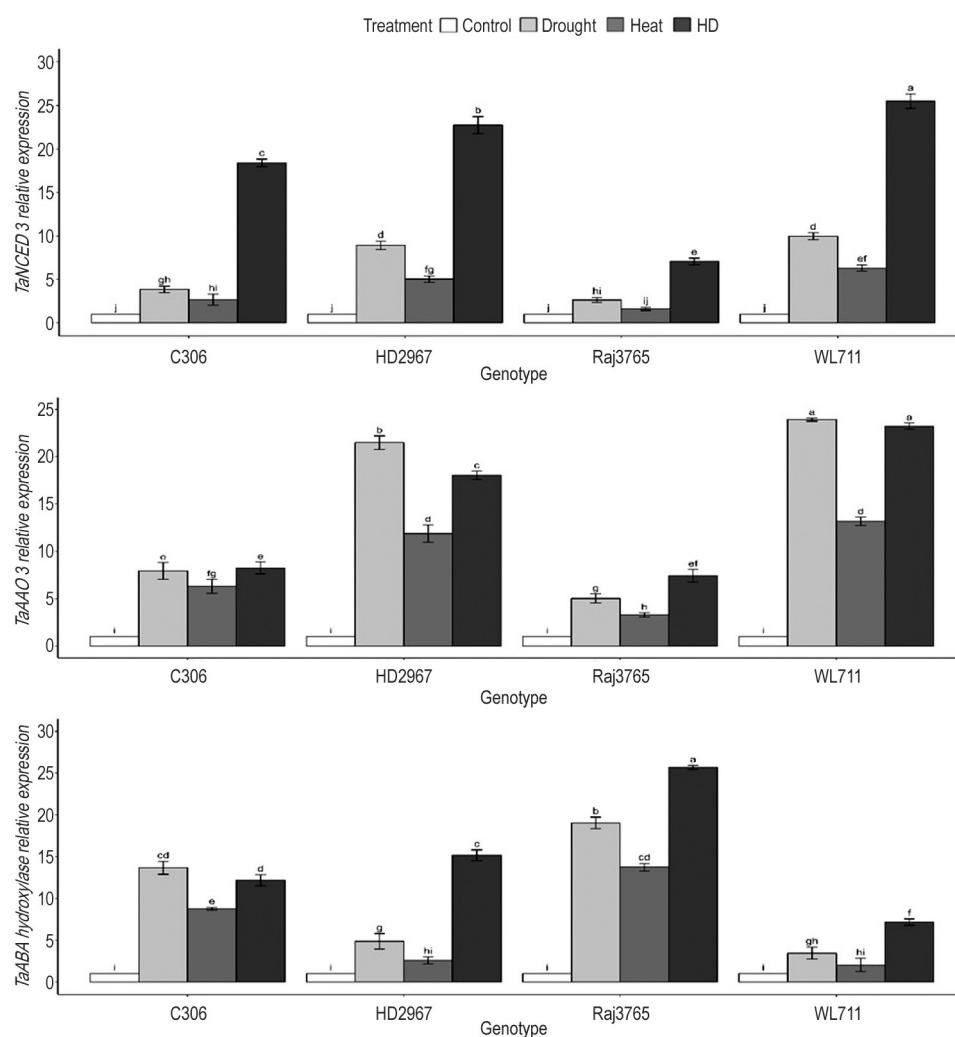


Fig 2 Relative expression of *TaNCED3* (A), *TaAAO3* (B) and *ABA8'OH* (C) in all 4 contrasting wheat genotypes under control, drought, heat and combined stress conditions.

Pn, RWC, MSI, TAC, proline, LA and TGY showed positive associations (Fig 3), indicating their collective contribution to wheat productivity. Under drought conditions, chlorophyll content exhibited a significant positive correlation with Pn (0.99**), LA (0.99*), TGY (0.99*), MSI (0.99*), RWC (0.96*), and TAC (0.98*) at a significance level of $P < 0.05$. H_2O_2 showed a negative correlation ($P < 0.05$) with Pn (-0.97*), TAC (-0.98*), LA (-0.99*), and a significant negative correlation ($P < 0.01$) with chlorophyll (-0.99**). ABA also demonstrated significant negative correlations with proline (-1.00**) at $P < 0.01$ and with POX (-0.96*), PV (-0.96*), and MSI (-0.96*) at a significance level of $P < 0.05$.

Under heat stress, TGY exhibited a negative correlation ($P < 0.05$) with H_2O_2 (-0.99*), ABA (-0.97*), and malondialdehyde (MDA) (-0.97*), and showed positive correlations with chlorophyll (0.98*) and peroxidase (POX) (0.98*). Pn showed positive correlations with chlorophyll (0.96*), POX (0.97*), peroxide value (PV) (0.99*), MSI (0.98*), RWC (0.98*) and growth rate (GR) (0.99*). Proline levels were negatively correlated with H_2O_2 (-0.96*) and ABA (-0.96*) at a significance level of $P < 0.05$, and with MDA (-0.99**) at a significance level of $P < 0.01$. Under combined stress conditions, MDA exhibited a significant negative correlation ($P < 0.01$) with proline and with POX, GR, RWC, and TAC at a significance level of $P < 0.05$. Furthermore, TGY showed a significant positive correlation with H_2O_2 (-0.99**).

Notably, we found a strong negative correlation between ABA concentration in the flag leaf during anthesis and TGY, suggesting that higher ABA levels can negatively impact grain yield. Proline content showed a positive correlation with TGY, indicating its role in stress tolerance and grain production. Conversely, ABA content displayed negative correlations with several physiological traits, indicating its potential negative impact on these traits. Additionally, higher PV at the anthesis stage under controlled conditions was positively correlated with TGY, highlighting its importance for grain production. Overall, these findings emphasize the interconnectedness of physiological and biochemical traits and their influence on grain yield in wheat during the anthesis stage.

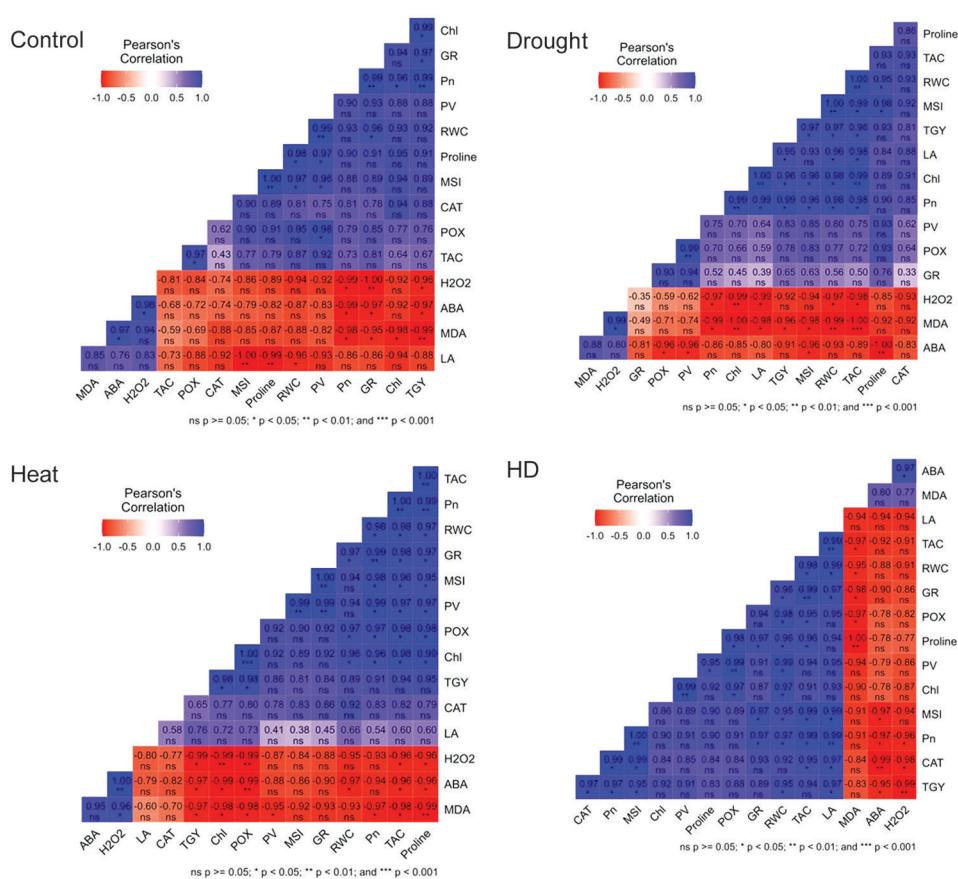


Fig 3 Pearson correlations between physio-biochemical parameters and total grain yield in wheat under control, drought, heat and combined stress conditions.

Our study highlights the adverse effects of increasing heat and drought stresses on wheat productivity, with combined stress having a greater impact. Wheat plants showed the ability to adapt their physiological and biochemical traits to cope with the stresses. WL711 was more susceptible, while Raj3765 showed better tolerance. This research contributes to understanding stress interactions in crops and informs future efforts to improve stress tolerance in wheat. Further research is needed to uncover the genetic mechanisms underlying these responses.

REFERENCES

Abdelrahman M, Burritt D J, Gupta A, Tsujimoto H and Tran L S P. 2020. Heat stress effects on source-sink relationships and metabolome dynamics in wheat. *Journal of Experimental Botany* **71**(2): 543–54.

Aebi H. 1984. Catalase in vitro. *Methods in Enzymology* **105**: 121–26.

Alexieva V, Sergiev I, Mapelli S and Karanov E. 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant, Cell and Environment* **24**(12): 1337–44.

Ali S, Hayat K, Iqbal A and Xie L. 2020. Implications of abscisic acid in the drought stress tolerance of plants. *Agronomy* **10**(9): 1323.

Baidya A, Atta K, Ali MA, Shah MH, Adhikary S, Mondal S, Maitra S and Hossain A. 2023. Abiotic stress-induced ROS production in wheat: Consequences, survival mechanisms, and

- mitigation strategies. *Abiotic Stresses in Wheat*, pp. 131–40. Academic Press.
- Barrs H D and Weatherly P E. 1962. Physiological indices for high yield potential in wheat. *Indian Journal of Plant Physiology* 25: 352–57.
- Bates L S, Waldren R P and Teare I D. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil* 39(1): 205–07.
- Benzie I F and Szeto Y T. 1999. Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry* 47(2): 633–36.
- Bheemanahalli R, Sunoj V J, Saripalli G, Prasad P V, Balyan H S, Gupta P K, Grant N, Gill K S and Jagadish S K. 2019. Quantifying the impact of heat stress on pollen germination, seed set and grain filling in spring wheat. *Crop Science* 59(2): 684–96.
- Bhunia P, Das P and Maiti R. 2020. Meteorological drought study through SPI in three drought prone districts of West Bengal, India. *Earth Systems and Environment* 4(1): 43–55.
- Black C A. 1965. Methods of soil analysis. Agronomy No. 9, part 2. Sponsored by the American Society of Agronomy and the American Society for Testing Materials. American Society of Agronomy, Madison, Wisconsin, USA.
- Castillo F J, Penel C and Greppin H. 1984. Peroxidase release induced by ozone in *Sedum album* leaves: involvement of Ca²⁺. *Plant Physiology* 74(4): 46–851.
- Farooq M, Bramley H, Palta J A and Siddique K H. 2019. Heat stress in wheat during reproductive and grain-filling phases. *Critical Reviews in Plant Sciences* 30(6): 491–507.
- Food Outlook. 2021. Food Outlook - Biannual Report on Global Food Markets. FAO
- Heath R L and Packer L. 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* 125(1): 189–98.
- Hiscox J D and Israelstam G F. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. *Canadian Journal of Botany* 57(12): 1332–34.
- Kumar R, Barman D, Ghimire O P, Gurumurthy S, Singh P K, Chinnusamy V, Padaria J C and Arora A. 2022. Stay-green trait serves as yield stability attribute under combined heat and drought stress in wheat (*Triticum aestivum* L.). *Plant Growth Regulation*, 1–12.
- Kumar R, Harikrishna H A, Ghimire O, Barman D, Chinnusamy V, Singh P K, Padaria J C, Suman A and Arora A. 2021. Stay-green trait ameliorates combined heat and drought stress in wheat. *Indian Journal of Agricultural Sciences* 1: 91.
- Long S P, Farage P K and Garcia R L. 1996. Measurement of leaf and canopy photosynthetic CO₂ exchange in the field. *Journal of Experimental Botany* 47(11): 1629–42.
- Mansoor S, Ali Wani O, Lone J K, Manhas S, Kour N, Alam P, Ahmad A and Ahmad P. 2022. Reactive oxygen species in plants: from source to sink. *Antioxidants* 11(2): 225.
- Premachandra G S, Saneoka H and Ogata S. 1990. Cell membrane stability, an indicator of drought tolerance, as affected by applied nitrogen in soyabean. *The Journal of Agricultural Science* 115(1): 63–66.
- Qaseem M F, Qureshi R and Shaheen H. 2019. Effects of pre-anthesis drought, heat and their combination on the growth, yield and physiology of diverse wheat (*Triticum aestivum* L.) genotypes varying in sensitivity to heat and drought stress. *Scientific Reports* 9(1): 1–12.
- Rollins J A, Habte E, Templer S E, Colby T, Schmidt J and Von Korf M. 2013. Leaf proteome alterations in the context of physiological and morphological responses to drought and heat stress in barley (*Hordeum vulgare* L.). *Journal of Experimental Botany* 64(11): 3201–12.
- Seiler C, Harshavardhan V T, Rajesh K, Reddy P S, Strickert M, Rolletschek H and Sreenivasulu N. 2011. ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. *Journal of Experimental Botany* 62(8): 2615–32.
- Shao H B, Chu L Y, Shao M A, Jaleel C A and Hong-mei M. 2008. Higher plant antioxidants and redox signaling under environmental stresses. *Comptes rendus biologiques* 331(6): 433–41.
- Silva-Ortega C O, Ochoa-Alfaro A E, Reyes-Agüero J A, Aguado-Santacruz G A and Jiménez-Bremont J F. 2008. Salt stress increases the expression of p5cs gene and induces proline accumulation in cactus pear. *Plant Physiology and Biochemistry* 46(1): 82–92.
- Smith N C, Bryant C and Boreham P F L. 1988. Possible roles for pyruvate: ferredoxin oxidoreductase and thiol-dependent peroxidase and reductase activities in resistance to nitroheterocyclic drugs in *Giardia intestinalis*. *International Journal for Parasitology* 18(7): 991–97.
- Valipour M, Bateni S M and Jun C. 2021. Global surface temperature: A new insight. *Climate* 9(5): 81.
- WHEAT Annual Technical Report. 2020. WHEAT 2020 Technical Annual Report – WHEAT.
- Zdunek-Zastocka E. 2008. Molecular cloning, characterization and expression analysis of three aldehyde oxidase genes from *Pisum sativum* L. *Plant Physiology and Biochemistry* 46(1): 19–28.
- Zeevaart J A. 1999. Abscisic acid metabolism and its regulation. *Biochemistry and Molecular Biology of Plant Hormones* 189–209.