

Foliar L-Ascorbic Acid (Vit-C) Application Enhances Antioxidant Defense and Alleviates Heat-Induced Oxidative Stress in Wheat (*Triticum aestivum* L.) Genotypes

Abstract

High temperature stress disrupts cellular metabolism in plants by increasing the accumulation of reactive oxygen species (ROS) resulting in oxidative injury and impaired physiological function. This study investigated the mitigative role of foliar-applied L-ascorbic acid (Vit-C) in two contrasting wheat genotypes HUW-510 (heat-tolerant) and HUW-468 (heat-susceptible) subjected to normal and late-sown heat stress conditions. Heat stress significantly elevated ROS levels by 35–55%, while reducing chlorophyll content (20–30%), nitrate reductase activity (18–28%), total protein (25–35%), membrane stability index (MSI) and relative water content (RWC) indicating marked oxidative and metabolic impairment. Activities of key antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidase (APX) also declined under heat stress suggesting suppression of the enzymatic defense system.

Foliar application of L-ascorbic acid at 1.0, 5.0 and 10.0 mM substantially alleviated oxidative stress with 10.0 mM exhibiting the strongest mitigative effect. Ascorbic acid reduced ROS accumulation by 25–35%, enhanced CAT, SOD and APX activities by 30–50%, improved chlorophyll and NR activity and increased osmolyte levels (proline and soluble sugars). The tolerant genotype HUW-510 consistently displayed superior antioxidant recovery compared to HUW-468 demonstrating genotype-specific responsiveness to AA. Overall L-ascorbic acid effectively strengthened antioxidant defense, stabilized cellular metabolism and mitigated heat-induced oxidative damage highlighting its potential as a practical strategy for improving thermotolerance in wheat.

Keywords

Antioxidant defense; Ascorbate peroxidase (APX); Catalase (CAT); Heat stress; L-ascorbic acid (Vit C); Nitrate reductase (NR); Oxidative stress; Proline; Reactive oxygen species (ROS); Soluble sugars; Superoxide dismutase (SOD)

1. Introduction

Wheat (*Triticum aestivum* L.) is highly sensitive to rising temperatures, particularly during its reproductive and grain-filling phases where heat stress severely limits growth, physiology and yield (Pratap *et al.*, 2025). Exposure to high temperature disrupts cellular homeostasis and accelerates the overproduction of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl ions (Mamatkulova, 2025; Suresh *et al.*, 2025a). Excessive ROS

accumulation leads to oxidative damage of lipids, proteins and nucleic acids, causing membrane destabilization, enzyme inactivation, chlorophyll degradation and impaired metabolic processes (Djanaguiraman *et al.*, 2020). The severity of oxidative injury often depends on the intrinsic heat tolerance of the genotype, with susceptible cultivars exhibiting higher ROS levels and greater metabolic disruption (Kumar *et al.*, 2025; He & Huang, 2010).

Plants possess a complex antioxidant defense system comprising both enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) and non-enzymatic antioxidants such as ascorbic acid, glutathione and phenolics (Rao *et al.*, 2025). These components work synergistically to detoxify ROS and maintain redox balance. However, under severe or prolonged heat stress the endogenous antioxidant capacity becomes insufficient leading to oxidative imbalance and metabolic impairment (Ying *et al.*, 2025). Therefore, external supplementation of antioxidants has emerged as an effective strategy to enhance stress resilience (Sharma *et al.*, 2012; Das & Roychoudhury, 2014; Dadarwal *et al.*, 2025).

L-ascorbic acid (Vit-C) is a key non-enzymatic antioxidant involved in ROS scavenging, regeneration of other antioxidants and protection of photosynthetic and metabolic machinery (Akram *et al.*, 2017; Dadarwal *et al.*, 2022b). Exogenous application of ascorbic acid has been reported to improve chlorophyll stability, enhance antioxidant enzyme activity, strengthen membrane integrity and regulate osmolyte accumulation under various abiotic stresses (Gallie, 2013; Dadarwal 2022). However, detailed investigations on how exogenous L-ascorbic acid modulates oxidative stress and antioxidant defense in contrasting wheat genotypes under field-imposed heat stress are still limited (Dadarwal *et al.*, 2022a; Chen *et al.*, 2023; De Queiroz *et al.*, 2023).

The present investigation evaluated the effectiveness of foliar-applied L-ascorbic acid in mitigating heat-induced oxidative stress in heat-tolerant (HUW-510) and heat-susceptible (HUW-468) wheat genotypes. Specifically, the study assessed its impact on ROS accumulation, antioxidant enzymes (CAT, SOD, APX), chlorophyll content, nitrate reductase (NR) activity, protein content and osmolyte levels under normal and late-sown conditions. This work provides insight into the physiological and biochemical basis of ascorbic acid-mediated thermotolerance and its potential application for sustaining wheat productivity under warming climates.

2. Materials and Methods

2.1 Experimental Site and Growth Conditions

The field experiment was conducted during the Rabi season at the Agricultural Research Farm, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India (25°18' N, 83°03' E; 75.7 m AMSL). The region has a subtropical climate characterized by cool winters and hot summers, with an average annual rainfall of ~1100 mm. Timely-sown (normal) and late-sown conditions were used to impose natural heat stress during the reproductive phase. Meteorological

data recorded during the crop season confirmed significantly higher temperatures during grain filling in the late-sown crop.

2.2 Plant Material and Experimental Design

Two contrasting wheat genotypes were selected: HUW-510 (heat-tolerant) and HUW-468 (heat-susceptible). The experiment was laid out in a randomized block design (RBD) with three replications. Plants were grown under recommended agronomic practices to ensure uniform crop establishment.

Commented [P1]: Could elaborate the RBD design. Weather the growth was in field or in lab condition in pots needs to be explained.

2.3 Treatments, Foliar Application of L-Ascorbic Acid, and Sampling Stages

Four treatments were imposed: T1: Control (water spray with 0.1% Tween-20 surfactant), T2: 1.0 mM L-ascorbic acid, T3: 5.0 mM L-ascorbic acid, and T4: 10.0 mM L-ascorbic acid. L-ascorbic acid solutions were prepared fresh using analytical grade Vit-C dissolved in distilled water and 0.1% Tween-20 was added as a surfactant to ensure uniform leaf wetting. Foliar sprays were applied twice at 45 days after sowing (DAS) and 65 DAS covering the entire plant canopy uniformly.

Leaf samples were collected at two critical growth stages representing distinct physiological and stress phases: 65 DAS (vegetative–pre-reproductive stage) and 85 DAS (reproductive grain filling stage) the latter corresponding to peak heat stress conditions in late-sown crops. Samples were collected from the uppermost fully expanded leaves, immediately frozen in liquid nitrogen and stored at -80°C for subsequent biochemical and antioxidant analyses.

2.4 Biochemical and Physiological Measurements

2.4.1 Chlorophyll Content

Chlorophyll content was measured using a SPAD-502 chlorophyll meter (Konica Minolta, Japan). Readings were recorded as SPAD units and converted to chlorophyll concentration (mg g^{-1} FW) using crop-specific calibration equations described by Jat *et al.* (2024).

2.4.2 Nitrate Reductase Activity (NRA)

Nitrate reductase activity was estimated using the *in vivo* assay described by Jaworski (1971). Fresh leaf discs (0.5 g) were incubated in phosphate buffer (pH 7.4) containing 0.1 M KNO_3 and 1% n-propanol at 30°C in darkness. The reaction was terminated by boiling, and nitrite accumulation was quantified calorimetrically at 540 nm using sulphanilamide and N-1-naphthyl ethylenediamine (NED). Results were expressed as $\text{nM NO}_2 \text{ h}^{-1} \text{ g}^{-1}$ FW.

2.4.3 Soluble Protein Content

Total soluble protein content was determined using the Lowry method (Lowry *et al.*, 1951). Absorbance was measured at 660 nm and protein concentration was calculated using bovine serum albumin (BSA) as the standard. Values were expressed as mg protein g^{-1} FW.

2.4.4 Proline Content

Proline accumulation was quantified following the method of Bates *et al.* (1973). Fresh leaf tissue (0.5 g) was homogenized in 3% sulfosalicylic acid, and the extract was reacted with acid ninhydrin. The chromophore was extracted in toluene and absorbance recorded at 520 nm. Proline concentration was expressed as $\mu\text{mol g}^{-1}$ FW.

2.4.5 Soluble Sugar Content

Soluble sugars were estimated using the anthrone method (Hansen & Møller, 1975). Ethanol extracts were treated with anthrone reagent in concentrated sulfuric acid, heated in a boiling water bath and absorbance measured at 620 nm. Results were expressed as mg g^{-1} FW.

2.5 Antioxidant Enzyme Assays

2.5.1 Superoxide Dismutase (SOD; EC 1.15.1.1)

SOD activity was determined based on inhibition of nitro blue tetrazolium (NBT) photoreduction, following the method of Dhindsa and Matowe (1981). One unit of SOD activity represented 50% inhibition of NBT reduction. Results were expressed as Units mg^{-1} protein.

2.5.2 Catalase (CAT; EC 1.11.1.6)

CAT activity was measured by monitoring the rate of H_2O_2 decomposition at 240 nm, according to Janda *et al.* (1999). Enzyme activity was expressed as Units mg^{-1} protein min^{-1} .

2.5.3 Ascorbate Peroxidase (APX; EC 1.11.1.11)

APX activity was estimated following Nakano and Asada (1981) by measuring the decline in absorbance at 290 nm due to ascorbate oxidation, using an extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. Values were expressed as Units mg^{-1} protein.

2.6 Measurement of ROS Content

ROS content was quantified using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay (Able *et al.*, 1998), with modifications. Absorbance was recorded at 470 nm and corrected for background chlorophyll interference. ROS concentration was calculated using an extinction coefficient of $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. This method is widely used for quantifying ROS generation in plant tissues under abiotic stress (Wang & Joseph, 1999).

2.8 Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) in a randomized block design. Treatment means were compared using Duncan's Multiple Range Test (DMRT) or LSD at $\mathbf{p} \leq 0.05$. All biochemical assays were performed in triplicate, and results were expressed as mean \pm SE.

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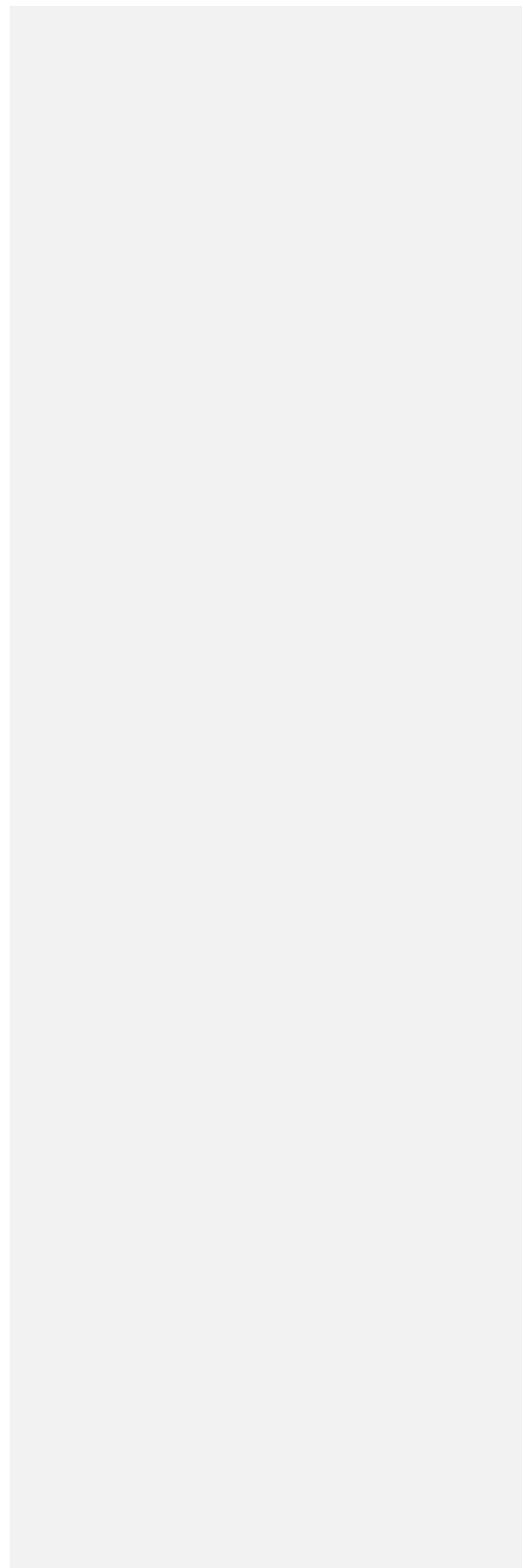


Table 1. Effect of L-Ascorbic Acid (Vit-C) foliar treatments on total chlorophyll content, nitrate reductase activity, soluble protein content and soluble sugar content in wheat (*Triticum aestivum* L.) under early and late sowing conditions at 65 DAS.

TREATMENTS	Total Chlorophyll Content (mg g ⁻¹ FW)			Nitrate Reductase Activity (nM h ⁻¹ g ⁻¹ FW)			Soluble Protein Content (mg g ⁻¹ FW)			Soluble Sugar Content (mg g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	35.28	27.03	31.15	46.40	34.60	40.50	3.98	2.34	3.16	3.34	1.14	2.24
T2 - 1.0 mM AA	36.73	28.17	32.45	65.05	43.25	54.15	5.20	3.02	4.11	4.08	1.99	3.03
T3 - 5.0 mM AA	37.78	29.19	33.48	69.90	52.05	60.98	6.49	3.46	4.98	4.97	2.39	3.68
T4 - 10.0 mM AA	39.32	30.72	35.02	75.75	55.15	65.45	6.68	4.02	5.35	5.47	3.23	4.35
MEAN	37.27	28.78		64.28	46.26		5.59	3.21		4.46	2.18	
	T	S	TXS	T	S	TXS	T	S	TXS	T	S	TXS
CD	0.508	0.719	NS	0.637	0.901	1.274	0.063	0.089	0.125	0.050	0.070	0.099
SE(d)	0.235	0.332	0.469	0.294	0.416	0.588	0.029	0.041	0.058	0.023	0.032	0.046
SE(m)	0.166	0.235	0.332	0.208	0.294	0.416	0.020	0.029	0.041	0.016	0.023	0.032
<i>p-value</i>	< 0.001	<0.001	0.945	< 0.001	<0.001	<0.001	< 0.001	<0.001	<0.001	< 0.001	<0.001	<0.001

Table 2. Effect of L-Ascorbic Acid (Vit-C) foliar treatments on reactive oxygen species (ROS), antioxidant enzyme activities (APX, SOD, CAT) and proline content in wheat (*Triticum aestivum* L.) under early and late sowing conditions at 65 DAS.

TREATMENTS	ROS (μmol g ⁻¹ FW)			APX (Units mg ⁻¹ protein min ⁻¹)			SOD (Units mg ⁻¹ protein min ⁻¹)			CAT (Units mg ⁻¹ protein min ⁻¹)			Proline Content (μmol g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	6.64	8.02	7.33	0.292	0.343	0.317	0.992	1.213	1.103	2.78	2.90	2.84	0.513	0.932	0.723
T2 - 1.0 mM AA	5.98	6.85	6.41	0.369	0.440	0.405	1.545	1.847	1.696	3.01	2.99	3.00	0.618	1.160	0.889
T3 - 5.0 mM AA	5.32	6.28	5.80	0.433	0.526	0.479	1.753	1.998	1.876	3.08	3.15	3.11	0.735	1.304	1.020
T4 - 10.0 mM AA	4.61	5.45	5.03	0.504	0.642	0.573	2.053	2.305	2.179	4.35	4.42	4.38	0.890	1.472	1.181
MEAN	5.63	6.65		0.400	0.488		1.586	1.841		3.30	3.36		0.689	1.217	
	T	S	TXS	T	S	TXS	T	S	TXS	T	S	TXS	T	S	TXS
CD	0.088	0.125	0.176	0.008	0.011	0.015	0.021	0.030	NS	0.056	0.080	N/A	0.016	0.023	0.033
SE(d)	0.041	0.058	0.081	0.004	0.005	0.007	0.010	0.014	0.020	0.026	0.037	0.052	0.008	0.011	0.015
SE(m)	0.029	0.041	0.058	0.003	0.004	0.005	0.007	0.010	0.014	0.018	0.026	0.037	0.005	0.008	0.011
<i>p-value</i>	< 0.001	<0.001	0.001	< 0.001	<0.001	<0.001	< 0.001	<0.001	0.072	0.034	<0.001	0.272	< 0.001	<0.001	<0.001

*CD = Critical Difference, SE(d) = Standard Error of Difference, SE(m) = Standard Error of Mean, T = Treatment effect, S = Sowing time effect, and T×S = Treatment × Sowing time interaction.

Table 3. Effect of L-Ascorbic Acid (Vit-C) foliar treatments on total chlorophyll content, nitrate reductase activity, soluble protein content and soluble sugar content in wheat (*Triticum aestivum* L.) under early and late sowing conditions at 85 DAS.

TREATMENTS	Total Chlorophyll Content (mg g ⁻¹ FW)			Nitrate Reductase Activity (nM h ⁻¹ g ⁻¹ FW)			Soluble Protein Content (mg g ⁻¹ FW)			Soluble Sugar Content (mg g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	37.67	28.67	33.17	53.50	38.90	46.20	4.27	2.51	3.39	3.68	1.38	2.53
T2 - 1.0 mM AA	39.22	29.74	34.48	68.40	48.90	58.65	5.47	3.26	4.37	4.72	2.18	3.45
T3 - 5.0 mM AA	40.79	30.78	35.78	76.50	55.85	66.18	6.57	3.69	5.13	5.36	2.55	3.96
T4 - 10.0 mM AA	42.33	32.34	37.33	79.90	61.10	70.50	7.01	4.32	5.66	5.70	2.88	4.29
MEAN	40.00	30.38		69.58	51.19		5.83	3.44		4.86	2.25	
	T	S	TXS	T	S	TXS	T	S	TXS	T	S	TXS
CD	0.334	0.472	NS	0.720	1.018	1.439	0.067	0.095	0.135	0.041	0.058	0.083
SE(d)	0.154	0.218	0.308	0.332	0.470	0.665	0.031	0.044	0.062	0.019	0.027	0.038
SE(m)	0.109	0.154	0.218	0.235	0.332	0.470	0.022	0.031	0.044	0.014	0.019	0.027
p-value	< 0.001	< 0.001	0.106	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Table 4. Effect of L-Ascorbic Acid (Vit-C) foliar treatments on reactive oxygen species (ROS), antioxidant enzyme activities (APX, SOD, CAT) and proline content in wheat (*Triticum aestivum* L.) under early and late sowing conditions at 85 DAS.

TREATMENTS	ROS (μmol g ⁻¹ FW)			APX (Units mg ⁻¹ protein min ⁻¹)			SOD (Units mg ⁻¹ protein min ⁻¹)			CAT (Units mg ⁻¹ protein min ⁻¹)			Proline Content (μmol g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	7.82	8.93	8.37	0.315	0.394	0.355	1.102	1.397	1.249	3.35	3.66	3.50	0.627	1.027	0.827
T2 - 1.0 mM AA	7.08	7.30	7.19	0.401	0.475	0.438	1.690	1.992	1.841	3.92	4.46	4.19	0.724	1.278	1.001
T3 - 5.0 mM AA	6.22	6.31	6.26	0.457	0.551	0.504	1.868	2.252	2.060	4.28	4.41	4.35	0.833	1.460	1.147
T4 - 10.0 mM AA	5.37	5.60	5.49	0.538	0.667	0.602	2.190	2.512	2.351	4.70	5.22	4.96	1.040	1.663	1.352
MEAN	6.62	7.03		0.428	0.522		1.713	2.038		4.06	4.44		0.806	1.357	
	T	S	TXS	T	S	TXS	T	S	TXS	T	S	TXS	T	S	TXS
CD	0.164	0.232	0.329	0.009	0.012	0.018	0.039	0.055	NS	0.085	0.120	0.170	0.017	0.024	0.034
SE(d)	0.076	0.107	0.152	0.004	0.006	0.008	0.018	0.025	0.036	0.039	0.055	0.078	0.008	0.011	0.016
SE(m)	0.054	0.076	0.107	0.003	0.004	0.006	0.013	0.018	0.025	0.028	0.039	0.055	0.006	0.008	0.011
p-value	< 0.001	< 0.001	0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	0.326	< 0.001	< 0.001	0.007	< 0.001	< 0.001	< 0.001

*CD = Critical Difference, SE(d) = Standard Error of Difference, SE(m) = Standard Error of Mean, T = Treatment effect, S = Sowing time effect, and T×S = Treatment × Sowing time interaction.

Table 5. Comparative response of wheat (*Triticum aestivum* L.) genotypes HUW-468 and HUW-510 to foliar-applied L-Ascorbic Acid (Vit-C) in terms of chlorophyll content, NR activity, soluble protein and soluble sugar content under early and late sowing conditions at 65 DAS.

TREATMENTS	Total Chlorophyll Content (mg g ⁻¹ FW)			Nitrate Reductase Activity (nM h ⁻¹ g ⁻¹ FW)			Soluble Protein Content (mg g ⁻¹ FW)			Soluble Sugar Content (mg g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	29.53	32.78	31.15	37.00	44.00	40.50	2.55	3.77	3.16	1.65	2.83	2.24
T2 - 1.0 mM AA	30.74	34.16	32.45	50.40	57.90	54.15	3.41	4.81	4.11	2.38	3.68	3.03
T3 - 5.0 mM AA	31.78	35.19	33.48	56.00	65.95	60.98	4.59	5.36	4.98	2.82	4.54	3.68
T4 - 10.0 mM AA	32.82	37.22	35.02	60.75	70.15	65.45	4.89	5.82	5.35	3.82	4.88	4.35
MEAN	31.22	34.83		51.04	59.50		3.86	4.94		2.67	3.98	
	T	G	TXG	T	G	TXG	T	G	TXG	T	G	TXG
CD	0.648	0.916	NS	0.991	1.402	NS	0.090	0.127	0.180	0.053	0.075	0.107
SE(d)	0.299	0.423	0.598	0.458	0.647	0.916	0.041	0.059	0.083	0.025	0.035	0.049
SE(m)	0.212	0.299	0.423	0.324	0.458	0.647	0.029	0.041	0.059	0.017	0.025	0.035
p-value	< 0.001	< 0.001	0.531	< 0.001	< 0.001	0.107	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Table 6. Comparative response of wheat (*Triticum aestivum* L.) to foliar-applied L-Ascorbic Acid (Vit-C) in terms of reactive oxygen species (ROS), antioxidant enzyme activities (APX, SOD, CAT) and proline content under early and late sowing conditions at 65 DAS.

TREATMENTS	ROS (μmol g ⁻¹ FW)			APX (Units mg ⁻¹ protein min ⁻¹)			SOD (Units mg ⁻¹ protein min ⁻¹)			CAT (Units mg ⁻¹ protein min ⁻¹)			Proline Content (μmol g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	7.75	6.90	7.33	0.237	0.397	0.317	0.802	1.403	1.103	2.60	3.08	2.84	0.813	0.632	0.723
T2 - 1.0 mM AA	7.00	5.83	6.41	0.280	0.530	0.405	1.400	1.992	1.696	2.78	3.22	3.00	1.005	0.773	0.889
T3 - 5.0 mM AA	6.38	5.22	5.80	0.334	0.625	0.479	1.555	2.197	1.876	2.90	3.33	3.11	1.128	0.912	1.020
T4 - 10.0 mM AA	5.48	4.58	5.03	0.405	0.741	0.573	1.854	2.503	2.179	3.47	5.30	4.38	1.318	1.044	1.181
MEAN	6.65	5.63		0.314	0.573		1.403	2.024		2.94	3.73		1.066	0.840	
	T	G	TXG	T	G	TXG	T	G	TXG	T	G	TXG	T	G	TXG
CD	0.072	0.101	0.143	0.007	0.010	0.015	0.030	0.042	NS	0.043	0.060	0.086	0.019	0.026	0.037
SE(d)	0.033	0.047	0.066	0.003	0.005	0.007	0.014	0.019	0.027	0.020	0.028	0.039	0.009	0.012	0.017
SE(m)	0.023	0.033	0.047	0.002	0.003	0.005	0.010	0.014	0.019	0.014	0.020	0.028	0.006	0.009	0.012
p-value	< 0.001	< 0.001	0.006	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.388	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.014

*CD = Critical Difference, G= Genotype, SE(d)= Standard Error of Difference, SE(m) = Standard Error of Mean, T = Treatment effect, S = Sowing effect, and T×G = Treatment × Genotype interaction.

Table 7. Comparative response of wheat (*Triticum aestivum* L.) genotypes HUW-468 and HUW-510 to foliar-applied L-Ascorbic Acid (Vit-C) in terms of chlorophyll content, NR activity, soluble protein and soluble sugar content under early and late sowing conditions at 85 DAS.

TREATMENTS	Total Chlorophyll Content (mg g ⁻¹ FW)			Nitrate Reductase Activity (nM h ⁻¹ g ⁻¹ FW)			Soluble Protein Content (mg g ⁻¹ FW)			Soluble Sugar Content (mg g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	31.15	35.18	33.17	40.05	52.35	46.20	2.70	4.08	3.39	1.89	3.17	2.53
T2 - 1.0 mMAA	32.24	36.72	34.48	52.90	64.40	58.65	3.66	5.07	4.37	2.52	4.38	3.45
T3 - 5.0 mMAA	33.79	37.78	35.78	61.45	70.90	66.18	4.67	5.59	5.13	3.00	4.92	3.96
T4 - 10.0 mMAA	34.85	39.82	37.33	66.60	74.40	70.50	5.18	6.15	5.66	4.04	4.55	4.29
MEAN	33.01	37.37		55.25	65.51		4.05	5.22		2.86	4.25	
	T	G	TXG	T	G	TXG	T	G	TXG	T	G	TXG
CD	0.367	0.519	NS	0.782	1.106	1.564	0.075	0.106	0.149	0.062	0.087	0.123
SE(d)	0.170	0.240	0.339	0.361	0.511	0.722	0.035	0.049	0.069	0.028	0.040	0.057
SE(m)	0.120	0.170	0.240	0.255	0.361	0.511	0.024	0.035	0.049	0.020	0.028	0.040
<i>p-value</i>	< 0.001	< 0.001	0.189	< 0.001	< 0.001	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Table 8. Comparative response of wheat (*Triticum aestivum* L.) to foliar-applied L-Ascorbic Acid (Vit-C) in terms of reactive oxygen species (ROS), antioxidant enzyme activities (APX, SOD, CAT) and proline content under early and late sowing conditions at 85 DAS.

TREATMENTS	ROS (μmol g ⁻¹ FW)			APX (Units mg ⁻¹ protein min ⁻¹)			SOD (Units mg ⁻¹ protein min ⁻¹)			CAT (Units mg ⁻¹ protein min ⁻¹)			Proline Content (μmol g ⁻¹ FW)		
	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN	EARLY	LATE	MEAN
T1 - Control	8.86	7.88	8.37	0.270	0.440	0.355	0.957	1.542	1.249	3.50	3.51	3.50	0.915	0.739	0.827
T2 - 1.0 mMAA	7.57	6.81	7.19	0.316	0.561	0.438	1.540	2.142	1.841	3.77	4.61	4.19	1.134	0.868	1.001
T3 - 5.0 mMAA	6.55	5.98	6.26	0.362	0.646	0.504	1.725	2.395	2.060	4.08	4.62	4.35	1.270	1.023	1.147
T4 - 10.0 mMAA	5.75	5.22	5.49	0.443	0.762	0.602	2.000	2.702	2.351	4.37	5.55	4.96	1.468	1.235	1.352
MEAN	7.18	6.47	6.348	0.602	0.602	0.602	1.555	2.195	1.555	3.93	4.57	4.19	1.197	0.966	1.197
	T	G	TXG	T	G	TXG	T	G	TXG	T	G	TXG	T	G	TXG
CD	0.115	0.163	0.230	0.009	0.013	0.019	0.033	0.047	NS	0.072	0.102	0.144	0.019	0.027	0.038
SE(d)	0.053	0.075	0.106	0.004	0.006	0.009	0.015	0.022	0.030	0.033	0.047	0.066	0.009	0.013	0.018
SE(m)	0.038	0.053	0.075	0.003	0.004	0.006	0.011	0.015	0.022	0.024	0.033	0.047	0.006	0.009	0.013
<i>p-value</i>	< 0.001	< 0.001	0.037	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.051	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

*CD = Critical Difference, G= Genotype, SE(d) = Standard Error of Difference, SE(m) = Standard Error of Mean, T = Treatment effect, S = Sowing effect, and T×G = Treatment × Genotype interaction.

4. Results and Discussion

4.1 Reactive Oxygen Species (ROS) Accumulation

Late-sown heat stress caused a pronounced elevation of ROS accumulation in both genotypes. The susceptible HUW-468 showed a rise of approximately 45–55%, while HUW-510 exhibited a more moderate increase of about 35–40%. Such heat-induced ROS surges correspond with recent findings indicating that heat stress disrupts photosynthetic electron transport and enhances photorespiration, thus driving ROS overproduction in wheat (Suresh *et al.*, 2025c; Abidin *et al.*, 2024). Foliar application of L-ascorbic acid significantly suppressed ROS levels with 10.0 mM AA reducing ROS by about 28–35% compared to untreated stressed plants. This mitigation mirrors observations of antioxidant supplementation reducing ROS under heat stress in wheat and other cereals (Khazada *et al.*, 2024; Yadav *et al.*, 2024).

4.2 Antioxidant Enzyme Activities (SOD, CAT, APX)

Heat stress substantially lowered the activities of SOD, CAT and APX in both genotypes, with declines around 20–30% in HUW-468 and 10–18% in HUW-510. This suppression aligns with recent reviews showing that elevated temperature down-regulates antioxidant systems and compromises redox homeostasis in wheat (Mahajan *et al.*, 2025). Application of L-ascorbic acid restored enzyme activities markedly: SOD increased by 35–48%, CAT by 30–45% and APX by 40–50% under 10.0 mM AA compared to stressed control. Enhanced antioxidant enzyme function following AA application supports its role in upstream ROS detoxification and stabilization of the ASC–GSH cycle (Abidin *et al.*, 2024).

4.3 Chlorophyll Content

Heat-induced chlorophyll losses ranged between 20–30% with HUW-468 showing reductions of ~28–30% and HUW-510 ~20–22%. These losses reflect membrane disintegration and pigment degradation commonly reported under elevated temperature in wheat (Yadav *et al.*, 2024). L-ascorbic acid increased chlorophyll retention by 18–25% particularly under 10.0 mM AA. Similar improvements under antioxidant treatment have been reported elsewhere highlighting AA's protective role on photosynthetic apparatus (Abidin *et al.*, 2024).

4.4 Nitrate Reductase (NR) Activity

Heat stress reduced NR activity by 18–28% in late-sown plants, especially in HUW-468. Elevated temperatures inhibit NR by altering enzyme stability and substrate availability (Mahajan *et al.*, 2025; Suresh *et al.*, 2025b). Foliar AA application increased NR activity by 22–30% at 10 mM; this improvement aligns with reports of antioxidant amelioration of nitrogen metabolism under abiotic stress (Khazada *et al.*, 2024).

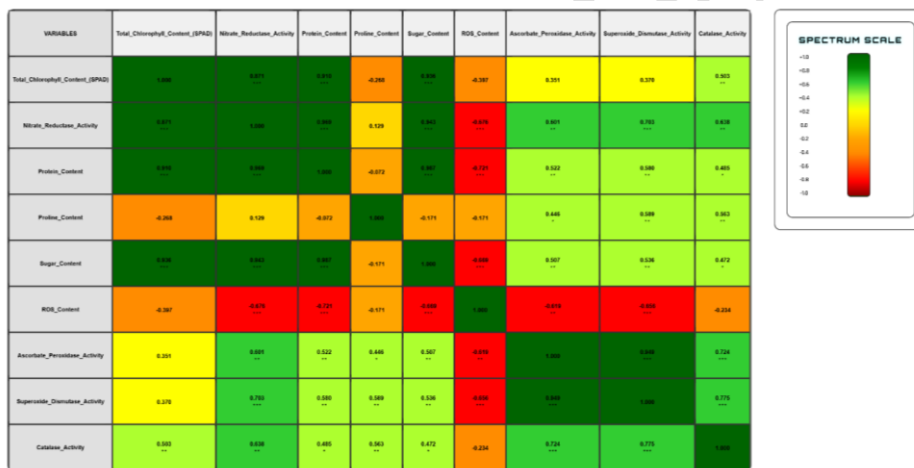
4.5 Soluble Protein Content

Protein content declined by 25–35% under heat stress reflecting oxidative denaturation and inhibited synthesis. HUW-468 lost more protein than HUW-510. AA application restored protein levels by 18–30% consistent with studies showing AA’s stabilization of proteins and reduction of heat-induced proteolysis (Yadav *et al.*, 2024).

4.6 Proline and Soluble Sugars

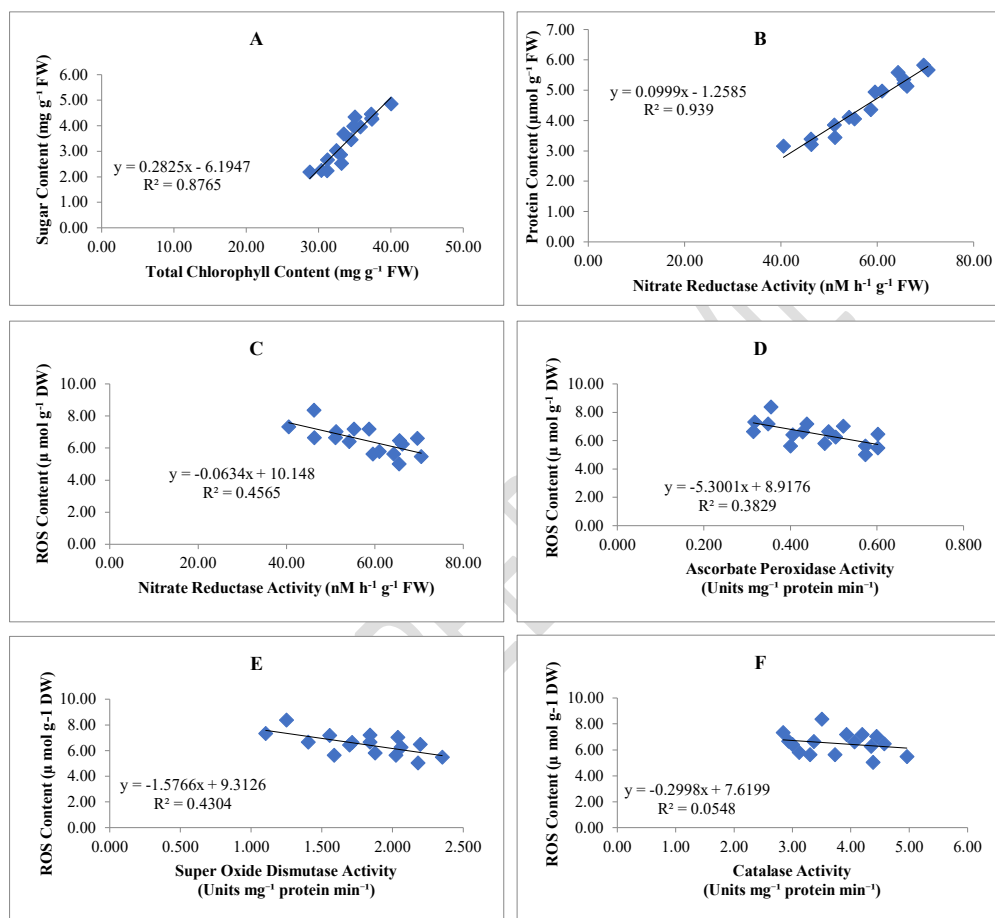
Osmolyte accumulation increased under heat stress: proline by 40–55%, soluble sugars by 30–45% with greater accumulation in HUW-468. Exogenous AA further enhanced osmolytes by 10–20% supporting osmotic adjustment and cellular protection. Recent reviews confirm that heat-tolerant wheat genotypes often show higher osmolyte accumulation under antioxidant treatments (Mahajan *et al.*, 2025).

Fig. 1. Correlation matrix among biochemical traits and antioxidant enzyme activities in wheat under L-Ascorbic Acid (Vit-C) foliar treatments



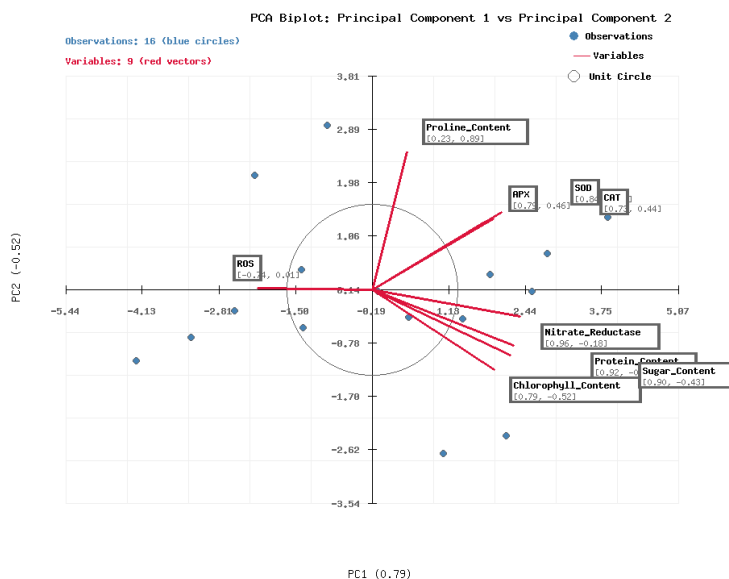
The correlation matrix revealed clear physiological interrelationships among the measured traits. Total chlorophyll content exhibited a strong positive association with nitrate reductase activity, protein content and sugar content reflecting coordinated enhancement of photosynthetic capacity and nitrogen assimilation. In contrast, ROS content demonstrated negative correlations with most growth promoting biochemical parameters indicating oxidative stress as a limiting factor for cellular functioning. Antioxidant enzymes such as SOD, APX and CAT showed moderate associations with ROS levels suggesting a compensatory detoxification response to counteract oxidative damage. These patterns collectively highlight the integrated metabolic adjustments induced across treatments supporting the role of L-Ascorbic Acid in strengthening biochemical efficiency and redox homeostasis in wheat.

Fig. 2. Regression relationships among biochemical traits and ROS content in wheat (*Triticum aestivum* L.) under L-Ascorbic Acid (Vit-C) foliar treatments



Regression analysis showed a positive relationship between Total Chlorophyll Content and Sugar Content (A) and between Nitrate Reductase Activity and Protein Content (B). ROS Content shown negative correlations with Nitrate Reductase Activity (C), Ascorbate Peroxidase Activity (D), Superoxide Dismutase Activity (E) and Catalase Activity (F) indicating that increased antioxidant enzyme activities reduced oxidative stress. Each point represents mean (\pm SD) of three replicates. Regression equations and R^2 values indicate that L-Ascorbic Acid enhances antioxidant defense and suppresses ROS accumulation.

Fig. 3. Principal Component Analysis (PCA) biplot of biochemical traits and antioxidant enzyme activities in wheat (*Triticum aestivum* L.) under L-Ascorbic Acid (Vit-C) foliar treatments.



Principal Component Analysis effectively reduced the dimensionality of the dataset and captured the major variance patterns across treatments. The first two components accounted for most of the variability, driven primarily by chlorophyll, protein, sugar content, and nitrate reductase activity loading strongly and positively on PC1 (0.79). ROS content loaded in the opposite direction, emphasizing its antagonistic influence on physiological traits. Antioxidant enzyme variables contributed mainly to PC2 (-0.52), indicating a distinct biochemical axis reflecting stress-response capacity. The PCA biplot thus clearly separated high-performance and stress-responsive profiles, offering a concise multivariate interpretation of the dataset.

5. Conclusion

Late-sown heat stress substantially impaired biochemical stability and redox homeostasis in wheat, as reflected by increased ROS accumulation, reduced antioxidant enzyme activities, chlorophyll degradation, inhibited nitrate reductase activity and loss of soluble proteins. These effects were

more severe in the heat-susceptible HUW-468, while the tolerant HUW-510 maintained comparatively better metabolic function. Foliar application of L-ascorbic acid effectively mitigated these adverse effects with the 10 mM concentration exhibiting the strongest protective response. Ascorbic acid significantly reduced ROS, restored SOD, CAT and APX activities, enhanced chlorophyll retention, improved NR and protein levels, and promoted osmolyte accumulation (proline and soluble sugars). These improvements collectively strengthened membrane stability and cellular hydration contributing to enhanced physiological resilience under heat stress. The stronger positive response of HUW-510 to ascorbic acid highlights genotype-specific efficiency in utilizing exogenous antioxidants for redox regulation and metabolic protection. Overall, the findings demonstrate that foliar L-ascorbic acid is an effective, low-cost and eco-friendly approach for enhancing thermotolerance in wheat supporting its potential application in regions increasingly affected by rising temperatures.

Abbreviations

Abbreviation	Full Form
AA	L-Ascorbic Acid / Vitamin-C
APX	Ascorbate Peroxidase
CAT	Catalase
SOD	Superoxide Dismutase
ROS	Reactive Oxygen Species
NR	Nitrate Reductase
MSI	Membrane Stability Index
RWC	Relative Water Content
FW	Fresh Weight
DW	Dry Weight
DAS	Days After Sowing
T1, T2, T3, T4	Foliar Treatment Levels

References

- 1 Abidin, Z. U., Mahmood, A., Alawadi, H. F., Ullah, M. S., Shahid, A., Khan, B. A., Al-Khayri, J. M., Aldaj, M. I., Al-Dossary, O., Alsubaie, B., Shehata, W. F., & Rezk, A. A. (2024). Heat stress responses and mitigation strategies in wheat: an updated and comprehensive review. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 52(3), 13636. <https://doi.org/10.15835/nbha52313636>
- 2 Akram, N. A., Shafiq, F., & Ashraf, M. (2017). Ascorbic Acid-A potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Frontiers in Plant Science* <https://doi.org/10.3389/fpls.2017.00613>
- 3 Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil*, 39(1), 205–207. <https://doi.org/10.1007/bf00018060>
- 4 Chen, X., Han, H., Cong, Y., Li, X., Zhang, W., Wan, W., Cui, J., Xu, W., Diao, M., & Liu, H. (2023). The Protective Effect of Exogenous Ascorbic Acid on Photosystem Inhibition of Tomato Seedlings Induced by Salt Stress. *Plants*. <https://doi.org/10.3390/plants12061379>
- 5 Dadarwal, B. K., Yadav, N., Chauhan, J., & Singhal, R. K. (2025). Plant Growth and Development Under High Night Temperatures. In R. K. Singhal, R. Bheemanahalli, S. Pandey, & M. D. Pratibha (Eds.), *Impact of High Night Temperature on Plant Biology* (pp. 31-50). Apple Academic Press. <https://doi.org/10.1201/9781003503415>
- 6 Dadrwal, B. K. (2022). Effect of elevated fluoride levels on hydrolysis of starch during seed germination and alteration in antioxidant system in wheat *Triticum aestivum* L plants. Department of Physiology, Banaras Hindu University, Varanasi (Doctoral dissertation,)
- 7 Dadrwal, B. K., Bagdi, D. L., Kakralya, B. L., & Sharma, M. K. (2022). Foliar treatment with ascobin reduces the adverse effects of salt stress on physiological and biochemical parameters in wheat. *The Pharma Innovation Journal*, 11(5), 2117-2120. <https://www.thepharmajournal.com/archives/2022/vol11issue5/PartAC/11-5-100-700.pdf>
- 8 Dadrwal, B. K., Bagdi, D. L., Kakralya, B. L., & Sharma, M. K. (2022). Alleviation of adverse effects of salt stress on growth, yield and yield attributes in wheat by foliar treatment with ascobin. *The Pharma Innovation Journal*, 11(5), 2112-2116. <https://www.thepharmajournal.com/archives/2022/vol11issue5/PartAD/11-5-10-800.pdf>
- 9 Das, K., & Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science* <https://doi.org/10.3389/fenvs.2014.00053>
- 10 De Queiroz, A. R., Hines, C., Brown, J., Sahay, S., Vijayan, J., Stone, J. M., Bickford, N., Wuellner, M., Glowacka, K., Buan, N. R., & Roston, R. L. (2023). The effects of exogenously applied antioxidants on plant growth and resilience. *Phytochemistry Reviews*, 22(2), 407–447. <https://doi.org/10.1007/s11101-023-09862-3>
- 11 Djanaguiraman, M., Narayanan, S., Erdayani, E., & Prasad, P. V. V. (2020). Effects of high temperature stress during anthesis and grain filling periods on photosynthesis, lipids and grain yield in wheat. *BMC Plant Biology*, 20(1), 268. <https://doi.org/10.1186/s12870-020-02479-0>

- 12 Gallie, D. R. (2013). L-Ascorbic acid: a multifunctional molecule supporting plant growth and development. *Scientifica* <https://doi.org/10.1155/2013/795964>
- 13 Hansen, J., & Møller, I. (1975). Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. *Analytical Biochemistry*, 68(1), 87–94. [https://doi.org/10.1016/0003-2697\(75\)90682-x](https://doi.org/10.1016/0003-2697(75)90682-x)
- 14 He, Y., & Huang, B. (2010). Differential responses to heat stress in activities and isozymes of four antioxidant enzymes for two cultivars of Kentucky bluegrass contrasting in heat tolerance. *Journal of the American Society for Horticultural Science*, 135(2), 116–124. <https://doi.org/10.21273/jashs.135.2.116>
- 15 Jat, M., Ray, M., Ahmad, M. A., & Prakash, P. (2024). Unravelling the photosynthetic dynamics and fluorescence parameters under ameliorative effects of 24-epibrassinolide in wheat (*Triticum aestivum* L.) grown under heat stress regime. *Scientific Reports*. <https://doi.org/10.1038/s41598-024-79676-6>
- 16 Jaworski, E. G. (1971). Nitrate reductase assay in intact plant tissues. *Biochemical and Biophysical Research Communications*, 43(6), 1274–1279. [https://doi.org/10.1016/s0006-291x\(71\)80010-4](https://doi.org/10.1016/s0006-291x(71)80010-4)
- 17 Khanzada, A., Wang, X., Malko, M. M., Wu, Y., Samo, A., & Dong, J. (2024). Response of the persistence of heat stress tolerance in winter wheat seedling to heat priming at early growth stages. *Plant Stress*, 11, 100323. <https://doi.org/10.1016/j.stress.2023.100323>
- 18 Kumar, A., Muthuramalingam, P., Kumar, R., Tiwari, S., Verma, L., Park, S., & Shin, H. (2025). Adapting crops to rising temperatures: Understanding heat stress and plant resilience mechanisms. *International Journal of Molecular Sciences*. <https://doi.org/10.3390/ijms262110426>
- 19 Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193(1), 265–275. [https://doi.org/10.1016/s0021-9258\(19\)52451-6](https://doi.org/10.1016/s0021-9258(19)52451-6)
- 20 Mahajan, S., Thakur, P., Das, S., Sharma, R. P., Manuja, S., Jha, P. K., Saini, A., Sahoo, C., & Fayeziadeh, M. R. (2025). Impression of contemporary heat stress complexities in agricultural crops: a review. *Plant Growth Regulation*. <https://doi.org/10.1007/s10725-025-01382-8>
- 21 Mamatkulova, G. F., Erjigitov, D. S. H., Mukhammadiev, O. A., Sokiboyeva, D. B., Kholova, M. D., Oripova, B. B., Ernazarova, D. K., Baboev, S. K., Turaev, O. S., & Kushanov, F. N. (2025). Assessment of heat tolerance in wheat (*Triticum aestivum* L.) at the seedling stage. *SABRAO Journal of Breeding and Genetics*, 57(3), 1041–1049. <https://doi.org/10.54910/sabrao2025.57.3.16>
- 22 Pratap, A., Taylor, N. L., Pal, M., Chinnusamy, V., & Siddique, K. H. M. (2025). Heat tolerance mechanisms in bread wheat: Insights from flag leaves and spike tissues. *Plant Stress*, 16, 100876. <https://doi.org/10.1016/j.stress.2025.100876>
- 23 Rao, M. J., Duan, M., Zhou, C., Jiao, J., Cheng, P., Yang, L., Wei, W., Shen, Q., Ji, P., Yang, Y., Conteh, O., Yan, D., Yuan, H., Rauf, A., Ai, J., & Zheng, B. (2025). Antioxidant defense system in plants: reactive oxygen species production, signaling, and scavenging during abiotic

Stress-Induced oxidative damage. Horticulturae, 11(5), 477
<https://doi.org/10.3390/horticulturae11050477>

- 24 Riaz, M. W., Yang, L., Yousaf, M. I., Sami, A., Mei, X. D., Shah, L., Rehman, S., Xue, L., Si, H., & Ma, C. (2021). Effects of Heat Stress on Growth, Physiology of Plants, Yield and Grain Quality of Different Spring Wheat (*Triticum aestivum* L.) Genotypes. *Sustainability*, 13(5), 2972. <https://doi.org/10.3390/su13052972>
- 25 Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany* <https://doi.org/10.1155/2012/217037>
- 26 Suresh, G., Sharma, P., Prakash, P., Dadrwal, B. K., Kumari, A., & Teja, M. S. V. R. (2025). Exogenous 24-Epibrassinolide (24-EBL) Enhances Morpho-physiological Traits in Wheat (*Triticum aestivum* L.) Genotypes under High Temperature Stress. *Journal of Advances in Biology & Biotechnology*, 28(10), 1499–1510. <https://doi.org/10.9734/jabb/2025/v28i103165>
- 27 Suresh, G., Sharma, P., Prakash, P., Dadrwal, B. K., Kumari, A., & Teja, M. S. V. R. (2025b). 24-Epibrassinolide (24-EBL) Enhances Antioxidant Defense to Mitigate Heat-induced Oxidative Stress in wheat (*Triticum aestivum* L.). *Journal of Scientific Research and Reports*, 31(10), 805–816. <https://doi.org/10.9734/jsrr/2025/v31i103625>
- 28 Suresh, G., Sharma, P., Prakash, P., Dadrwal, B. K., Kumari, A., & Teja, M. S. V. R. (2025). 24-Epibrassinolide (24-EBL) Mediated Regulation of Chlorophyll, Protein and Osmolyte Metabolism in Wheat (*Triticum aestivum* L.) Genotypes under Heat Stress. *Plant Cell Biotechnology and Molecular Biology*, 26(9–10), 333–342. <https://doi.org/10.56557/pcbmb/2025/v26i9-109873>
- 29 Yadav, S. B. K., & Bhatt, S. (2024). Heat stress and its tolerance in wheat. *Cogent Food & Agriculture*. <https://doi.org/10.1080/23311932.2024.2413398>
- 30 Ying, Z., Fu, S., & Yang, Y. (2025). Signaling and scavenging: Unraveling the complex network of antioxidant enzyme regulation in plant cold adaptation. *Plant Stress*, 16, 100833. <https://doi.org/10.1016/j.stress.2025.100833>