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ORIGINAL RESEARCH

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Do diverse wheat genotypes unleash their biochemical arsenal differentially to conquer cold stress? A comprehensive study in the Western Himalayas

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Abstract

Wheat is one of the most important cereal crops in the world. Cold stress is a major constraint in production of wheat grown in cold climate regions. In this study, we conducted a comprehensive assessment of cold stress tolerance in wheat genotypes through field screening, cell membrane stability through electrolyte leakage assay and biochemical profiling. A core set comprising 4560 genotypes was evaluated for two years (2021–2022), revealing substantial genetic variation for cold stress tolerance. Most genotypes exhibited moderate tolerance, while a smaller proportion showed susceptibility to cold stress. Based on the cold screening data in the field, a mini-core set of 350 genotypes was selected for membrane stability analysis using electrical conductivity assays. Significant differences were observed in membrane stability among the genotypes, indicating the presence of genetic variation for this trait. Furthermore, a mini-core set was narrowed down to 50 diverse candidate genotypes that were subsequently profiled for various biochemicals, including reactive oxygen species (ROS) like lipid peroxidation (MDA) and hydrogen peroxide (H_20_2), osmoprotectant (proline) and enzymatic antioxidants including ascorbate peroxidase (APX), superoxide dismutase (SOD), guaiacol peroxidase (GPX), and catalase (CAT). Correlation analysis of the biochemicals revealed negative associations between antioxidants and reactive oxygen species (ROS), highlighting their role in mitigating oxidative damage under cold stress. This study enhances our understanding of the physiological and biochemical mechanisms underlying cold stress tolerance in wheat. The identified genotypes with superior cold stress tolerance can serve as valuable genetic resources for wheat breeding.

1 | INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops and is grown extensively in many regions around the world (Cuong et al., 2020; Chang et al., 2022). Belonging to the Poaceae family, wheat thrives at latitudes ranging from 30°N to 60°N and 27°S to 40°S and up to a maximum altitude of 3000 meters above sea level (Deng et al., 2005). Globally, wheat covers an area of approximately

217 million hectares with an annual yield of approximately 764 million tonnes (USDA, 2021), which accounts for two-thirds of human food consumption and thus represents the pillars of global food security (Reeves et al., 2016). The wheat production of India contributes to approximately 14% of the total world wheat area and produced 107.59 million tonnes during the year 2020–2021 with a record average productivity of 3.58 qt/ha production (Gupta et al., 2021). However, increasing climatic fluctuations over the past years have severely impacted agricultural production, as they are the primary drivers of abiotic and biotic stresses (Rosenzweig et al., 2014). Abiotic stresses such as episodes of excessive cold or heat, precipitation or drought, and soil salinity or sodicity represent some of the most common types of stresses that plants experience in response to climate change (Ashraf et al., 2018; Soren et al., 2020; Varshney et al., 2021a; Barmukh et al., 2022). Among the various abiotic stresses, extreme weather events are becoming more common as a result of climate change, causing severe episodes of freezing injury in our modern crop cultivars, exposing them to low-temperature conditions for which they were not bred or for which native plants had not time to adapt through selection pressures (Solanke et al., 2008; Janksa et al., 2010; Kumar et al., 2013). Under freezing stress conditions, the formation of intracellular or extracellular ice crystals occurs, which exerts a profound impact on the structural integrity of the cellular framework of plants. As these ice crystals expand, they can ultimately lead to cell death (Jahed et al., 2023). However, the cascade of events culminating in cell or plant death upon cold stress is primarily attributed to the heightened production of diverse reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Petrov et al., 2023). These ROS accumulate significantly within cellular organelles like chloroplasts, mitochondria, and peroxisomes, consequently resulting in oxidative harm to proteins and DNA, peroxidation of membrane lipids, enzyme inhibition, or even cellular death. These changes result in a combination of symptoms such as poor germination, reduced seedling vigor or stunted growth, reduced leaf size, leaf yellowing and withering, reduced tillering, poor root proliferation, disturbed plant water relations, impeded nutrient uptake, premature heading, increased seed abortion, and reduced seed size, leading to reduced yield (Shimono et al., 2002; Andaya and Tai, 2006; Oliver et al., 2007; Wang et al., 2013; Li et al., 2015; Hassan et al., 2021).

Every year, 85% of the wheat sown area in the world is affected by spring frost, which usually takes place during March and April at the early booting stage (Yue et al., 2016). In the spring season, when the wheat canopy temperature falls to 0°C or below, severe frost damage occurs (Frederiks et al., 2015; Zheng et al., 2015). Winter wheat initially suffers low-temperature stress when tillering begins and when photosynthate assimilation and nutrient absorption sites are under development (Rinalducci et al., 2011). In India, wheat is mainly grown in northern plains such as Uttar Pradesh, Madhya Pradesh, Punjab, Haryana, Rajasthan, Bihar, Gujarat and Maharashtra during the winter season (October to March) when the average winter temperature ranges between 10–15°C. However, it can be well grown in tropical and subtropical, temperate and cold zones even beyond 67°N of Jammu and Kashmir. In Jammu and Kashmir, wheat-growing areas have remained confined to subtropical areas of the Jammu division; however, the temperate climate of the Kashmir valley and higher hills are more conducive for realizing higher yields of wheat crops because the valley of Kashmir and higher hills receive most of the annual precipitation during the months of December to May, which coincides with the critical growth period of the crop in the valley. However, the occurrence of low temperatures (0°C or below) during the winter months significantly reduces germination and subsequent seedling emergence, thus significantly affecting wheat production in the temperate region of the Kashmir valley.

To mitigate the negative effects of cold stress on plant growth and development and ensure agricultural production in regions with cold climates, the development of cold-tolerant varieties is urgently needed. Studying the physiology of cold stress tolerance in plants is crucial for developing cold-tolerant varieties, as activation of specific enzymes, such as peroxidases and catalases, which can scavenge ROS and protect cells from oxidative damage, is the key physiological response to cold stress. Furthermore, plants can produce antioxidants, such as ascorbate and glutathione, to prevent ROS accumulation and maintain redox homeostasis. By understanding the physiological changes occuring in response to cold stress, researchers must develop strategies to enhance cold tolerance and improve agricultural productivity in regions with cold climates. Keeping this in mind, the current study was carried out to characterize diverse wheat germplasm in the Western Himalayas using key physiological traits to identify genotypes with improved cold tolerance that could be used in future breeding programs. To the best of our knowledge, this is the first study that comprehensively evaluated the cold tolerance of diverse wheat germplasm in this region using physiological traits. Our findings provide valuable insights into the mechanisms underlying cold tolerance in wheat and can be used to develop new strategies for improving cold tolerance in wheat varieties.

2 | MATERIALS AND METHODS

2.1 | Location of experiment and plant material

A core set of 4560 genotypes, which included the NBPGR core set, IIWBR core set, USDA core set, IC set, EC set, Mexican set, Iranian set, Indian set and BISA genomic selection genotypes, was used for the evaluation of cold tolerance in the field at the Experimental Research Field, Division of Genetics and Plant Breeding, Faculty of Agriculture, Wadura, Sopore, Kashmir, India. The experimental design was augmented block design (ABD) with 4 genotypical benchmarks (SKUA_52, SKUA_118, SKUA_4301, SKUA_1701). These genotypes were strategically chosen based on their known cold tolerance characteristics: SKUA_52 and SKUA_118, identified as cold-tolerant genotypes through our previous studies, served as benchmarks for superior cold resilience. In contrast, SKUA_4301 and SKUA_1701, identified as cold-susceptible genotypes, were included to provide a contrasted response. Genotypes refered to as "SKUA" in this study correspond to their original IC or EC numbers, provided in Table S1. However, SKUA is the local designation for these genotypes in this study.

2.2 | Weather conditions at the experimental site

The cold weather conditions in the Kashmir valley prevailing during the winter season were found ideal for the screening of 4560 wheat genotypes for cold stress tolerance. During the year 2020–2021 (October–February), the average high/day temperatures ranged from 5.8°C to 18.23°C, while the range of average low/night temperature recorded was – 5.9°C to 5.36°C. During the year 2021–2022 (October–February), the average high/day temperatures ranged from 6.2°C to 21.45°C, while the range of average low/night temperature recorded was – 1.0°C to 6.3°C. Furthermore, during the year 2022–2023 (October–February), the average high/day temperatures ranged from 8°C to 22°C, while the range of average low/night temperature recorded was – 3°C to 7°C (Figure 1).

2.3 | Cold stress tolerance screening under natural conditions in the field

The core set of 4560 genotypes was sown in the field in October in the year 2020 and year 2021, and the crop was subjected to cold stress in the fourth winter. The data on cold tolerance were recorded using the scale proposed by Zhao et al. (2019). A score of 0 indicated no frozen parts on the leaves, resulting in a predominantly green field. A score of 1 represented only frozen tips of leaves, with older leaves remaining largely unfrozen. At a score of 2, the majority of leaf areas, particularly young leaves, remained unfrozen, while dead yellow leaves were scattered on the ground. A score of 3 indicated a significant freezing of leaf areas, with whole dead leaves making up the majority of intact leaves on the ground. Lastly, a score of 4 was assigned to completely frozen and dead leaves, or in severe cases, to an entire damaged plant (Figure 2).

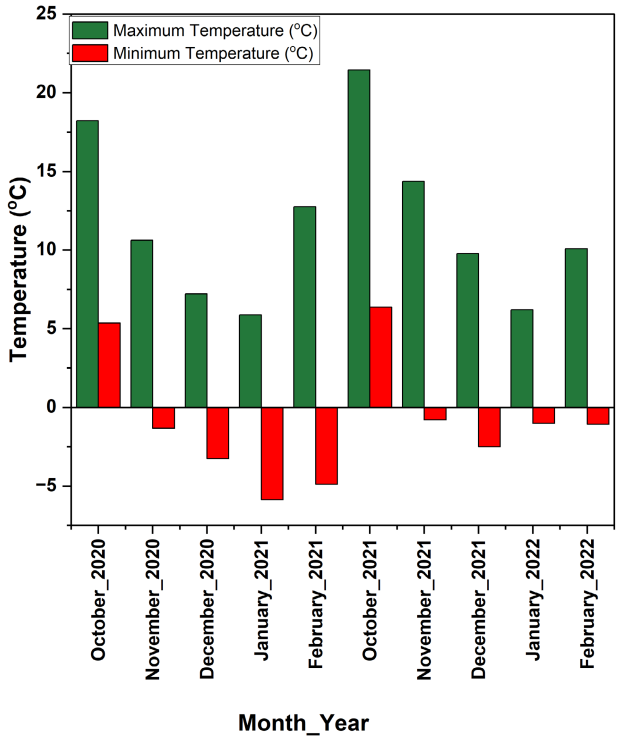

FIGURE 1 Histogram showing variation in the temperature during the growing season of wheat (October_2020 to February_2021, October_2021 to February_2022, October_2022 to February_2023) at Faculty of Agriculture, SKUAST-K, Sopore.

2.4 | Membrane stability analysis of mini-core set of wheat in response to cold stress

On the basis of field-based cold screening data, a mini-core set comprising of 350 genotypes, which showed consistent performance over two years was selected. In October 2022, 350 genotypes were sown in an ABD design and then subjected to cold stress under natural conditions. Using the protocol of Nejadsadeghi et al. (2014) and Mir et al. (2021), the impacts of low temperature on the membrane integrity of the whole plant were determined by measuring the electrolyte leakage index (ELI) from damaged leaves. Fresh mass (FM, 100 mg) of leaf fragments were cut into two pieces and then deposited in glass tubes containing 10 mL of distilled water. The tubes were sealed and shaken at 250 rpm for 90 min on an electric shaker. At 25°C, the electrical conductivity (μS^{-1}) of an extract containing released ions was measured using a digital conductivity meter (Thermofisher ECtestr11+). In the second step, the tubes and their contents were placed in a boiling water bath for 10 min, followed by 30 min of stirring, and their electrical conductivity was measured. The ELI (%) was calculated using the following formula: I = [(Lt–L0)/(Lb–L0)]*100, where Lt is the sample's electrical conductivity after thermal treatments, L0 is the sample's electrical conductivity under control conditions, and Lb is the sample's electrical conductivity after boiling.

2.5 | Biochemical Profiling for ROS, osmoprotectants and antioxidants in mini-core set in response to cold stress

Based on the cold screening in the field and membrane stability assessed by electrical conductivity assay, 50 diverse wheat genotypes were selected and analyzed for various stress-responsive biochemical parameters at different cold treatments: T0 = control, T1 = acclimation phase at 4°C for 14 days, T2 = cold stress at −5°C after acclimation, and T3 = cold stress at −5°C without acclimation treatments.

Seeds of these genotypes were sown in pots containing a mixture of soil, sand, and farmyard manure. The plants were grown in a controlled growth chamber under specific conditions, a temperature of 25°C, an irradiance of 200 µmol m^{-2} s^{-1} from white light luminescent lamps, a 16/8 h light/dark photoperiod, and 75% relative humidity for 14 days. Following this, the plants were exposed to an acclimation temperature of 4–5°C for 14 days, maintaining the same photoperiod and irradiance (T1). Leaf samples were collected after 14 days under

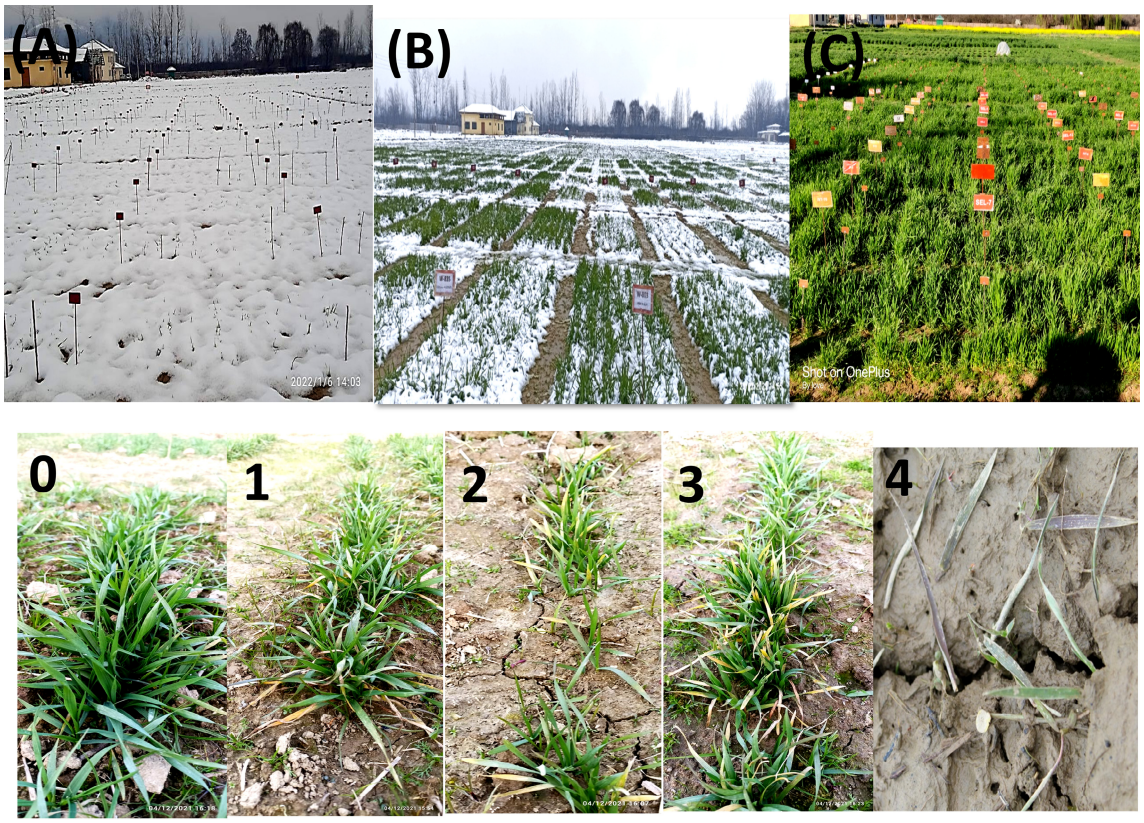

FIGURE 2 Overview of wheat field at Faculty of Agriculture (FoA), Wadura. (A,B) The figure shows wheat germplasm (4560 wheat genotypes) under snow during winters subjected to cold/freezing stress followed by (C) its regrowth in summer. Representative pics of the 0–4 scale of Zhao et al. (2019) used during the present study for screening wheat germplasm for cold tolerance. In the scale, 0 = No cold injury; 1 = only tips injured; 2 = majority of old leaves damaged; 3 = majority of leaves dead and fallen on ground and 4 = whole plant dead.

these acclimated conditions. Subsequently, the plants were transferred to a climatic chamber with a preliminary chilling temperature of 0°C. The temperature was gradually decreased to −5°C at a rate of 0.5°C per minute, and the plants were incubated at this temperature for 24 hours (T2). Non-acclimated plants were directly exposed to −5°C (T3). The control group remained at 25°C under normal conditions (T0). Physiological analyses were conducted on leaf samples harvested immediately after removing the plants from the cold exposure room. Measurements were taken from the middle parts of the first leaves of wheat seedlings in two replications (Nazari et al., 2012).

2.5.1 | Lipid peroxidation (MDA) analysis

Thiobarbituric acid (TBA) testing, which identifies MDA as a byproduct of lipid peroxidation, was used to measure lipid peroxidation in leaves (Heath et al., 1968). FM leaflets (250 mg) were homogenized in 2 mL of trichloroacetic acid (TCA) extraction buffer at 1% (w/v) before being centrifuged at 13,000 × *g* for 15 min. Then, 1 mL of the supernatant was added to 2 mL of 5% (w/v) TBA in 20% (w/v) TCA. After 30 min of incubation in boiling water, the samples were placed in an ice bath to cease the process. The samples were then centrifuged at 10,000 × *g* for 10 min, and a spectrophotometer was used to determine the absorbance of the supernatants at 532 and 600 nm. OD600 values were subtracted from the MDA-TBA complex values at 532 nm. The MDA concentration was determined using the formula C = D/E× L, where L is the thickness of the layer of solution in the vessel (1 cm), E is the coefficient of molar extinction (1.56×10^5 cm^{-1} M^{-1}), and C is the concentration of MDA in μmol g^{-1} FM.

2.5.2 | Estimation of the hydrogen peroxide (H_2O_2) content

The amount of H_2O_2 was determined based on Loreto and Velikova (2001). FM (0.20 g) of leaf fragments was homogenized with 5 mL of 0.1% TCA in an ice bath after being pulverized in liquid nitrogen with a mortar and pestle. After centrifuging the homogenate at 12,000 × *g* for 15 min, 0.5 mL of the supernatant was added to 1 mL of 1 M potassium iodide and 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0). Using a spectrophotometer, the absorbance of the supernatant was determined at 390 nm. The amount of H_2O_2 was determined through comparison to a standard calibration curve that had previously been created using various H_2O_2 concentrations and was expressed in μmol g^{-1} FM.

2.5.3 | Proline content

The proline content was measured according to Bates et al. (1973). Sulphosalicylic acid was used to homogenize samples (0.2 g leaflets), which were then filtered via filter paper. As soon as the acid ninhydrin and glacial acetic acid were added, the mixture was heated at 100°C for 1 hour in a water bath. After that, an ice bath prevented the reaction to continue. Toluene was used to extract the mixture, and the absorbance of the toluene-aspirated fraction from the liquid phase was measured at 520 nm. Using a calibration curve, the concentration of proline was calculated and expressed as μmol g^{-1} FM.

2.5.4 | Soluble protein content and antioxidant enzyme activity

The amount of total soluble protein was calculated and expressed in mg ml^{-1} protein (Bradford, 1976). Enzyme extract for ascorbate peroxidase (EC 1.11.1.11), guaiacol peroxidase (EC 1.11.1.7) and catalase (EC 1.11.1.6) was prepared by crushing 0.1 g of fresh leaf tissue with 2 mL of ice-cold 0.1 M potassium phosphate buffer (pH = 7.5 containing 0.5 mM EDTA and 5% PvPP). The homogenate was centrifuged for 20 min at 15000 × *g* at 4°C and the supernatant was used as crude enzyme extract for enzyme activity assay.

For ascorbate peroxidase (APX) activity, the reaction mixture contained 1.5 mL potassium phosphate buffer, 0.5 mL ascorbic acid, 0.1 mL EDTA, 0.1 mL hydrogen peroxide, 100 μL of enzyme extract and 0.7 mL distilled water. The reaction started with addition of 0.1 mL hydrogen peroxide. The H_2O_2-dependent oxidation of ascorbic acid was measured by the decrease in absorbance at 290 nm for 3 min at the interval of 30 seconds in UV–visible spectrophotometer. The amount of ascorbate oxidized protein $minute^{-1}$ μmol^{-1} of APX activity was expressed.

Catalase activity (CAT), was measured immediately in fresh extract as described by Scebba et al. (1973). The reaction mixture contained 1.5 mL phosphate buffer, 0.5 mL H_2O_2, 200 μL enzyme extract and 1 mL distilled water. The reaction started when H_2O_2 was added. For measurement of catalase activity, the decline in absorbance at 240 nm was recorded for 3 min with 30-second intervals. Assuming an extinction coefficient of 39.4 cm^{-1} mM^{-1}, CAT specific activity was expressed in μM of H_2O_2 decomposed min^{-1} mg $protein^{-1}$.

For guaiacol peroxidase activity (GPX), the reaction mixture contains 1.5 mL potassium phosphate buffer, 0.5 mL guaiacol, 0.5 mL hydrogen peroxide, 100 μL of enzyme extract and 0.4 mL of distilled water. The addition of H_2O_2 started the reaction. For measurement of guaiacol peroxidation, the increase in absorbance at 480 nm was recorded for 3 min at the interval of 30 seconds. Assuming an extinction coefficient of 26.6 cm^{-1} mM^{-1}, GPX specific activity was expressed as μM of guaiacol oxidized min^{-1} mg $protein^{-1}$.

Superoxide dismutase (SOD) activity (EC 1.15.1.1) was determined according to the protocol of Beyer and Fridovich (1987). Phosphate buffer 100 mM (pH 7.8), EDTA 0.1 mM, methionine 12 mM, NBT 75 μM, and triton ×100 0.025% (v/v) made up the reaction medium. The reaction medium was supplemented with 100 μL of crude enzyme extraction and 1 μL of riboflavin buffer. After 20 min of illumination at 25°C, turning off the lights put an end to the reaction and the reaction was observed at 560 nm. Blanks were non-illuminated solutions with minimal enzyme extraction. The following equation was used to determine the activity: SOD (U ml^{-1}) = (Px 1,000)/(50x mg protein) and P = (V - v/v) × 1000, where V is the rate of reaction in the absence of the enzyme and v is the rate of reaction in its presence. P represents the percentage of prohibition. The specific activity of SOD was represented as U min^{-1} mg^{-1} protein for each sample, and the activity of SOD was expressed in relation to the volume (ml) of enzyme extraction corresponding to a 50% prohibition rate.

2.6 | Statistical analysis

The cold screening data collected during this study under in vivo conditions was analyzed using the R-software. Frequency distribution analysis was performed to examine the distribution patterns and characteristics of the data. Furthermore, an augmented block design (ABD) analysis was conducted to assess the effect of cold stress on EC (electrolyte leakage) levels. The mean values of EC among the different cold tolerance groups were calculated using one-way ANOVA in the R-software. For the biochemical data, analysis of variance (ANOVA) was used to calculate the mean differences between cold stress levels and genotypes and their interaction at 0.05 and 0.01 significance levels in R-software. Pearson's correlation coefficient was assessed to find the linear relation between various biochemical parameters in R-software. The differentiated genotypes were selected based on principal component analysis (PCA) and radar analysis. For this purpose, Origin Pro software was used. The statistically significant PCs were selected using eigenvalue standards as established by Kaiser (1960).

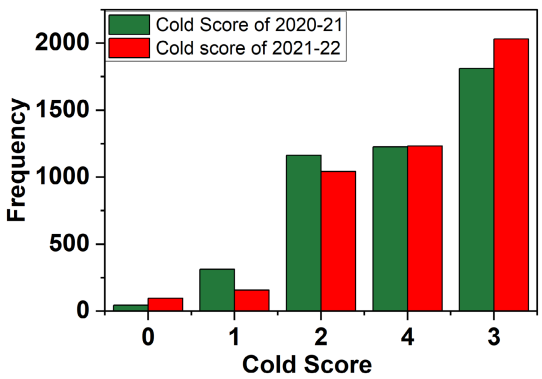

FIGURE 3 Histogram showing the frequency distribution of 4560 wheat genotypes for cold stress tolerance in the year 2020–2021 and year 2021–2022.

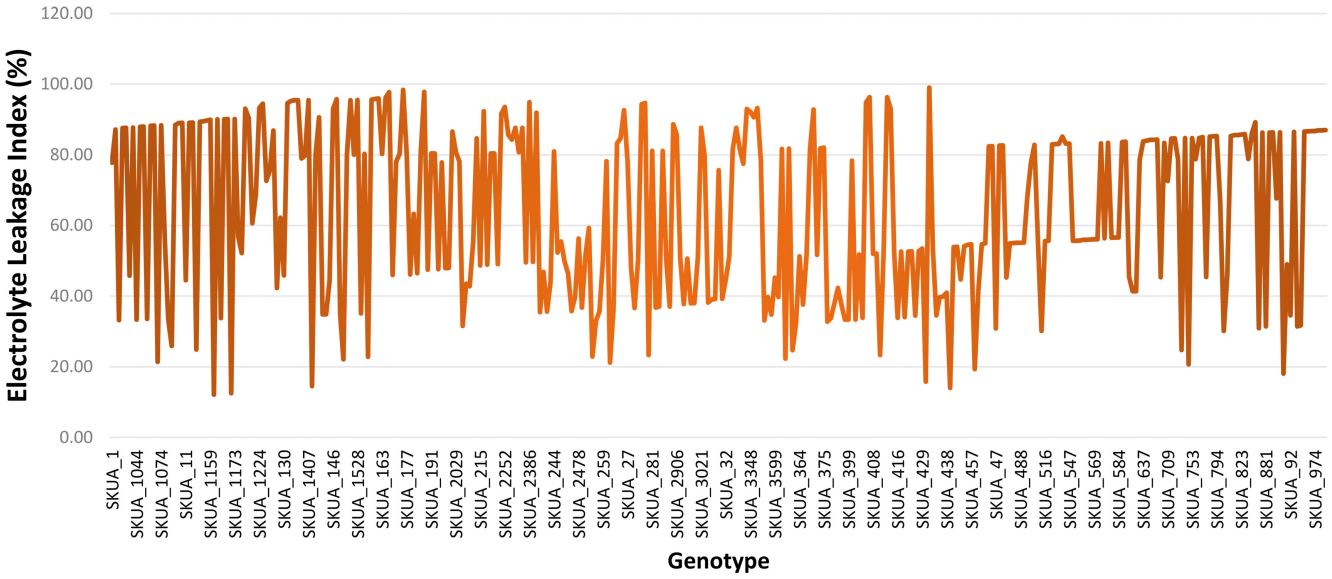

FIGURE 4 Line graph showing variation in membrane stability accessed by the measure of electrical conductivity (EC) in the mini-core set (350 wheat genotypes) used in this study. SKUA is the local designation for these genotypes in this study. The original IC/EC numbers of these genotypes is provided in Table S1.

TABLE 1 Analysis of variance (ANOVA) mean squares of 350 genotypes for membrane stability assessed by electrolyte leakage index under cold stress conditions prevailing in the field

Sources of variation	DF	Mean value
Block (ignoring Treatments)	5	2547.08 **
Block (eliminating Treatments)	5	4.7e-25 ns
Treatment (eliminating Blocks)	350	675.15 **
Treatment (ignoring Blocks)	350	711.53 **
Treatment: Check	3	16762.06 **
Treatment: Test vs. Check	1	2682.54 **
Residuals	16	9.00E-02

DF = degrees of freedom;
**= Significance at the P-value of 0.001, ns = non-significant.

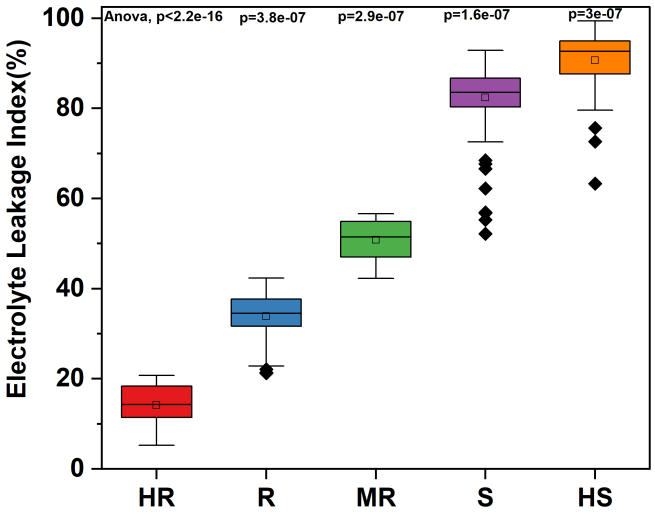

FIGURE 5 Box plots depicting the increasing ELI trend from highly resistant (HR) to highly susceptible (HS) genotypes. Significant differences (P-value<0.05) among HR, resistant (R), moderately resistant (MR), susceptible (S), and HS genotypes were determined using grouped one-way ANOVA.

3 | RESULTS

3.1 | Evaluation of 4560 genotypes for cold tolerance under natural conditions in the field

A total of 4560 genotypes were screened for cold tolerance in the two environments, *i.e*, year 2020 and year 2021 at the Faculty of Agriculture at Wadura, Kashmir, India. is shown in Figure 3. The. The frequency distribution results (Figure 3 and Table S1 for individual cold score for each genotype) show that the majority of wheat genotypes screened in both years had a cold score of 2 (moderately resistant), with 1164 genotypes in 2020 and 2012 genotypes in 2021 falling into this category. A small number of genotypes had cold scores of 1 (cold tolerant), 3 (susceptible), or 4 (highly susceptible). In 2020, 311 genotypes had a cold score of 1, while in 2021, 1043 genotypes had this score. Similarly, 1812 genotypes in 2020 and 1233 genotypes in 2021 had a cold score of 3, while 1227 genotypes in 2020 and 166 genotypes in 2021 had a cold score of 4. Only a few genotypes, 46 genotypes in 2020 and 106 genotypes in 2021, were classified as highly cold tolerant (cold score of 0). Overall, the results revealed that the majority of wheat genotypes screened in both years had a cold score of 2. In contrast, a smaller

number of genotypes had a cold score of 1, which indicates that they are cold tolerant, and an even smaller number had a cold score of 0, which indicates that they are highly cold tolerant. On the other hand, a significant number of genotypes had a cold score of 3 or 4, which indicates that they are susceptible to cold temperatures.

3.2 | Evaluation of mini-core collection for cell membrane stability under cold stress

The ELI values of 350 genotypes having diverse responses to cold stress conditions presented a wide range of variation(Table S2; Figure 4). The analysis of variance (ANOVA) showed that the ELI

TABLE 2 Analysis of variance (ANOVA) mean squares of 50 genotypes for various biochemical traits recorded under normal and different cold stress conditions in this study

SOV	DF	Protein	MDA	H_2O_2	APX	PROLINE	SOD	CAT	GPX
Genotype	49	23.00***	84.90***	43.00***	69424.00***	9.80***	108472.00***	14738.00***	9294.00***
Treatment	3	3974.00***	2859.90***	4740.00***	839339.00***	1460.00***	3805111.00***	4830630.00***	53005.00***
Genotype*Treatment	147	1.00***	5.20***	7.00^{ns}	37.00**	0.60***	4391.00***	77.00***	4.00^{ns}
Error	200	0.05	0.05	6.00	25.00	0.05	4.00	3.00	3.00

DF = degrees of freedom; Sign P-value codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1; MDA = malondialdehyde; H_2O_2 = hydrogen peroxide; SOD = superoxide dismutase; CAT = catalase; GPX = glutathione peroxidase.

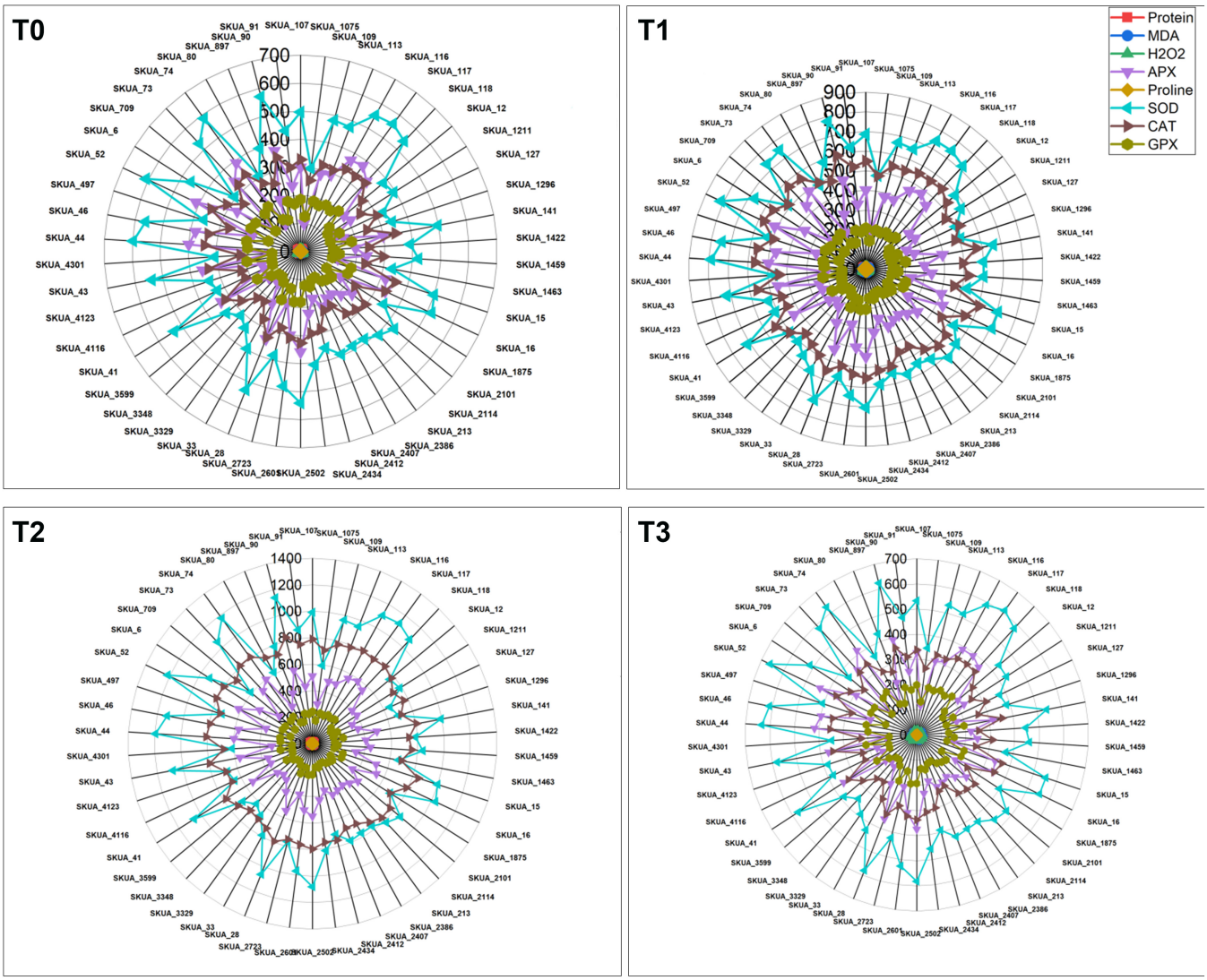

FIGURE 6 Radar graphs showing variations in various biochemicals under (T0) normal conditions, (T1) acclimation phase at 4°C for 14 days, (T2) cold stress at −5°C after acclimation, (T3) cold stress at −5°C without acclimation treatments. SKUA is the local designation for these genotypes in this study. The original IC/EC numbers of these genotypes is provided in Table S1.

variation among treatments (genotypes) was highly significant, while the variation among blocks (experimental units) was not significant. There were significant differences observed between the benchmark genotypes and other treatments, as well as between the test and check genotypes (Table 1). Overall, the ANOVA results suggest that the genotypes have a significant impact on ELI in the field.

Furthermore, the 350 wheat genotypes were grouped into five categories based on their cold scores in the field screening: highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S), and highly susceptible (HS). We calculated the mean ELI values for each group to compare their membrane stability under cold stress conditions. The analysis of the mean ELI values for each group revealed significant differences ($p < 0.001$) between the groups. The mean ELI values of the genotypes in the highly resistant (HR) group were the lowest (14.2%), while the mean ELI values of the genotypes in the highly susceptible (HS) group were the highest (90.6%). We further compared the mean values of ELI among the different cold tolerance groups using one-way ANOVA, which revealed a significant difference between the groups (Figure 5). The box plots showed an increasing trend from the HR to HS groups, with the HR group having the lowest mean ELI value (14.2%) and the HS group having the highest mean EC value (90.6%). Interestingly, we observed higher variability within each group in the more tolerant groups (HR, R, and MR) compared to the more susceptible groups (S and HS). Subsequently, we performed posthoc tests using the HR group as a control and found significant differences between the HR and R, HR and S, and HR and HS groups (Figure 5).

3.3 | Evaluation of diverse candidate genotypes for biochemicals induced by cold stress

Malondialdehyde (MDA) levels, hydrogen peroxide (H_2O_2) levels, protein content, antioxidant enzyme activities (APX, SOD, CAT, and GPX)

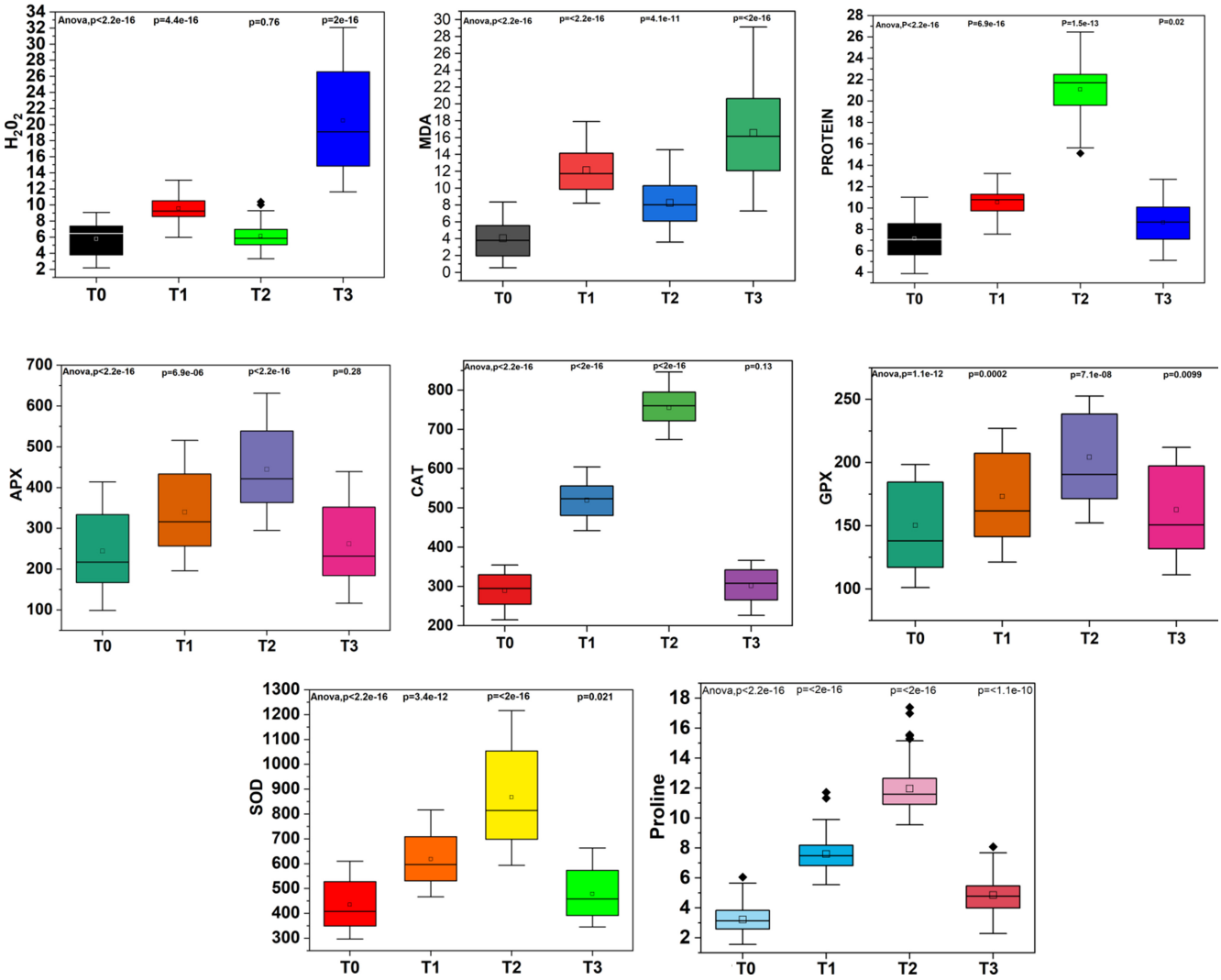

FIGURE 7 Box plots revealing significant differences in the mean values of various biochemical traits assayed in this study under (**T0**) normal conditions, (**T1**) acclimation phase at 4°C for 14 days, (**T2**), cold stress at −5°C after acclimation (**T3**) cold stress at −5°C without acclimation treatments. Significant differences (P-value<0.05) among the treatments were determined using grouped one-way ANOVA.

TABLE 3 Correlation matrix among biochemical traits under (T0) normal conditions, (T1) acclimation phase at 4°C for 14 days, (T2) cold stress at −5°C after acclimation, (T3) cold stress at −5°C without acclimation treatments

Traits	Level	Protein	MDA	H_2O_2	APX	Proline	SOD	CAT
MDA	T0	−0.89*						
	T1	−0.73*						
	T2	−0.73*						
	T3	−0.84*						
H_2O_2	T0	−0.8*	0.82*					
	T1	−0.70*	0.86*					
	T2	−0.58*	0.79*					
	T3	−0.80*	0.90*					
APX	T0	0.87*	−0.82*	−0.90*				
	T1	0.56	−0.77*	−0.85*				
	T2	0.57*	−0.75*	−0.90*				
	T3	0.87*	−0.75*	−0.74*				
Proline	T0	0.87*	−0.84*	−0.85*	0.84*			
	T1	0.58*	−0.63*	−0.60*	0.73*			
	T2	0.46*	−0.43*	-0.16^{ns}	0.56*			
	T3	0.67*	−0.59*	−0.55*	0.66*			
SOD	T0	0.87*	−0.86*	−0.90*	0.10*	0.85*		
	T1	0.58*	−0.78*	−0.68*	0.99*	0.73*		
	T2	0.56*	−0.76*	−0.58*	0.10*	0.55*		
	T3	0.86*	−0.75*	−0.74	0.99*	0.63*		
CAT	T0	0.91*	−0.96*	−0.87*	0.88*	0.90*	0.87*	
	T1	0.66*	−0.91*	−0.83*	0.84*	0.66*	0.86*	
	T2	0.68*	−0.87*	−0.74*	0.83*	0.48*	0.84*	
	T3	0.90*	−0.90*	−0.92*	0.87*	0.63*	0.88*	
GPX	T0	0.93*	−0.92*	−0.88*	0.90*	0.87*	0.90*	0.95*
	T1	0.70*	−0.90*	−0.76*	0.90*	0.70*	0.90*	0.92*
	T2	0.70*	−0.89*	−0.68*	0.90*	0.50*	0.90*	0.91*
	T3	0.92*	−0.882	−0.83*	0.90*	0.60*	0.90*	0.95*

P-value = 0.01′*′; MDA = malondialdehyde; H_2O_2 = hydrogen peroxide; SOD = superoxide dismutase; CAT = catalase; GPX = glutathione peroxidase.

and osmoprotectant (proline content) were evaluated for each genotype at different treatments (T0, T1, T2 and T3) (Figure 6).The ANOVA revealed significant differences in the genotypes for all analyzed biochemical parameters (Table 2). Additionally, the effects of various cold treatments on the biochemical profiles of the genotypes were highly significant. Furthermore, the interaction between genotype and treatment demonstrated significant effects on all biochemical parameters except for H_2O_2 and GPX ($p > 0.05$). Post-hoc LSD (least significant difference) analysis was performed to determine specific differences among genotypes and among the treatments. The LSD values for each parameter, representing the minimum significant difference required for pairwise comparisons between genotypes and between the treatments, are provided in Tables S3 and S4).

One-way ANOVA was conducted to assess the significance of differences in the mean values of various biochemical parameters among different cold treatments. The results of the one-way ANOVA indicate that there are significant differences in the mean values of biochemical parameters among the different treatments (Figure 7). The highest mean values for protein synthesis, oxidative stress, hydrogen peroxide accumulation, and antioxidant activity were generally observed in the cold stress after acclimation treatment. The box plots revealed interesting insights into the variations among the different cold treatments.

Examining oxidative stress markers, the MDA level under normal conditions (T0) was 4.0 μmol g^{-1}, while it increased to 12.13 μmol g^{-1} during acclimation (T1). Interestingly, cold stress after acclimation (T2) reduced MDA levels to 8.27 μmol g^{-1}, while MDA levels significantly rose to 16.58 μmol g^{-1} without acclimation (T3). Likewise, H_2O_2 levels at T0 were 5.78 μmol g^{-1}, escalating to 9.57 μmol g^{-1} during acclimation (T1). Post-acclimation cold stress (T2) reduced H_2O_2 levels to 6.13 μmol g^{-1}, whereas in the absence of acclimation (T3), levels surged to 20.50 μmol g^{-1}.

Focusing on enzymatic responses, APX activity at T0 was 244.19 U min^{-1} mg^{-1} protein, escalating to 340.09 U min^{-1} mg^{-1}

protein during acclimation (T1). Cold stress after acclimation (T2) intensified this response to 445.34 U min^{-1} mg^{-1} protein. However, without acclimation (T3), APX activity dropped to 262.09 U min^{-1} mg^{-1} protein. SOD activity at T0 was 434.47 U min^{-1} mg^{-1} protein, increasing to 618.34 U min^{-1} mg^{-1} protein during acclimation (T1). After acclimation, cold stress (T2) further intensified SOD activity, peaking at 867.58 U min^{-1} mg^{-1} protein. Without acclimation (T3), SOD activity decreased to 478.27 U min^{-1} mg^{-1} protein. CAT activity started at 289.69 U min^{-1} mg^{-1} protein (T0), surging to 519.45 U min^{-1} mg^{-1} protein during acclimation (T1). Cold stress after acclimation (T2) led to the peak activity of 755.80 U min^{-1} mg^{-1} protein, while CAT activity reduced to 301.70 U min^{-1} mg^{-1} protein without acclimation (T3). Similarly, GPX activity at T0 was 150.36 U/mg protein, increasing to 173.22 U min^{-1} mg^{-1} protein during acclimation (T1). After acclimation, cold stress (T2) further intensified GPX activity, reaching 204.15 U min^{-1} mg^{-1} protein. However, without acclimation (T3), GPX activity decreased to 162.60 U min^{-1} mg^{-1} protein.

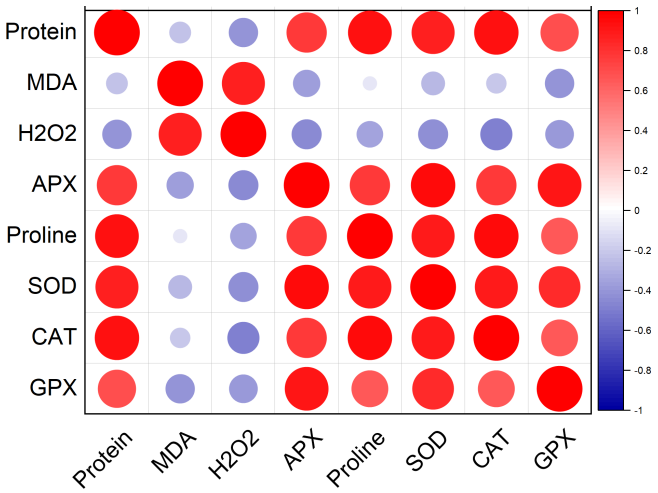

FIGURE 8 Correlation network analysis showing the biochemical interactions underlying cold stress tolerance in wheat in this study. The size of each circle is proportional to its degree of correlation with other parameters; larger circles indicate stronger correlations. The color intensity of circles corresponds to the magnitude of correlation, with deeper shades indicating a stronger correlation.

The study also delved into the osmotic stress response of wheat plants by examining the proline content. Under normal conditions (T0), the baseline proline content was 3.22 μmol g^{-1}. During acclimation (T1), proline levels significantly increased to 7.00 μmol g^{-1}, indicating a response to osmotic stress. This response was further heightened under cold stress after acclimation (T2), reaching 11.96 μmol g^{-1}. Conversely, without the preparatory acclimation phase (T3), proline content decreased to 4.86 μmol g^{-1}. Likewise, in the control group (T0), total soluble protein content maintained a baseline level of 7.13 mg ml^{-1} protein. During acclimation (T1), there was a significant increase to 10.55 mg ml^{-1} protein, indicating a response to preparatory stress adaptation. The most substantial change occurred during cold stress after acclimation (T2), where protein content surged to 21.07 mg ml^{-1} protein. In contrast, exposure to cold stress without prior acclimation (T3) led to a decline, reaching 8.65 mg ml^{-1} protein.

The correlation analysis revealed significant associations among the various biochemical parameters studied (Table 3). The correlation results highlighted the interconnectedness of the antioxidant system in response to the different cold treatments and control conditions in this study (Figure 8). Protein levels exhibited strong negative correlations with MDA levels and H_2O_2 content; however, it showed a positive correlation with APX, GPX, SOD, CAT and proline. H_2O_2 levels showed positive correlations with MDA and negative correlations with APX, GPX, SOD, CAT and proline. Likewise, MDA content showed a negative correlation with CAT, SOD, APX, GPX and proline. The antioxidants (viz, APX, GPX, CAT, SOD) and proline exhibited positive correlations with each other.

TABLE 4 Eigenvalues, variability, and cumulative of wheat seedling traits (T0) normal conditions, (T1) acclimation phase at 4°C for 14 days, (T2) cold stress at −5°C after acclimation, (T3) cold stress at −5°C without acclimation treatments

	Treatment	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Eigen vector	T0	4.17	2.34	1.18	0.13	0.11	0.05	0.03	0.018
	T1	3.30302	2.67	1.46	0.30	0.14	0.08	0.05	0.01
	T2	3.84	1.93	1.60	0.30	0.17	0.11	0.05	0.01
	T3	4.64	1.58	1.44	0.16	0.09	0.06	0.03	0.01
Percentage of Variance (%)	T	85.60%	6.14%	4.22%	1.68%	1.48%	0.56%	0.27%	0.05%
	T1	75.79%	10.27%	6.67%	3.70%	1.78%	1.06%	0.59%	0.14%
	T2	72.96%	11.55%	7.45%	3.76%	2.14%	1.45%	0.66%	0.03%
	T3	82.93%	7.17%	5.49%	1.96%	1.19%	0.83%	0.39%	0.04%
Cummulative (%)	T0	85.60%	91.74%	95.96%	97.64%	99.12%	99.68%	99.95%	100.00%
	T1	75.79%	86.06%	92.73%	96.43%	98.21%	99.27%	99.86%	100.00%
	T2	72.96%	84.51%	91.96%	95.72%	97.86%	99.31%	99.97%	100.00%
	T3	82.93%	90.10%	95.59%	97.55%	98.73%	99.57%	99.96%	100.00%

3.4 | Principal component analysis

Principal component analysis (PCA) was performed to investigate the relationships between genotypes and biochemical traits in our study. We generated biplots based on the PCA results to visually explore the relationships between genotypes and traits in the reduced-dimensional space and to select the parents for breeding programs (Figure S1).

We obtained a total of eight eigenvalues, representing the principal components, with varying degrees of contribution to the overall variance (Table 4). Out of 8 principal components of eigenvalues, the first three PCs with eigenvalues larger than 1 under normal and stress conditions were selected. The other three PCs data were considered non-significant and were not used for further analysis due to eigenvalues less than 1. The first three PCs showed 95.96% of the total variation under normal conditions (T0). Under cold acclimation (T1), the first three PCs accounted for 92.73% of the total variation. Meanwhile, under cold stress after acclimation (T2), the first three PCs showed 91.96% of the total variation, and under cold stress without acclimation, the first three PCs showed 95.93% of the total variation. The 1st PCs accounted for 85.60% of the variance at T0, 75.79% of the variance at T1, 72.96% of the variance at T2, and 82.93% of the variance at T3 (Table 4).

4 | DISCUSSION

Cold stress is a significant abiotic factor that severely affects crop productivity and poses a major challenge to agricultural systems, particularly in regions with low temperatures. Wheat, one of the world's most important cereal crops, is particularly susceptible to the detrimental effects of cold stress during various growth stages, including germination, seedling establishment, and reproductive development (Hassan et al., 2021). In response to the growing demand for cold-tolerant wheat varieties, extensive efforts have been made to understand the genetic basis of cold stress tolerance and to develop effective strategies for screening and selecting cold-tolerant genotypes (Rinalducci et al., 2011; Díaz et al., 2019; Zhao et al., 2020; Wang et al., 2023). Cold stress tolerance in wheat is a complex trait governed by multiple genetic and physiological mechanisms, including the regulation of gene expression, enzymatic activity, and osmoprotectant accumulation (Hassan et al., 2021; Manasa et al., 2022).

Screening large populations of wheat genotypes for cold stress tolerance is of paramount importance, especially in regions prone to low temperatures, as it directly impacts crop productivity and food security (Li et al., 2010; Mohammadi et al., 2015). In this study, field screening of a large diverse set of wheat genotypes in the challenging climatic conditions of Wadura, Kashmir, India, provided valuable insights into the distribution of cold stress tolerance among the screened wheat genotypes over two consecutive years, 2020 and 2021. It is well-documented that wheat cultivars often exhibit a range of responses to cold stress (Li et al., 2010; Zhao et al., 2019). The prevalence of moderate cold tolerance among the majority of the genotypes across the two years underscores the stability of this trait in the evaluated genotypes, reinforcing the importance of moderate cold tolerance as a baseline trait in wheat breeding programs. The identification of genotypes with a cold score of 1, indicating cold tolerance, is a promising finding of this study. The observed increase in the number of cold-tolerant genotypes from 2020 to 2021 may be attributed to the relatively milder cold conditions during 2021, allowing for a broader spectrum of genotypic responses to cold stress. Remarkably, some genotypes exhibited exceptional cold tolerance, with a cold score of 0 over the two years, holding substantial promise for breeding programs focused on developing highly cold-tolerant wheat varieties. Their capacity to thrive under severe cold stress conditions suggests the presence of valuable genetic traits related to cold tolerance. These traits hold great promise for breeding programs aimed at developing highly cold-tolerant wheat varieties. Utilizing these genetic strengths could enhance the resilience of wheat crops, making them better able to withstand adverse cold weather conditions (Lopes et al., 2015). On the flip side, a significant number of genotypes exhibited susceptibility to cold temperatures, as indicated by cold scores of 3 and 4, highlighting the importance of identifying and excluding these susceptible genotypes from breeding programs aiming at enhancing cold tolerance. These cold screening findings of diverse wheat germplasm under field conditions contribute to the broader goal of improving wheat crop resilience in the face of increasingly variable and unpredictable climatic conditions.

While simple phenotyping or field screening methods are valuable for initial screening and identifying potential candidates, they may have limitations in terms of reliability and accuracy due to environmental variability, subjectivity in scoring, and the inability to capture the full range of genetic and environmental interactions (Ghanemet al., 2015). However, the results of our two-year field screening of wheat germplasm for cold stress tolerance coupled with membrane stability assessment have provided more valuable information about the genetic basis of cold tolerance in wheat. Electrolyte leakage index (ELI) is a commonly used measure in plant physiology and stress research to assess membrane stability, particularly under abiotic stress conditions such as cold stress in plants (Svetlana et al., 2023). Cold stress disrupts cell membranes, leading to increased electrolyte leakage (Sanghera et al., 2011). The observed wide range of ELI values among the 350 selected wheat genotypes is a clear indicator of the substantial genetic diversity within the germplasm under study. This variation offers potential opportunities for selecting genotypes with enhanced membrane stability and cold stress tolerance. Furthermore, the highly significant variation among treatments (genotypes) in the ANOVA analysis underscores the strong influence of genotype on ELI in field conditions. Genetic factors play a pivotal role in shaping a plant's response to environmental stressors, and cold stress is no exception (Heidarvand et al., 2010). The unique genetic makeup of each genotype dictates its ability to withstand cold stress and maintain membrane integrity. The strong association between cold tolerance and membrane stability is underpinned by the molecular and physiological mechanisms that underlie a plant's response to cold stress (Yanli et al., 2023). The occurrence of highly resistant genotypes exhibiting the lowest mean ELI values suggests they have better membrane stability and enhanced tolerance to cold stress. This finding aligns with the expectation that plants with superior

cold tolerance have evolved mechanisms to mitigate membrane damage and, consequently, exhibit lower EC values (Amini et al., 2021). Conversely, the genotypes in the HS group displayed the highest mean ELI values, indicating the lowest membrane stability under cold stress. These findings are consistent with the anticipated trend of increased membrane damage and reduced stability in more susceptible genotypes (Murata, 1990; Pukacki et al., 1991). Furthermore, the higher variability observed within the more tolerant groups (HR, R, and MR) is a reflection of adaptive plasticity, where genotypes within these groups exhibit diverse responses to varying cold stress conditions (Chevin et al., 2017). Genotypes with higher cold tolerance possess the flexibility to respond to a wider range of cold stress intensities.

Furthermore, the comprehensive biochemical profiling undertaken in this study aligns with the overarching objective of unraveling the intricacies of cold stress tolerance mechanisms in wheat genotypes by examining osmoprotectant accumulation (proline), and antioxidant enzyme activities (such as SOD, CAT, APX, GPX), and oxidative damage by assessment of lipid peroxidation and hydrogen peroxidation responses of genotypes to low temperatures under different cold treatments. The substantial variation observed in biochemical parameters across different genotypes and treatments highlights the potential for identifying genotypes with superior cold stress resilience and the capacity to maintain membrane integrity, mitigate oxidative damage, and regulate osmotic balance under varying cold stress scenarios. One-way ANOVA revealed significant variations in the mean values of each biochemical parameter across the treatments, indicating distinct effects on the plant's biochemistry. The protein levels, which serve as indicators of cellular integrity and stress response (Flick et al., 2012; Hamann, 2012), showed notable changes across the treatment levels. The control group (T0) exhibited relatively stable protein levels, indicating steady-state conditions under normal growth conditions. However, during the acclimation phase (T1), the protein levels significantly increased, suggesting the activation of adaptive mechanisms to enhance stress tolerance. This increase in protein levels may be attributed to the synthesis of stress-related proteins and enzymes involved in cold acclimation processes (Mohsen et al., 2015).

The levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), which are indicative of lipid peroxidation and oxidative stress, respectively (Sairam and Srivastava, 2000; Apostolova et al., 2008), exhibited contrasting patterns across the treatments. The control group (T0) displayed relatively low levels of MDA and H_2O_2, signifying minimal oxidative damage. Under normal conditions, wheat genotypes maintain membrane integrity and redox homeostasis (Awasthi et al., 2015). However, during cold stress without acclimation (T3), the MDA and H_2O_2 levels significantly increased, reflecting heightened oxidative stress. This finding aligns with previous research that has demonstrated the adverse effects of rapid cold stress on membrane integrity and redox balance (Dreyer et al., 2018). In contrast, the acclimation phase (T1) and cold stress after acclimation (T2) showed lower MDA and H_2O_2 levels than T3, indicating that acclimation provides some level of protection against oxidative damage. During acclimation, plants undergo physiological adjustments, such as the accumulation of osmoprotectants and the activation of antioxidant defenses (Thomashow, 2001). These adaptations likely contribute to the observed reduction in lipid peroxidation and ROS accumulation.

Under cold stress, antioxidant enzymes play a crucial role in mitigating oxidative stress by scavenging reactive oxygen species (ROS) like superoxide radicals ($O2^{\bullet-}$) and hydrogen peroxide (H_2O_2), thus conferring cold tolerance (Lascano et al., 2001). The activities of APX, SOD, CAT, and GPX were significantly influenced by the treatments in this study. The upregulation of SOD leads to dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen. Subsequently, hydrogen peroxide, although less reactive than superoxide radicals, can still pose a threat. To neutralize this, the plant employs enzymes such as ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). APX, GPX, and CAT are responsible for the detoxification of hydrogen peroxide. APX catalyzes the reduction of hydrogen peroxide to water, using ascorbate as an electron donor. GPX utilizes glutathione as a reducing agent to convert hydrogen peroxide into water and oxygen molecules. CAT, on the other hand, directly converts hydrogen peroxide into water and molecular oxygen, providing an efficient way to eliminate this potentially harmful molecule (Gill et al., 2010). This orchestrated action of SOD, APX, GPX, and CAT ensures that reactive oxygen species are effectively neutralized and cellular damage due to oxidative stress is minimized, allowing the plant to maintain its physiological functions even under stressful cold conditions. In our study, we observed increased activities of these enzymes during acclimation phase and after subjecting the plants to cold stress following phase.

These findings suggest that acclimation primed the plant genotypes for improved antioxidant capacity, thereby enabling them to cope better with subsequent cold stress (Kim et al., 2005; Kazemi et al., 2013). Proline, an osmoprotectant and compatible solute, has been associated with enhanced cold stress tolerance, as it helps maintain cell turgor and stabilize cell structures (Hayat et al., 2012). In our study, the control group (T0) and cold stress without acclimation phase (T3) showed relatively low proline levels, suggesting a limited response to osmotic stress. However, during the acclimation phase (T1) and cold stress after acclimation phase (T2), proline levels increased significantly. This indicates that acclimation promotes the accumulation of proline. Consistent with our biochemical profiling results, the correlation analysis of the biochemical parameters also revealed meaningful associations that support our understanding of cold stress tolerance in wheat. Consistent with previous studies (Xu et al., 2013), we found positive correlations between osmoprotectants (such as proline), protein and antioxidant enzyme activities (such as SOD, CAT, and peroxidase), suggesting a coordinated response between enzyme activity and protein synthesis. Moreover, we observed a negative association between antioxidants and damage indices (MDA and H_2O_2), which is consistent with the concept that increased antioxidant capacity can lead to a reduction in ROS levels and oxidative stress (Xu et al., 2013; Nejadsadeghi et al., 2014; Ashraf et al., 2019; Hasanuzzaman et al., 2019). The physiological measurements between the cold tolerant and cold susceptible genotypes depicted the contrasting responses between the two groups. In this

TABLE 5 List of cold-tolerant genotypes selected on the basis of various biochemical parameters recorded under (T0) normal conditions, (T1) acclimation phase at 4°C for 14 days, (T2) cold stress at −5°C after acclimation, (T3)cold stress at −5°C without acclimation treatments. SKUA is the local designation for these genotypes in this study. The original IC/EC numbers of these genotypes are provided in Table S1.

Treatment	Genotypes	Protein	Genotypes	MDA	Genotypes	H_2O_2	Genotypes	APX	Genotypes	Proline	Genotypes	SOD	Genotypes	CAT	Genotypes	GPX
T0	SKUA_52	10.82	SKUA_52	0.69	SKUA_46	2.24	SKUA_52	412.18	SKUA_52	5.85	SKUA_52	608.18	SKUA_52	358.00	SKUA_52	202.20
	SKUA_44	9.77	SKUA_107	1.44	SKUA_44	2.47	SKUA_44	399.68	SKUA_74	4.81	SKUA_44	597.68	SKUA_43	353.00	SKUA_118	197.10
	SKUA_116	9.74	SKUA_41	1.52	SKUA_52	2.52	SKUA_74	389.68	SKUA_16	4.61	SKUA_74	585.68	SKUA_90	346.35	SKUA_74	196.00
	SKUA_46	9.47	SKUA_44	1.55	SKUA_43	2.52	SKUA_46	380.90	SKUA_117	4.51	SKUA_90	569.18	SKUA_141	343.60	SKUA_46	195.60
	SKUA_43	9.43	SKUA_73	1.58	SKUA_74	2.6	SKUA_117	377.90	SKUA_90	4.49	SKUA_46	562.90	SKUA_44	343.10	SKUA_43	194.00
T1	SKUA_52	13.03	SKUA_52	8.32	SKUA_52	6.18	SKUA_52	514.18	SKUA_52	11.52	SKUA_52	814.54	SKUA_52	603.20	SKUA_52	226.00
	SKUA_127	12.33	SKUA_118	8.45	SKUA_46	7.52	SKUA_44	496.68	SKUA_116	9.69	SKUA_44	788.03	SKUA_90	596.35	SKUA_44	221.00
	SKUA_6	12.28	SKUA_16	8.65	SKUA_109	8.01	SKUA_74	491.68	SKUA_74	9.63	SKUA_90	775.54	SKUA_73	575.60	SKUA_74	220.40
	SKUA_91	12.23	SKUA_109	8.73	SKUA_107	8.27	SKUA_117	476.90	SKUA_28	9.41	SKUA_74	747.04	SKUA_46	575.00	SKUA_118	220.30
	SKUA_107	12.00	SKUA_90	8.96	SKUA_141	8.34	SKUA_90	468.18	SKUA_2101	9.14	SKUA_46	744.69	SKUA_141	571.60	SKUA_46	220.20
T2	SKUA_52	26.26	SKUA_52	3.64	SKUA_52	3.51	SKUA_52	621.68	SKUA_52	17.19	SKUA_52	1214.54	SKUA_52	845.20	SKUA_74	255.40
	SKUA_6	24.61	SKUA_109	4.11	SKUA_109	3.92	SKUA_44	599.68	SKUA_116	15.36	SKUA_44	1194.03	SKUA_90	828.35	SKUA_52	250.20
	SKUA_127	24.51	SKUA_46	4.4	SKUA_141	4.20	SKUA_74	599.18	SKUA_113	15.30	SKUA_74	1170.04	SKUA_44	809.10	SKUA_118	249.30
	SKUA_91	24.36	SKUA_118	4.78	SKUA_91	4.21	SKUA_117	582.40	SKUA_74	15.08	SKUA_90	1136.54	SKUA_73	807.60	SKUA_46	249.20
	SKUA_107	24.158	SKUA_16	4.81	SKUA_46	4.31	SKUA_90	571.18	SKUA_44	14.81	SKUA_46	1124.69	SKUA_74	807.00	SKUA_43	247.00
T3	SKUA_52	12.49	SKUA_52	7.33	SKUA_52	13.985	SKUA_52	437.18	SKUA_52	7.88	SKUA_52	661.18	SKUA_52	365.20	SKUA_52	210.50
	SKUA_43	11.27	SKUA_109	7.97	SKUA_43	14.29	SKUA_44	417.68	SKUA_46	7.20	SKUA_44	633.68	SKUA_43	364.00	SKUA_74	210.40
	SKUA_116	11.06	SKUA_46	8.85	SKUA_46	14.35	SKUA_74	414.68	SKUA_113	7.12	SKUA_74	626.68	SKUA_90	357.35	SKUA_118	209.30
	SKUA_44	11.01	SKUA_118	9.61	SKUA_16	14.41	SKUA_46	397.90	SKUA_116	7.04	SKUA_90	622.18	SKUA_44	355.10	SKUA_46	209.20
	SKUA_117	10.86	SKUA_16	9.87	SKUA_107	15.09	SKUA_90	389.18	SKUA_117	6.62	SKUA_46	611.90	SKUA_141	354.60	SKUA_43	206.20

MDA = malondialdehyde; H_2O_2 = hydrogen peroxide; SOD = superoxide dismutase; CAT = catalase; GPX = glutathione peroxidase.

TABLE 6 List of Cold susceptible genotypes selected on the basis of various biochemical parameters recorded under (T0) normal conditions, (T1) acclimation phase at 4°C for 14 days, (T2) cold stress at −5°C after acclimation, (T3) cold stress at −5°C without acclimation treatments . SKUA is the local designation for these genotypes in this study. The original IC/EC numbers of these genotypes is provided in Table S1.

Treatment	Genotypes	Protein	Genotypes	MDA	Genotypes	H202	Genotypes	APX	Genotypes	Proline	Genotypes	SOD	Genotypes	CAT	Genotypes	GPX
T0	SKUA_4301	4.12	SKUA_1463	7.74	SKUA_33	8.06	SKUA_3348	130.68	SKUA_2386	2.15	SKUA_3348	312.68	SKUA_1463	222.55	SKUA_4116	109.35
	SKUA_1422	4.40	SKUA_4123	7.82	SKUA_1463	8.09	SKUA_709	121.18	SKUA_1875	2.13	SKUA_80	306.90	SKUA_3348	221.10	SKUA_1463	108.00
	SKUA_1463	4.55	SKUA_4116	7.87	SKUA_3348	8.20	SKUA_80	110.90	SKUA_3329	2.07	SKUA_4301	306.68	SKUA_3329	220.60	SKUA_4123	106.55
	SKUA_3599	4.59	SKUA_1875	8.06	SKUA_4116	8.60	SKUA_4301	108.68	SKUA_1422	2.06	SKUA_1463	305.18	SKUA_4116	219.35	SKUA_2386	105.10
	SKUA_2386	4.60	SKUA_4301	8.09	SKUA_4301	8.87	SKUA_1463	99.90	SKUA_1463	2.05	SKUA_1075	297.9	SKUA_4301	215.95	SKUA_4301	101.95
T1	SKUA_1296	8.69	SKUA_1211	16.06	SKUA_2407	11.84	SKUA_709	220.18	SKUA_2386	6.27	SKUA_3599	505.04	SKUA_2407	452.00	SKUA_1875	132.05
	SKUA_1422	8.64	SKUA_1463	16.16	SKUA_1463	12.09	SKUA_3348	215.68	SKUA_4301	6.25	SKUA_3348	503.03	SKUA_1463	449.55	SKUA_3329	130.80
	SKUA_3599	8.28	SKUA_2386	16.57	SKUA_3348	12.20	SKUA_1463	212.90	SKUA_1422	6.07	SKUA_1463	488.69	SKUA_3348	446.20	SKUA_4123	129.75
	SKUA_1463	8.24	SKUA_4116	17.43	SKUA_4116	12.60	SKUA_4301	205.68	SKUA_3329	6.03	SKUA_1075	479.69	SKUA_2386	443.10	SKUA_2386	128.30
	SKUA_4301	7.81	SKUA_4301	17.65	SKUA_4130	12.87	SKUA_1075	196.90	SKUA_1463	5.80	SKUA_4301	468.04	SKUA_4301	429.00	SKUA_4301	122.15
T2	SKUA_1296	17.48	SKUA_4116	12.44	SKUA_2407	8.63	SKUA_709	325.68	SKUA_3348	10.44	SKUA_3348	624.03	SKUA_41	689.10	SKUA_2412	161.60
	SKUA_1422	17.13	SKUA_2407	12.5	SKUA_3348	8.98	SKUA_3348	320.8	SKUA_1422	10.39	SKUA_1463	612.69	SKUA_2407	687.35	SKUA_3348	160.8
	SKUA_3599	16.38	SKUA_2386	12.75	SKUA_1463	9.09	SKUA_1463	320.3	SKUA_3348	10.32	SKUA_4301	612.04	SKUA_2412	684.00	SKUA_2407	159.3
	SKUA_4301	16.31	SKUA_4301	13.31	SKUA_4301	10.20	SKUA_4301	308.68	SKUA_1463	10.08	SKUA_709	608.54	SKUA_4301	675.95	SKUA_4116	158.75
	SKUA_1463	15.37	SKUA_1463	14.31	SKUA_52	13.985	SKUA_1075	299.90	SKUA_4301	9.99	SKUA_1075	594.69	SKUA_1463	675.35	SKUA_4301	153.15
T3	SKUA_1211	6.22	SKUA_4123	25.02	SKUA_1463	29.09	SKUA_3348	147.68	SKUA_4116	3.59	SKUA_709	358.18	SKUA_1463	234.35	SKUA_2407	121.6
	SKUA_1422	6.07	SKUA_2407	25.1	SKUA_4116	29.60	SKUA_1075	135.90	SKUA_1422	3.57	SKUA_80	355.90	SKUA_4123	232.55	SKUA_1875	120.05
	SKUA_3599	5.83	SKUA_2386	25.55	SKUA_4123	29.75	SKUA_709	132.18	SKUA_709	3.23	SKUA_3348	348.68	SKUA_3329	231.60	SKUA_1463	118.75
	SKUA_4301	5.79	SKUA_1463	26.87	SKUA_1875	30.76	SKUA_4301	126.68	SKUA_4301	2.43	SKUA_1463	347.68	SKUA_2386	231.10	SKUA_2386	118.3
	SKUA_1463	5.36	SKUA_4301	28.87	SKUA_4301	31.87	SKUA_1463	117.90	SKUA_1463	2.39	SKUA_4301	346.90	SKUA_4301	226.95	SKUA_4301	112.15

MDA = malondialdehyde; H_2O_2 = hydrogen peroxide; SOD = superoxide dismutase; CAT = catalase; GPX = glutathione peroxidase.

study, several cold-tolerant lines among the genotypes studied were identified using several key criteria based on the field cold screening data, ELI and biochemical data (Tables 5 and 6). First, genotypes exhibiting lower cold score values (0) and low ELI values were regarded as having improved cold tolerance. Additionally, the selection process favored genotypes demonstrating low MDA value, low H_2O_2 content and higher activities of important antioxidant enzymes, including APX, SOD, CAT, and GPX, as these enzymes play a crucial role in mitigating oxidative damage. Lastly, genotypes characterized by increased accumulation of proline, a compatible solute with cryoprotective properties, were given preference. For cold-susceptible genotypes, certain characteristics were used to distinguish them from cold-tolerant lines. These included higher cold score values, indicating increased susceptibility to cold stress, and elevated ELI values, indicating compromised membrane stability under cold conditions. Furthermore, cold-susceptible genotypes exhibited higher levels of MDA and H_2O_2, indicating higher oxidative damage. The activities of antioxidant enzymes, such as APX, SOD, CAT, and GPX, were comparatively lower in cold-susceptible genotypes, suggesting a reduced capacity to counteract oxidative stress. Furthermore, the comparison between cold-tolerant and cold-susceptible genotypes based on the data obtained in this study revealed significant differences between cold-tolerant and cold-susceptible genotypes in response to cold stress conditions. Overall, cold-tolerant genotypes exhibited higher levels of antioxidants, such as SOD CAT, APX, GPX, and proline, while the opposite trend was observed in cold-susceptible genotypes. For treatments T1 and T2, the tolerant genotype exhibited higher protein levels, proline and antioxidants enzymatic activity than the susceptible genotypes. This indicates genotype-dependent variations of these biochemical parameters in response to different treatments. Furthermore, the susceptible genotype showed increased levels of malondialdehyde (MDA) and H_2O_2, compared to tolerant genotypes in all the treatments, indicating higher oxidative stress than the tolerant genotypes. These findings suggest that the susceptible genotype may be more susceptible to oxidative damage under the given experimental conditions.

In our study, we applied PCA to our biochemical data to gain insights into the underlying structure and identify key biochemical variables contributing to the observed variation. By performing PCA, we were able to condense the information from multiple biochemical variables into a smaller number of dominant principal components (PCs) while retaining the maximum amount of variation in the dataset. This dominant PC represents the major factors driving the variation in our dataset, offering a profound understanding of the biochemical mechanisms underlying cold stress tolerance.

5 | CONCLUSION

In conclusion, our study provides a comprehensive assessment of cold stress tolerance in a diverse set of wheat genotypes through rigorous field screening, electrical conductivity assays, and biochemical profiling. Our study highlights the importance of comprehensive screening efforts to evaluate the cold tolerance of wheat genotypes. The results of this study demonstrated significant genetic variation for cold stress tolerance within the studied germplasm, which indicates cold tolerance is a complex trait, and paves the way for further improvement of this trait. Notably, our biochemical profiling revealed distinct correlations among various antioxidants, MDA, and H_2O_2 levels, highlighting their crucial roles in cold stress response. Overall, our findings underscore the significance of acclimation period as a preconditioning strategy to enhance the plant's adaptive capacity against cold stress. During this acclimation period and subsequent exposure to cold stress, there was a significant increase in the activities of antioxidant enzymes. This rise indicates a reinforced antioxidant defense system triggered specifically during the acclimation phase, strengthening the plant's adaptive capacity against cold stress. These insights into the biochemical responses of plant genotypes under different treatments contribute to the development of strategies for breeding and selecting cold-tolerant varieties. The outcomes of this study have significant implications for wheat breeding programs. The identified genotypes with superior cold stress tolerance and favorable biochemical profiles can serve as valuable genetic resources for developing more resilient wheat varieties. Future research can delve deeper into the underlying molecular mechanisms and explore the potential use of marker-assisted selection to expedite the breeding process. Continued research and collaboration between breeding programs and geneticists are warranted to unlock the full potential of cold-tolerant genotypes and drive advancements in wheat breeding for cold-stress resilience.

AUTHOR CONTRIBUTIONS

Sofora Jan and Reyazul Rouf Mir conceptualized this research. Reyazul Rouf Mir supervised this research. Sofora Jan, Sundeep Kumar, Safoora Shafi, Munaza Yousuf, Ronak Majid, Asif Bashir Shikari and M. Anwar Khan conducted the experiments and collected field and Laboratory based data. Fehim Jeelani helped in analysis of data using statistical tools. Satinder Kaur, Sundeep Kumar, Sanjay Kalia, Kuldeep Singh, Manoj Prasad, Rajeev.K. Varshney and Reyazul Rouf Mir provided guidance, genetic resources, funding and helped in writing and editing of the manuscript. All authors contributed to manuscript revision.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study is available in the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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