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# Cold tolerance in *Cicer arietinum* L. seedlings: multivariate insights into freezing stress resistance

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**Cold Tolerance in *Cicer arietinum* Seedlings: Multivariate Insights into Freezing Stress Resistance**

**Abstract**

To enhance the cultivation of chickpeas (*Cicer arietinum* L.) in regions susceptible to low temperatures, it is imperative to comprehend the physiological and molecular mechanisms that enable plants to tolerate low-temperature stress. This study evaluated eight chickpea genotypes—KAKA and seven cold-resistant lines (MCC194, MCC605, MCC607, MCC613, MCC885, MCC901, and MCC911) from the Mashhad Chickpea Collection (MCC)—under a range of freezing temperatures (0, −6, −10, −12, and −14 °C) using a comprehensive physiological, biochemical, and molecular approach. The plants were cultivated under semi-controlled conditions and underwent a cold acclimation process prior to undergoing freezing treatments. Cold-tolerant genotypes showed higher survival, biomass, and pigment levels under freezing stress, with less electrolyte leakage and more negative osmotic potential. They accumulated more proline, total soluble carbohydrate, phenolic, and antioxidants, especially at –12°C. The results of qRT-PCR showed upregulated expression of *caCAT*, *caPOD*, and *caAPX* as antioxidant genes, particularly in genotypes that were tolerant. Chlorophyll fluorescence confirmed better maximum quantum efficiency of photosystem II in these genotypes after cold exposure. Principal component analysis (PCA) and the correlation matrix revealed significant relationships among the measured indices. Partial least squares structural equation modeling (PLS-SEM) indicated that enzymatic activity was the strongest positive predictor of plant dry weight (β = 0.404), while membrane damage had a strong negative effect on it (β = -0.913). Furthermore, comprehensive membership function analysis ranked genotype MCC911 with the highest evaluation value (0.737) as the most tolerant and genotype KAKA with the lowest value (0.222) as the most sensitive. The findings indicate that maintaining membrane integrity, accumulating osmotically compatible compounds, and, particularly, activating the enzymatic antioxidant system are key mechanisms of freezing tolerance in chickpeas. Genotypes MCC911 and MCC901 are

identified as promising candidates for use in breeding programs aimed at developing cold-tolerant cultivars for autumn planting. Collectively, these findings provide a detailed physiological and molecular framework for cold stress adaptation in chickpea and identify potential markers for breeding cold-resilient cultivars

**Keywords**: Antioxidants, Chickpeas, PLS-SEM

## 1. Introduction

The phenomena of global warming and climate change have heightened heat and drought stress and exacerbated other abiotic and biotic challenges, including freezing, salinity, and flooding [1, 2]. A key point is that unpredictable climate change is the main factor limiting chickpea production because it increases the chances of drought and extreme temperatures, high (over 30°C) and low (under 15°C), significantly lowering grain yields [3, 4, 5]. Therefore, developing chickpea cultivars with higher yields and greater resilience under these stressful conditions is imperative, with particular focus on cold-tolerant genotypes [6, 7].

Autumn chickpea (*Cicer arietinum* L.) is a cool-season legume that is cultivated on a global scale. In spring or summer, under rainfed circumstances, such as in Iran, chickpea cultivation results in diminished grain yields due to drought stress. Chickpeas are often sown in autumn to dodge cold stress and terminal drought [6]. Furthermore, experiments indicate that cold-tolerant autumn or winter-sown chickpeas can yield 50%–100% more than spring-sown crops [8, 9]. However, the scarcity of cold-tolerant genotypes limits the potential for high-yielding winter or autumn sowings [10, 5]. The different ways plants respond to cold across varieties offer opportunities to develop cold-tolerant or cold-resistant varieties. A similar integrated methodology, employing physiological–biochemical markers and molecular data, has been successfully applied to assess abiotic stress tolerance in other crops (11).

Numerous studies on chickpea frost tolerance have shown that plants acquire the ability to endure freezing temperatures when exposed to cold environments, demonstrating

physiological, biochemical, and genetic adaptations that enable them to counteract and overcome the adverse effects of cold stress [12-16]. These processes encompass alterations in the plasma membrane's composition, structure, and function; the production of cryoprotectant molecules (such as soluble sugars and proline); an improved ability to eliminate reactive oxygen species (ROS); and the expression of cold-responsive genes [17-20]. Likewise, cold-tolerant chickpea and legume genotypes demonstrated minimal membrane damage and lipid peroxidation [12], increased levels of antioxidants [21], proline [22], and soluble carbohydrates [23, 24], and minimal decreases in chlorophyll fluorescence [13]. These physiological changes could substantially enhance cold tolerance and facilitate the identification of freeze-tolerant chickpea genotypes.

Furthermore, it is necessary to conduct research to elucidate and inform by analyzing physiological, biochemical, and molecular traits to cultivate crops with robust tolerance to low temperatures. The objective of this investigation was to examine the physiological and molecular responses exhibited by *Cicer arietinum* seedlings under conditions of freezing stress. The current study used various methods, including PCA and PLS-SEM, to thoroughly examine trait relationships and systematically rank genotypes based on their freezing resistance using a comprehensive membership function. This study provides new insights into how chickpea seedlings tolerate cold, highlighting certain types that could be used in breeding programs to improve their ability to withstand freezing conditions under changing climates.

## 2. Material and Method

*Plant material*

Chickpea seedlings were cultivated in a semi-controlled greenhouse at the Research Center for Plant Science (RCPS) at Ferdowsi University of Mashhad, Iran (Latitude: 36°18'38" N - Longitude: 59°32'06" E). Eight chickpea types—cold-tolerant MCC194, MCC605, MCC607, MCC613, MCC885, MCC901, MCC911, and cold-sensitive KAKA—were selected for this

study. The selection of these genotypes was based on a multi-stage evaluation program. This program included severe field-freezing events and complementary morphophysiological and biochemical analyses [16]. The chickpea seeds were sourced from the Mashhad chickpea collection at Ferdowsi University of Mashhad, Iran.

*Growth condition*

Seeds were planted in the first week of September in 15-cm-diameter pots filled with field soil. To apply cold acclimation, the plants were grown under natural conditions until the seedling stage (Fig. 1). On nights when temperatures were likely to drop below 0ºC, the seedlings were protected from the cold with plastic covers. The potted plants were irrigated daily until 24 hours before the freezing treatment.

*Cold stress treatment*

After four months, the cold-acclimated plants were placed in a thermogradient freezer, starting at 5°C, with the temperature gradually decreasing by 2°C per hour. At −2 °C, ice crystal formation was induced by spraying plants with ice-nucleation-active bacteria, as described by Wisniewski et al. (2002) [24]. The pots were held at each freezing point (0, -6, -10, -12, and -14°C) for two hours before being transferred to a growth chamber at 4 ± 1°C for overnight storage to control the melting rate. After that, plants were transferred to standard conditions in an unheated greenhouse (22/18°C day/night temperature, 16-hour photoperiod, 60% relative humidity). After four weeks of regrowth, the survival rate of the plants at each temperature was recorded. A live plant was defined as one that exhibits visible green tissues and the ability to resume growth, such as producing new leaves or shoots, following the removal of stress.

The stress treatments followed a completely randomized design (CRD) with three replications. The second leaf was collected and immediately frozen in liquid nitrogen at each specified time point, then stored at -80°C for further analysis. Three biological replicates, each containing three plants, were used at each time point.

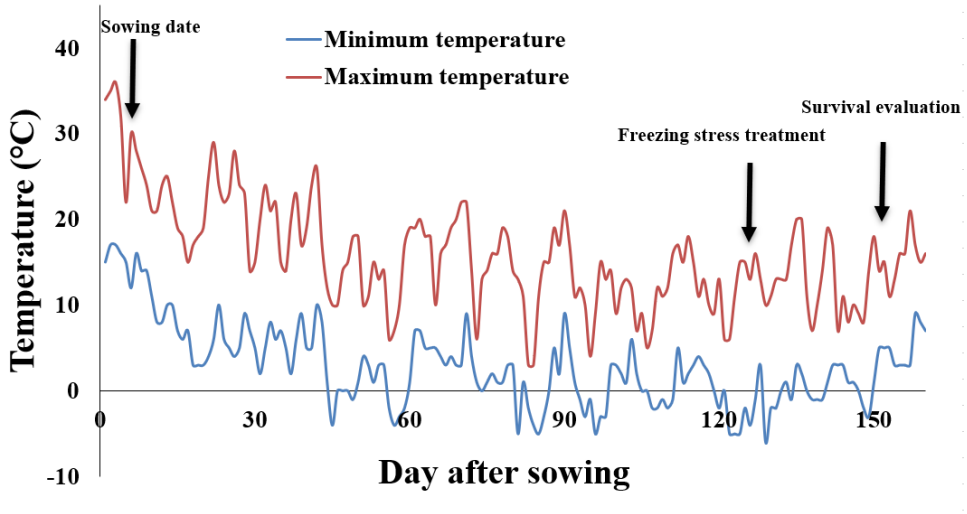


**Fig. 1**. Minimum and maximum daily temperatures at the experimental site under the natural photoperiod.

*Survival rate*

To assess survival percentage, the number of live plants was enumerated after establishment (n) and following the recovery period (m), with survival percentage (S) computed using the following equation [27]:

$$S(\%) = \frac{m}{n} \times 100$$

*Relative electrolyte leakage (EL)*

The youngest fully developed leaves were sectioned into small fragments, and roughly 0.2 grams of each sample was rinsed with deionized water to remove contaminants. The cleaned components were subsequently placed in a bottle containing 10 mL of distilled water and allowed to sit at room temperature for 24 hours. After being incubated for 24 hours, a conductance device (Jenway Model 4510) was used to measure the initial conductance (EC1). The samples were subjected to a 15-minute boiling process (autoclaved) to facilitate the greatest degree of leakage. After a cooling period at laboratory temperatures, the electrolyte

conductivity was remeasured and documented as EC2. The ultimate conductance was determined utilizing the subsequent formula [27, 28]:

$$EL(\%) = \frac{EC1}{EC2} \times 100$$

*Plant dry weight (DW)*

The dry weight of the plants was measured after a three-week recovery period following the freezing treatment by drying them at 80°C for 24 hours. The dry weight was then normalized to the number of plants per pot.

*Physiological and biochemical assessment*

The photosynthetic pigment content (chlorophyll a (Chl a), chlorophyll b (Chl b), carotenoids (Car), and total pigment (TP)) was assessed using the experimental method described by Dere et al. [29]. The sample's total phenolic content (TPC) was determined using the Folin-Ciocalteu method, following Singleton and Rossi. [30]. Free proline content (PC) was measured according to the procedure outlined by Bates et al [31]. The antioxidant capacity of the chickpea leaf extract was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, as described by Abe et al. [32]. Flavonoid content (FC) was quantified following the methodology of Chun et al. [33]. Total soluble carbohydrate (TSC) was determined using the method of Dubois et al. [34].

*Leaf osmotic potential*

Leaf osmotic potential measurements were conducted using an osmometer (OM802-D; Vogel, Germany) [35].

*Antioxidant enzyme activity assessment*

Enzyme extraction from 0.1 g of fresh leaf tissue was performed using a chilled mortar and pestle with 1 ml of extraction buffer (50 mM K-phosphate buffer, pH 7.6, and 0.1 mM $Na_2$-EDTA). The samples were centrifuged at 15,000 g for 15 minutes at 4 °C, and the supernatant

was used to measure catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) activities, following protocols by Velikova et al. [36], Sreenivasulu et al. [37], and Yamaguchi et al. [38].

*Measurement of chlorophyll fluorescence parameters*

Chlorophyll fluorescence parameters were assessed utilizing a pulse-modulated fluorometer (MINI-PAM Portable Chlorophyll Fluorometer, WALZ, Germany). All parameters were measured using the third true leaf in a light-adapted condition [39, 40].

*Quantitative RT-PCR*

Three stress-associated genes, *CAT, POD*, and *APX*, were used for analysis. The nucleotide sequences were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov). The Beacon Designer software was used to design the primer for polymerase chain reaction (PCR). Total RNA was extracted from chickpea seedlings subjected to freezing treatment or control treatment using the Paratous Kit (Iran, A101321) along with RNase-free DNase treatment (SinaClon, Iran, MO5401). 25 ng/µL of total RNA was utilized for first-strand cDNA synthesis in a 20 µL reaction volume. A first-strand cDNA synthesis kit (Parstous, Iran, A101162) was used, and amplification was performed with SYBR® Green Real-Time PCR Master Mix (Paratous, Iran, C101021) on a Bio-Rad real-time PCR machine (CFX96, Version 1.6, Germany) according to the manufacturer's instructions. The *Actin1* (NM_001278957.1) served as an internal control. The thermal cycling protocol was as follows: an initial incubation at 95°C for 1 min, then 40 cycles of 95°C for 30s, 60°C (a primer-specific Tm) for 30s, 72°C for 40s, and a final extension at 72°C for 5 min. The 2^−ΔΔCt method was applied to calculate the relative gene expression levels (41) (A1).

**Table 1**. Primer sequences used this study.

| Gene name | Sequence (5′– > 3′) of forward primer | Sequence (5′– > 3′) of reverse primer | Tm | Product size (bp) | mRNA accession number |
|---|---|---|---|---|---|
| *Actin1* | 5′-CTGTGCTCTCTCTCTTCCTCTC-3′ | 5′-CGTCTGCCATCTTCTAATATCTTCG-3′ | 60°C | 110 | NM_001278957.1 |

| | | | | | |
|---|---|---|---|---|---|
| *caCAT* | 5′-GAGAAGGGTGTAGTCTAGTGGT-3′ | 5′-AGAGGATGAGGAGAAACGAAGA-3′ | 60°C | 105 | XM_004500820.3 |
| *caAPX* | 5′-GCCAACGCTATATCAGTCACAC-3′ | 5′-AAACCAAACGAAGCACACCC-3′ | 60°C | 110 | XM_004501278.3 |
| *caPOD* | 5′-GGGTTGCGATGGGTCAGTATTA-3′ | 5′-AGACGATTCTTCCACACTGCTT-3′ | 60°C | 149 | XM_004496386.3 |

*Statistical analysis*

*Experiment design and data analysis*

An analysis of variance (ANOVA) was performed using Minitab 16.0, with mean separation performed using the least significant difference (LSD) procedure at the 0.05 and 0.01 levels of significance (A2 and A3). To ensure data uniformity, a normalization process was implemented prior to PCA and the subsequent generation of the heat map, and PLS-SEM analysis using the R environment (version 4.4.2). The data were uploaded to Origin (Origin Lab Corporation, v.2021) software for graphical representation. The membership function is calculated using normal and uniform data, as described by Cao et al. [42] and Sun et al. [43] (A4).

## 3. Results

*Physiological and biochemical indicators pattern*

*Survival rate and L50*

The tolerant genotype seedlings demonstrated 100% survival, whereas KAKA seedlings exhibited survival rates of 100%, 95%, 80%, and 70% under freezing stress at 0, –6, –10, and –12°C, respectively. At –14°C, both tolerant and sensitive genotype seedlings exhibited zero survival. The $LT_{50}$ values differed significantly among the genotypes. KAKA exhibited a higher (less negative) $LT_{50}$ value, while all other genotypes showed significantly lower values that were statistically similar (A5).

*Relative electrolyte leakage (EL)*

The extent of damage to the plants was assessed by measuring the electrolyte leakage index after freezing stress. As the temperature decreased, the EL leaf and crown of all seedlings exhibited a considerable rise, which was most pronounced at the -14°C treatment (Fig. 2a and b; $p < 0.01$). Leaf EL showed a marginal increase with a slight upward trend up to –10°C, except for MCC613 and KAKA; however, it showed a significant increase as the temperature decreased from –10 to –14°C, except for MCC911 and MCC194.

*Plant dry weight (DW)*

The genotypes' dry weight was significantly influenced by temperature, genotype, and their interaction (Fig. 2c; $p < 0.01$). The dry weight across all genotypes exhibits a declining trend, while the KAKA (sensitive genotype) shows a more pronounced reduction, particularly at 0°C, where it decreased by 1.98 - 1.59 times compared to tolerant genotypes.

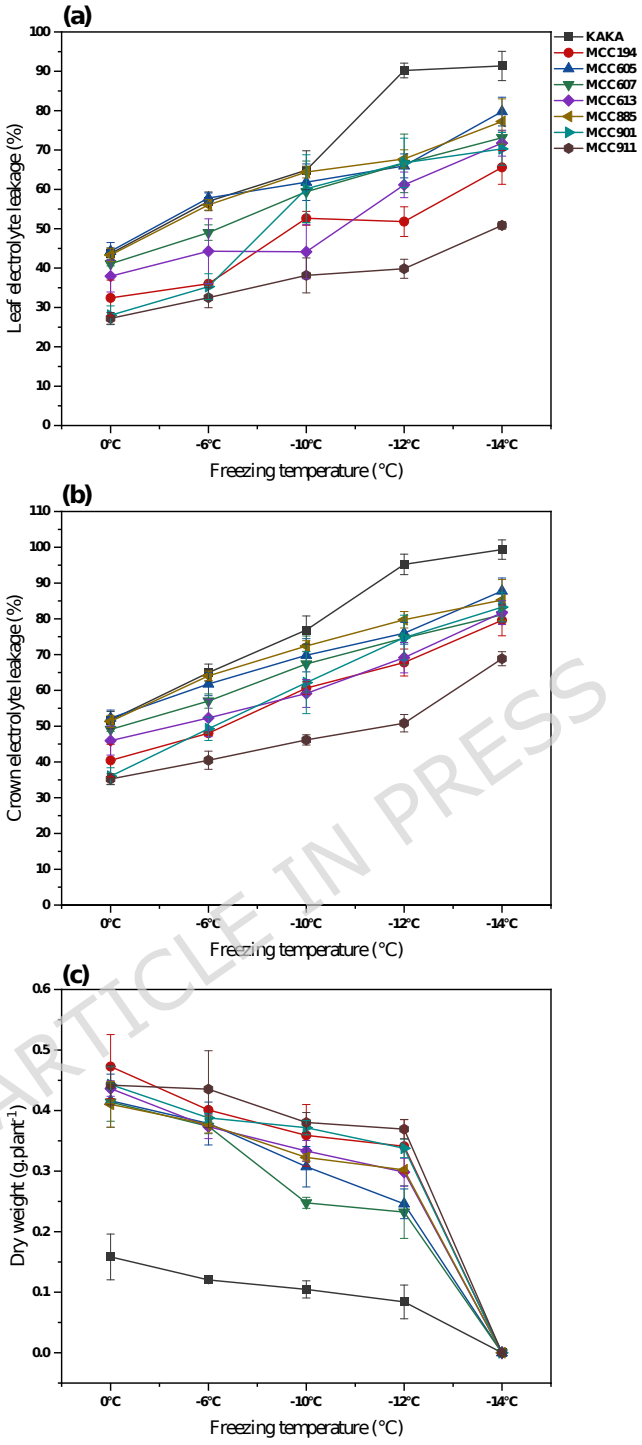


**Fig. 2.** Membrane damage as relative electrolyte leakage in leaves (a) and crown (b) in *Cicer arietinum* (KAKA, MCC194, MCC605, MCC607, MCC613, MCC885, MCC901, and MCC911)

under different freezing temperatures, and dry weight (c) evaluated three weeks following freezing stress. Vertical bars represent the least significant difference (LSD; $p = 0.05$) at each temperature.

*Photosynthetic pigments*

Chlorophyll a (Chla) and total pigment (TP) exhibited substantial variation across genotypes, temperatures, and their interaction ($p < 0.001$). Freezing stress (0°C, -6°C, -10°C,- 12 °C, and -14 °C) exerted a comparable influence on Chla and TP (Fig. 3a and b). In all plants, freezing stress markedly reduced Chla compared to their respective controls, with a greater decline observed in cold-sensitive genotypes (28.8%–1.4-fold) than in cold-tolerant genotypes (1.5%–81.5%). Freezing stress reduces total pigment levels in all eight genotypes, particularly in cold-sensitive genotypes. Tolerant genotypes exhibited a narrower range of 3.3–55.4% in pigmentation, while the genotype KAKA, which was more likely to be affected by cold stress, lost 20.1-90.8%. (relative to the control, Fig. 3b).

*Proline content (PC)*

The leaf PC outcomes showed significant variation by genotype and temperature (Fig. 3c; $p < 0.01$). The PC exhibited a repetitive fluctuation pattern, marked by increases at 0°C, –6°C, –10°C, and –12°C, followed by a reduction at –14°C. Compared with the control, freezing stress increased PC levels, especially in cold-tolerant genotypes (13.7–96%), whereas the cold-sensitive genotype KAKA showed lower increases (7.4–51.5%).

*Total soluble carbohydrate (TSC)*

The result showed that genotype, temperature, and the genotype × temperature interaction significantly affected soluble sugar levels in seedlings (Fig. 3d; $p < 0.001$). The amount of TSC varied over time, rising at 0°C, –6°C, –10°C, and –12°C, followed by falling at –14°C. All genotypes showed a markedly elevated TSS at –12°C (1.01-fold in KAKA and 2.75-fold in MCC607 compared to the control).

*Total phenol content (TPC)*

The leaf phenol data showed considerable variance across genotype, temperature, and the genotype-temperature interaction (Fig. 3e; $p < 0.05$). Total phenol content decreased initially on exposure to 0°C compared to the control in all genotypes (25.9% in KAKA and 2.07-13.85% in other genotypes). Subsequently, the TPC exhibited a significant increase at –6°C, –10°C, –12°C, and –14°C, reaching a maximum of 26.9% at –12°C in MCC901. The amount of TPC went up a little at –6, –10, –12, and –14, but it went down compared to the control in KAKA (5.7–29.6%) (Fig. 3e).

*Leaf osmotic potential (OP)*

The osmotic potential changed significantly depending on the genotype, temperature, and how they affected each other (Fig. 3f; $p < 0.001$). As the temperature decreased, the OP of all seedlings decreased considerably, most notably at 0°C, where KAKA declined by 42.5%. At the same time, other genotypes saw reductions ranging from 7.6% to 29.5% compared to the control.

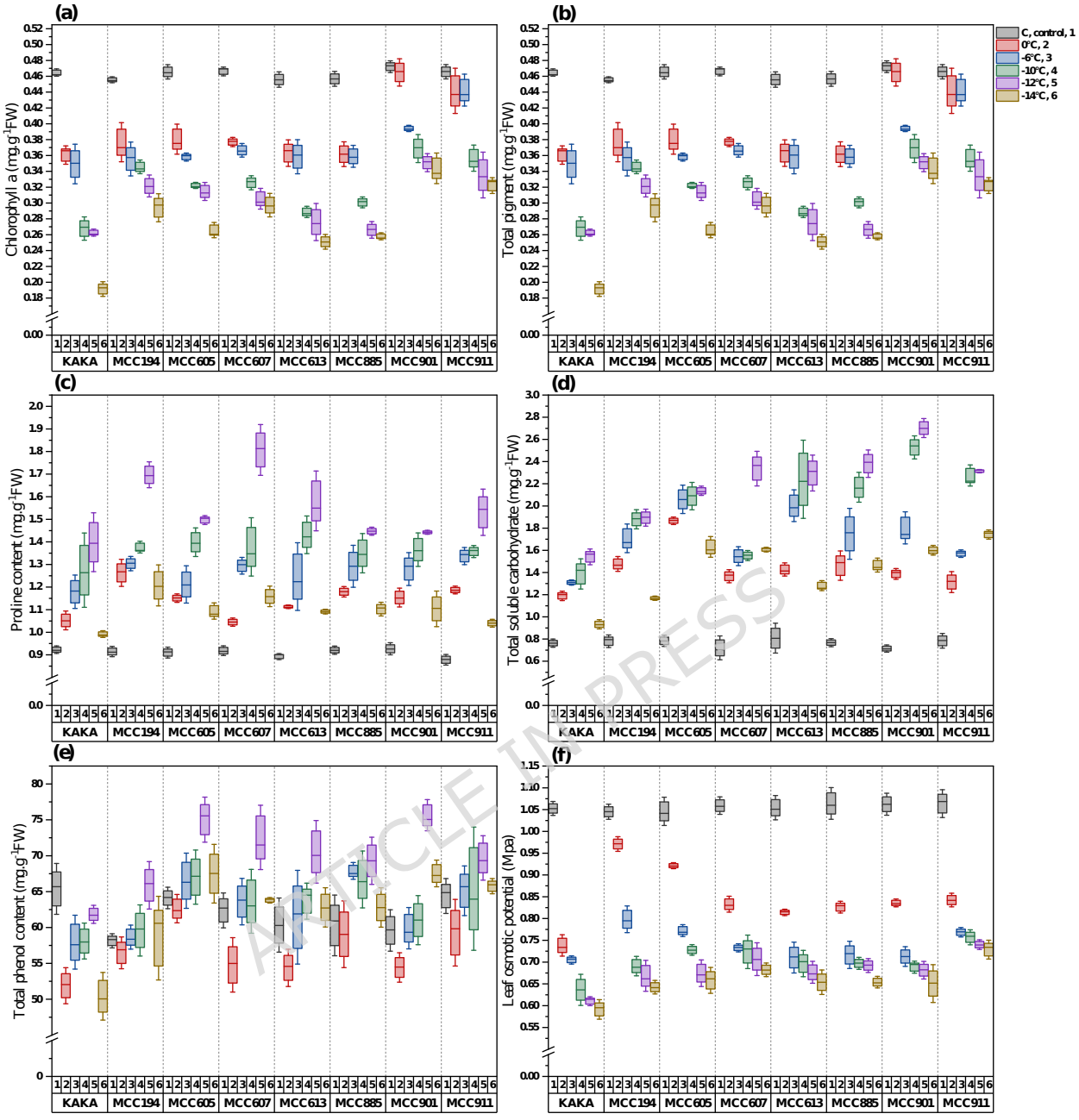


**Fig. 3.** Content of chlorophyll a (a), total pigment (b), proline content (c), total soluble carbohydrate (d), total phenol content (e), and osmotic potential of leaf (f) at different freezing temperatures (data shown are the mean ± SD).

*Enzymatic antioxidants activity*

The CAT, POD, and APX activities all changed in a similar way (sigmoidal response curve): initially rising at 0°C, –6°C, –10°C, and –12°C, and thereafter declining at –14°C (Fig. 4a, b, c). MCC901's CAT activity (3.1-fold vs. control) was highest at –12 °C, while KAKA's (1.6-fold) activity was lowest at –12 °C. The maximum and minimum POD activity levels were 83% and 38.3% for MCC911 and KAKA, respectively, at –12°C in comparison to the control (Fig. 4b). Likewise, MCC911 demonstrated the highest APX activity (4.1-fold relative to the control) at –12 °C, while KAKA exhibited the lowest activity (1.6-fold) at the same temperature (Fig. 4c). At a temperature of –14°C, the activities of CAT, POD, and APX showed decreasing trends in all eight genotypes. The CAT, POD, and APX levels dropped the least in KAKA when exposed to –14°C, by 46%, 17%, and 29%, respectively (Fig. 4a, b, c).

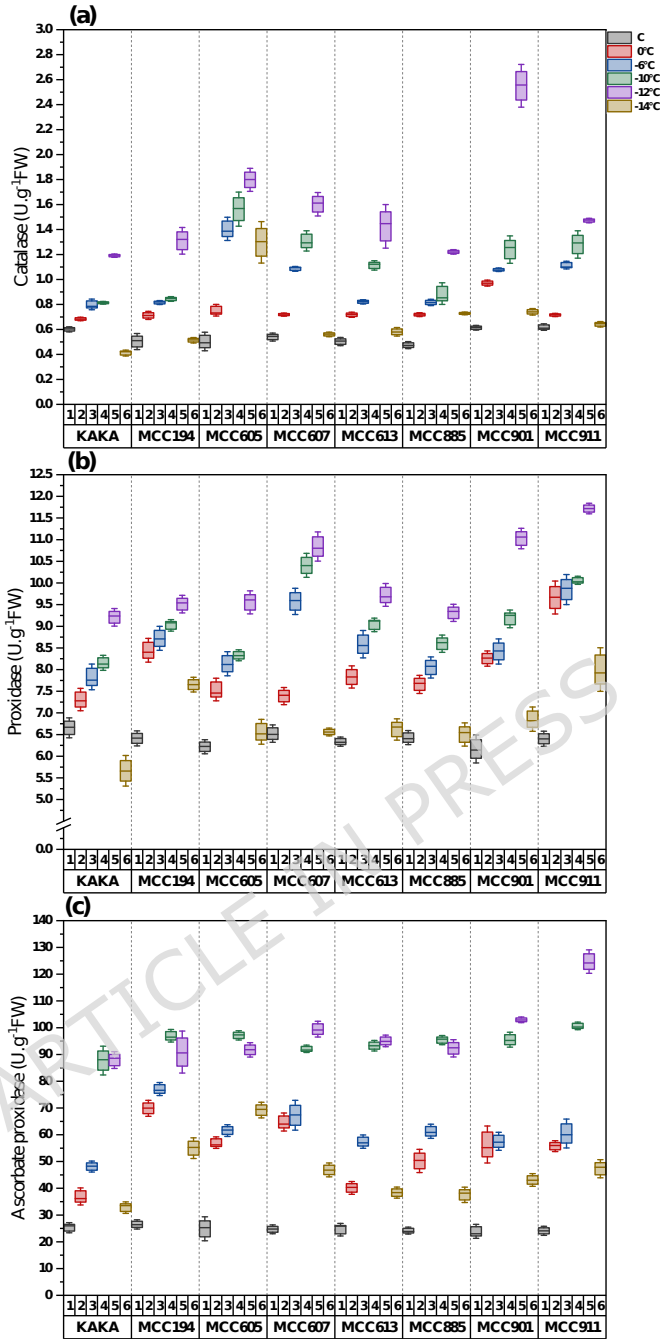


**Fig. 4.** CAT (a), POD (b), and APX (c) enzyme activity in eight genotypes at different freezing temperatures (mean ± SD).

*Chlorophyll fluorescence parameters*

The findings indicated that the maximum quantum efficiency of photosystem II (Fv′/Fm′) did not significantly differ among genotypes prior to the beginning of freezing stress (Fig. 5). The Fv′/Fm′ ratio markedly diminished after 24 hours at 0°C, –6°C, and –10°C, although it exhibited relative stability after 48 hours across all genotypes. A significant rise in Fv′/Fm′ was noted after 72 hours. At -12°C, all six genotypes showed the same trend: a decline after 24 hours, stability at 48 hours, and a pronounced increase after 72 hours, except for MCC613 and KAKA. The Fv′/Fm′ ratio of MCC613 and KAKA showed a significant decrease at 24 h after freezing stress compared to other genotypes. Afterward, it increased sharply. The distinctions among the cold-tolerant genotypes were evident at –14°C. KAKA is unable to recover 120 hours post-freezing stress, unlike other genotypes.

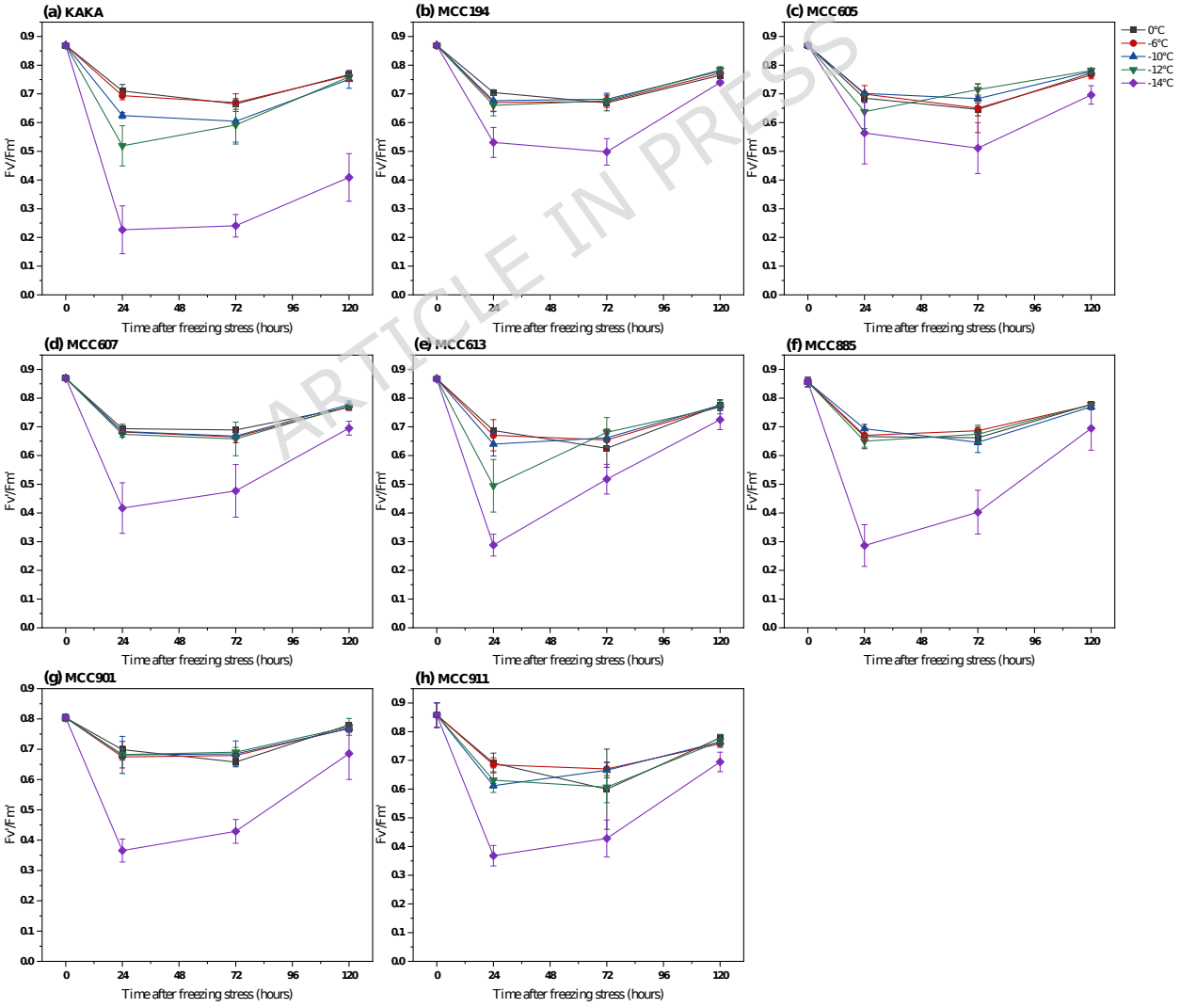

**Fig. 5.** The maximum quantum efficiency of photosystem II (F′v/F′m) of eight chickpea genotypes under different freezing temperatures. Vertical bars represent LSD (p = 0.05) at each temperature. 0 hours = before stress.

*Change in gene expression*

The antioxidant gene expression profile showed that CAT, POD, and APX transcription quickly increased at 0°C, –6°C, –10°C, and –12°C. However, expression dropped significantly at –14°C across all genotypes. The peak expression of *caCAT*, *caPOD*, and *caAPX* occurred at –12°C. MCC901, a cold-tolerant genotype, exhibited the highest levels of *caCAT*, *caPOD*, and *caAPX* (11.3, 9.7, and 11.29-fold higher than the control, respectively). In KAKA, a cold-sensitive genotype, the highest levels of *caCAT*, *caPOD*, and *caAPX* were also found at –12°C (3.7, 6.4, and 9.6-fold higher than the control, respectively) (Fig. 6a, b, c).

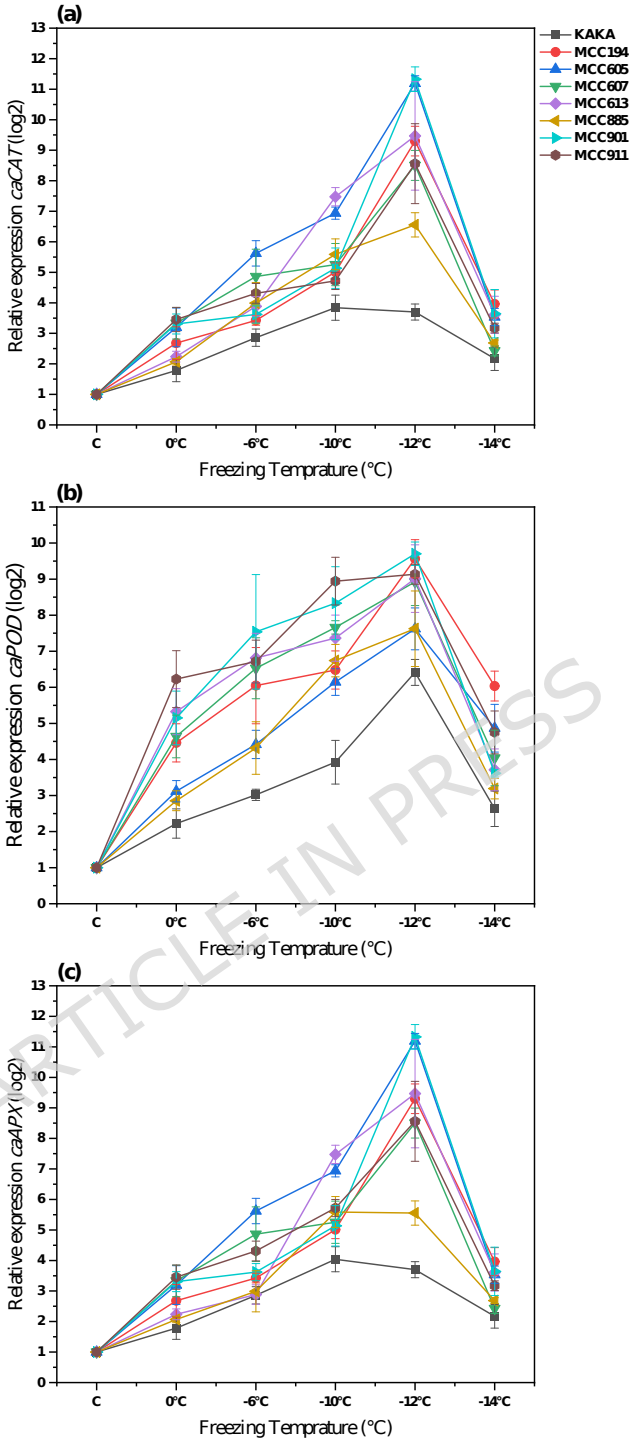


**Fig. 6.** The antioxidant gene expression profile *caCAT* (a), *caPOD* (b), and *caAPX* (c) in chickpeas at different freezing temperatures. Error bars represent SD (n=3)**.**

*Identification and comprehension of treatment-variable interrelations using PCA, and correlation analysis*

*Principle Component Analysis (PCA)*

PCA analysis and correlation were carried out to identify the links between the different parameters in the treatment groups, thereby facilitating a thorough understanding of the interactions among freezing tolerance indices across all freezing temperature treatments (Fig. 7). The PCA demonstrated that the first principal component (PC1) attained the maximum eigenvalue of 7.85, accounting for 60.4% of the variability. In contrast, the second principal component (PC2) had an eigenvalue of 3.18, accounting for 24.50% of the variance. PC1 exhibited substantial negative loadings for Chla, TP, OP, Fv′/Fm′, and DW, alongside positive loadings for the ELL, ELC, and APX parameters. PC2 was primarily defined by positive loadings on POD and CAT. TPC was the primary factor influencing PC3, which exhibited the most significant negative loading. The hue of the variables in the biplot indicates, based on the square of the cosine, that TPC is less significant than the other variables. Furthermore, the biplot illustrates the systematic classification of genotypes into six distinct groups, clearly differentiating between the control and five treatment groups (Fig. 7).

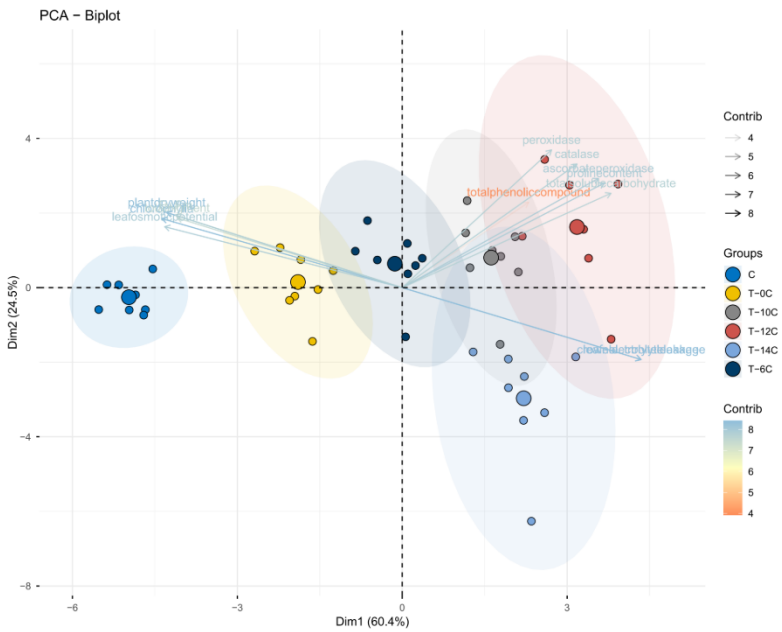


**Fig. 7**. Principal component analysis (PCA) illustrate treatment–variable relationships under different freezing temperatures

*Correlation matrix*

The Pearson correlation coefficients are displayed in the correlation matrix (Fig. 8). These values indicate the degree of linear relationship between two variables and enhance insights from the PCA analysis. Among the physiological and biochemical parameters, DW demonstrated significant positive correlations with OP (0.87), Chla (0.89), Fv′/Fm′ (0.92), and TP (0.86) ($p<0.001$) and negative correlations with APX (-0.36; $p<0.05$), CAT (-0.29; $p<0.05$), TSC (-0.47; $p<0.01$), PC (-0.38; $p<0.05$), ELL, and ELC (-0.91; $p<0.001$). Conversely, DW exhibited negative statistically insignificant associations with POD and TPC. There was a positive relationship between the expression levels of the caCAT, caAPX, and caPOD genes and the activities of the enzymes CAT (0.87), APX (0.86), and POD (0.89), respectively ($p<0.001$). Moreover, the antioxidant enzymes APX, POD, and CAT correlated highly with the non-enzymatic antioxidant parameters of TSC (0.88, 0.76, and 0.80), TPC (0.46, 0.48, and 0.61), and PC (0.87, 0.85, and 0.84), respectively ($p<0.001$). APX was negatively correlated with the photosynthetic parameters Chla, TP, and Fv′/Fm′ (-0.44, -0.40,

and -0.36, respectively, at $p<0.01$ and $p<0.05$), but CAT and POD showed insignificant negative correlations.

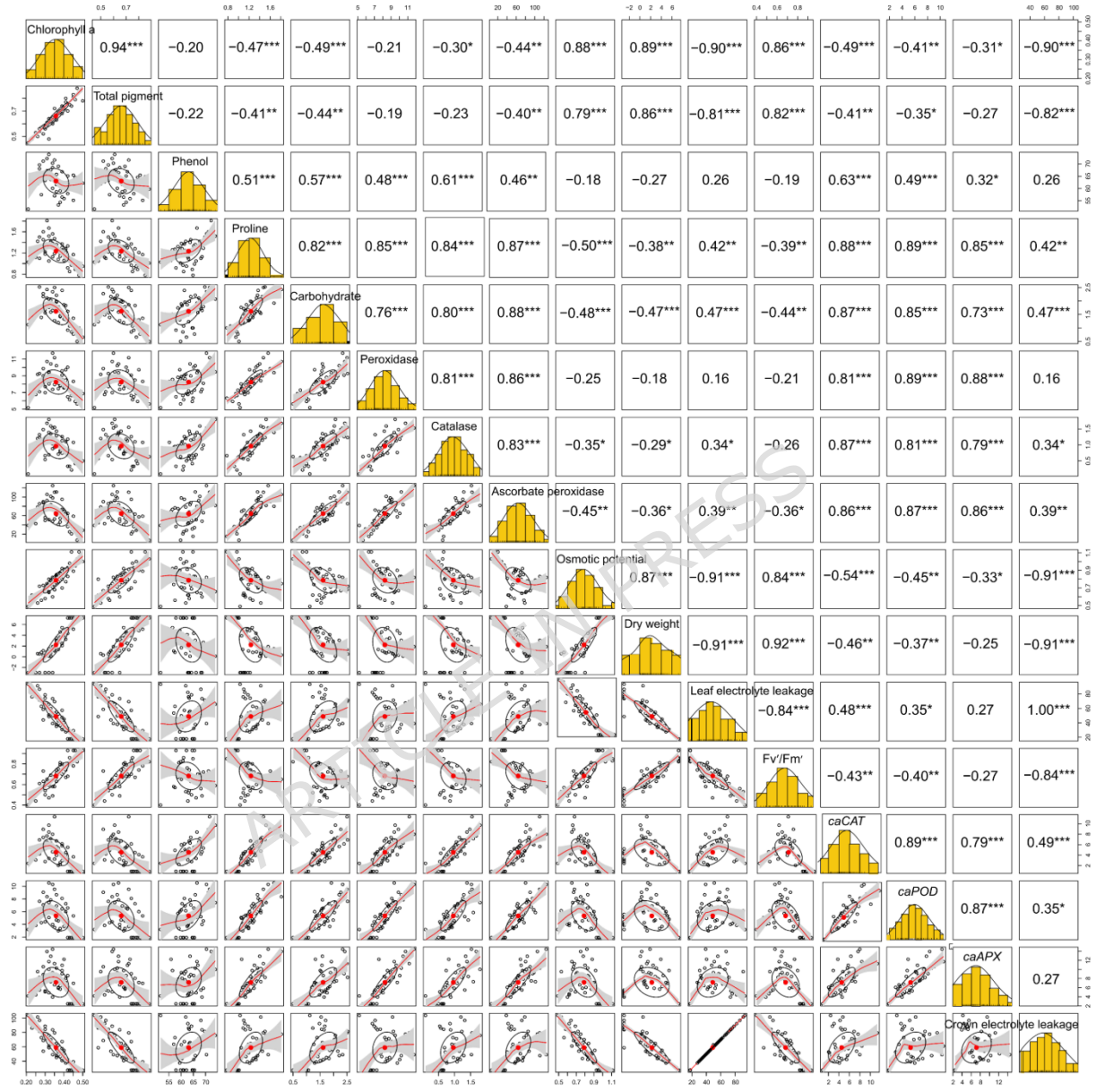


**Fig. 8.** Pearson correlation matrix shows variable relationships under different freezing temperatures.

*PLS-SEM analysis*

The partial least squares structural equation modeling (PLS-SEM) analysis was conducted with six latent variables (freezing temperature, membrane damage, enzyme activity, non-enzymatic compounds, photosynthetic pigments, and plant dry weight) and 11 visible variables. As demonstrated in Figure 9, the final PLS-SEM model is illustrated. The model's goodness-of-fit (GoF) index was 0.842 (A6-A9).

The coefficients of determination ($R^2$) for the endogenous variables indicated substantial explanatory power of the model: non-enzymatic compounds ($R^2 = 0.876$), enzyme activity ($R^2 = 0.867$), membrane damage ($R^2 = 0.819$), photosynthetic pigments ($R^2 = 0.748$), and plant dry mass ($R^2 = 0.618$).

Evaluation of the measurement (outer) model showed that the Average Variance Extracted (AVE) for all constructs exceeded the recommended threshold of 0.50, confirming convergent validity: non-enzymatic compounds (0.907), enzyme activity (0.862), membrane damage (0.928), photosynthetic pigments (0.930), and plant dry mass (1.000). Internal consistency reliability was also satisfactory, with Cronbach's alpha values ranging from 0.898 to 0.925 for multi-indicator constructs and 1.000 for single-indicator constructs. Composite reliability values (DG.rho) were all above 0.94, further supporting strong measurement reliability. In addition, all indicator loadings were high (≥ 0.90), demonstrating good indicator reliability.



In the structural model, freezing temperature exerted strong and statistically significant positive effects on membrane damage ($\beta = 0.905$, $p < 0.001$), enzyme activity ($\beta = 1.078$, $p < 0.001$), and non-enzymatic compounds ($\beta = 0.591$, $p < 0.001$), while significantly decreasing photosynthetic pigments ($\beta = -0.987$, $p < 0.001$). The direct effect of freezing temperature on plant dry mass was negative and statistically significant, though weaker in magnitude ($\beta = -0.529$, $p = 0.043$).

Membrane damage had a strong negative effect on plant dry mass ($\beta = -0.913$, $p < 0.001$) and photosynthetic pigments ($\beta = -0.427$, $p < 0.001$). It also showed a small but significant

negative effect on enzyme activity (β = −0.166, p = 0.039), while its direct effect on non-enzymatic compounds was not statistically significant. Enzyme activity positively influenced photosynthetic pigments (β = 0.619, p < 0.001), non-enzymatic compounds (β = 0.436, p < 0.001), and plant dry mass (β = 0.404, p = 0.036). Non-enzymatic compounds also had a strong positive effect on plant dry mass (β = 0.736, p < 0.001). In contrast, the direct effect of photosynthetic pigments on plant dry mass was not statistically significant (β = 0.179, p = 0.125).

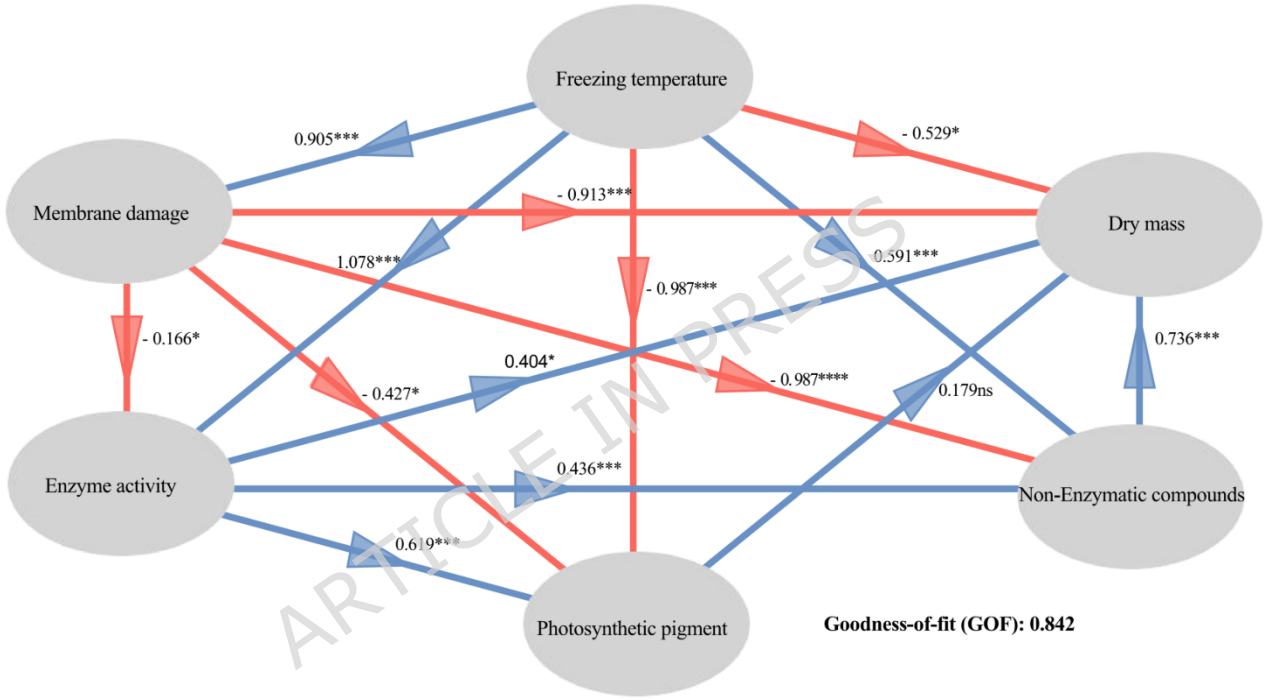


**Fig. 9**. The PLS-SEM model illustrates the causal relationship between the impacts of freezing temperatures on chickpeas. The red arrow denotes a negative correlation, while the blue arrow signifies a positive correlation. The significance of each correlation is indicated by ($^{ns}$ p > 0.05, $^{*}$ p < 0.05, $^{**}$ p < 0.001, $^{***}$ p < 0.001).

*Assessment of freezing resistance in chickpea genotypes by thorough, comprehensive membership value*

By applying PCA with CTC to 12 attributes (Chla, TP, PC, TSC, APX, CAT, POD, OP, ELL, ELC, FV′/FM′ , and DW) at freezing temperatures (0°C, -6°C, -10°C, -12°C, and -14°C), a comprehensive index was created from the first five principal components, which was then used in membership function analysis. Table 2 presents the comprehensive evaluation value (D) for the eight chickpea genotypes. As anticipated, the KAKA genotype demonstrated the lowest evaluation value (0.222), signifying its sensitivity. In contrast, the MCC911 genotype had the highest evaluation value (0.737). An evaluation was conducted to ascertain the cold-tolerance capabilities of the eight chickpea genotypes, which were subsequently ranked as follows:

KAKA = 0.222< MCC607 = 0.563 < MCC885 = 0.583 < MCC605 = 0.628 < MCC613 = 0.640 < MCC194 = 0.653, MCC901 = 0.657 < MCC911 = 0.737

**Table. 2.** Comparison of the comprehensive index, degree of membership (Ui), weight, and comprehensive evaluation value (D) for chickpea genotypes under different freezing temperatures

| Genotype | Comprehensive index | | | | | Ui value | | | | | D value at 0°C |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PC1 | PC2 | PC3 | PC4 | PC5 | U1 | U2 | U3 | U4 | U5 | |
| **KAKA** | -4.709 | -1.408 | -0.053 | -0.763 | 0.190 | 0.000 | 0.267 | 0.522 | 0.369 | 0.598 | 0.208 |
| **MCC194** | 1.152 | -0.272 | -2.307 | -0.003 | -0.318 | 0.848 | 0.501 | 0.000 | 0.605 | 0.409 | 0.544 |
| **MCC605** | -0.523 | 2.155 | -1.491 | 0.961 | 0.533 | 0.606 | 1.000 | 0.189 | 0.905 | 0.725 | 0.631 |
| **MCC607** | -1.386 | 0.995 | 0.896 | 0.717 | -1.420 | 0.481 | 0.761 | 0.742 | 0.829 | 0.000 | 0.562 |
| **MCC613** | 0.648 | -0.788 | 0.316 | -0.110 | 1.274 | 0.775 | 0.395 | 0.607 | 0.572 | 1.000 | 0.614 |
| **MCC885** | 0.754 | 1.876 | 2.011 | -0.121 | 0.520 | 0.790 | 0.943 | 1.000 | 0.568 | 0.720 | 0.790 |
| **MCC901** | 2.202 | 0.150 | -0.070 | -1.948 | -0.573 | 1.000 | 0.588 | 0.518 | 0.000 | 0.315 | 0.649 |
| **MCC911** | 1.862 | -2.709 | 0.699 | 1.266 | -0.207 | 0.951 | 0.000 | 0.696 | 1.000 | 0.450 | 0.618 |
| **Weight** | | | | | | 0.417 | 0.228 | 0.155 | 0.089 | 0.056 | |
| **Genotype** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** | **U1** | **U2** | **U3** | **U4** | **U5** | **D value at -6°C** |
| **KAKA** | -5.043 | -0.004 | -1.224 | 0.453 | -0.172 | 0.000 | 0.478 | 0.039 | 0.667 | 0.386 | 0.210 |
| **MCC194** | -0.031 | 1.429 | 0.322 | -1.227 | 1.375 | 0.697 | 0.730 | 0.419 | 0.129 | 1.000 | 0.603 |
| **MCC605** | -0.906 | -0.974 | 2.684 | -0.324 | -0.763 | 0.576 | 0.307 | 1.000 | 0.418 | 0.151 | 0.511 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **MCC607** | 0.135 | -1.209 | 0.096 | -0.386 | 0.665 | 0.721 | 0.266 | 0.364 | 0.398 | 0.718 | 0.501 |
| **MCC613** | 0.673 | -0.055 | 0.245 | 0.706 | 0.013 | 0.795 | 0.469 | 0.400 | 0.748 | 0.459 | 0.610 |
| **MCC885** | 2.144 | -2.724 | -0.925 | 0.914 | 0.233 | 1.000 | 0.000 | 0.113 | 0.814 | 0.546 | 0.551 |
| **MCC901** | 1.585 | 0.571 | -1.383 | -1.629 | -1.143 | 0.922 | 0.579 | 0.000 | 0.000 | 0.000 | 0.539 |
| **MCC911** | 1.442 | 2.967 | 0.185 | 1.493 | -0.208 | 0.902 | 1.000 | 0.386 | 1.000 | 0.371 | 0.806 |
| **Weight** | | | | | | 0.427 | 0.250 | 0.139 | 0.097 | 0.052 | |

| **Genotype** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** | **U1** | **U2** | **U3** | **U4** | **U5** | **D value at -10°C** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **KAKA** | -5.403 | -0.752 | -0.889 | -0.168 | 0.284 | 0.000 | 0.443 | 0.312 | 0.379 | 0.761 | 0.207 |
| **MCC194** | 0.425 | -0.183 | 0.237 | -1.395 | -1.466 | 0.729 | 0.572 | 0.555 | 0.000 | 0.000 | 0.553 |
| **MCC605** | 0.293 | 1.321 | 2.300 | -0.328 | 0.692 | 0.713 | 0.912 | 1.000 | 0.329 | 0.938 | 0.757 |
| **MCC607** | -0.442 | 0.628 | 0.870 | -0.104 | 0.257 | 0.621 | 0.755 | 0.692 | 0.399 | 0.749 | 0.627 |
| **MCC613** | 0.557 | 1.709 | -0.480 | 0.513 | -0.024 | 0.746 | 1.000 | 0.400 | 0.589 | 0.627 | 0.698 |
| **MCC885** | 0.345 | -1.538 | 0.836 | 1.845 | -0.670 | 0.719 | 0.265 | 0.684 | 1.000 | 0.346 | 0.594 |
| **MCC901** | 2.589 | -2.708 | -0.537 | -0.622 | 0.835 | 1.000 | 0.000 | 0.388 | 0.238 | 1.000 | 0.597 |
| **MCC911** | 1.637 | 1.523 | -2.336 | 0.258 | 0.093 | 0.881 | 0.958 | 0.000 | 0.510 | 0.678 | 0.685 |
| **Weight** | | | | | | 0.470 | 0.210 | 0.162 | 0.074 | 0.047 | |

| **Genotype** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** | **U1** | **U2** | **U3** | **U4** | **U5** | **D value at -12°C** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **KAKA** | -5.598 | -0.064 | -0.237 | 0.778 | 0.029 | 0.000 | 0.587 | 0.460 | 0.828 | 0.407 | 0.270 |
| **MCC194** | 0.820 | 1.592 | 1.883 | 0.883 | -0.667 | 0.844 | 1.000 | 1.000 | 0.864 | 0.156 | 0.800 |
| **MCC605** | 0.014 | 0.997 | 1.051 | -1.269 | 0.433 | 0.738 | 0.852 | 0.788 | 0.142 | 0.553 | 0.639 |
| **MCC607** | 0.547 | -0.155 | -0.738 | -1.691 | -1.099 | 0.809 | 0.565 | 0.333 | 0.000 | 0.000 | 0.522 |
| **MCC613** | 0.783 | 0.451 | -0.445 | -0.483 | 1.674 | 0.840 | 0.716 | 0.407 | 0.406 | 1.000 | 0.675 |
| **MCC885** | -0.399 | -1.631 | -0.225 | -0.670 | -0.288 | 0.684 | 0.197 | 0.463 | 0.343 | 0.292 | 0.474 |
| **MCC901** | 2.003 | -2.421 | 0.755 | 1.163 | 0.181 | 1.000 | 0.000 | 0.713 | 0.958 | 0.462 | 0.704 |
| **MCC911** | 1.829 | 1.231 | -2.043 | 1.289 | -0.264 | 0.977 | 0.910 | 0.000 | 1.000 | 0.301 | 0.751 |
| **Weight** | | | | | | 0.481 | 0.164 | 0.122 | 0.114 | 0.058 | |

| **Genotype** | **PC1** | **PC2** | **PC3** | **PC4** | **PC5** | **U1** | **U2** | **U3** | **U4** | **U5** | **D value at -14°C** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **KAKA** | -5.935 | 0.158 | 0.191 | 0.137 | 0.210 | 0.000 | 0.385 | 0.593 | 0.582 | 0.603 | 0.215 |
| **MCC194** | 1.512 | -0.024 | 0.799 | 1.312 | -0.922 | 0.972 | 0.344 | 0.760 | 1.000 | 0.061 | 0.766 |
| **MCC605** | 1.730 | -1.220 | -1.958 | -0.650 | 0.087 | 1.000 | 0.069 | 0.000 | 0.303 | 0.544 | 0.603 |
| **MCC607** | 0.099 | -0.123 | 1.256 | -1.501 | -1.050 | 0.787 | 0.321 | 0.886 | 0.000 | 0.000 | 0.601 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **MCC613** | -0.094 | 0.910 | -1.508 | 1.043 | -0.403 | 0.762 | 0.558 | 0.124 | 0.904 | 0.310 | 0.605 |
| **MCC885** | -0.455 | -1.521 | -0.479 | -0.304 | 0.369 | 0.715 | 0.000 | 0.408 | 0.426 | 0.679 | 0.507 |
| **MCC901** | 1.669 | -1.013 | 1.668 | 0.591 | 1.039 | 0.992 | 0.117 | 1.000 | 0.744 | 1.000 | 0.796 |
| **MCC911** | 1.475 | 2.835 | 0.031 | -0.627 | 0.671 | 0.967 | 1.000 | 0.549 | 0.311 | 0.824 | 0.825 |
| **Weight** | | | | | | 0.544 | 0.163 | 0.135 | 0.075 | 0.046 | |

**Average of D value:**
KAKA =0.222, MCC607=0.563, MCC885=0.583, MCC605=0.628, MCC613=0.640, MCC194=0.653, MCC901=0.657, MCC911=0.737

MCC, Mashhad Chickpea Collection

## 4. Discussion

Accordingly, it is evident that, based on the cultivation season, freezing stress significantly impedes the growth and development of chickpeas [44, 5]. A prior study involving genotypic screening of cold-tolerant genotypes with elevated yield identified high-performing genotypes (MCC607, MCC885, MCC605, MCC613, MCC194, MCC901, and MCC911) that performed well in field settings [16]. The present work analyzed the performance of seven top-freezing-tolerant chickpea genotypes, alongside KAKA, a sensitive genotype, under freezing stress at physiological, biochemical, and molecular levels. All genotypes exhibited substantial changes in character under stress compared to the control treatment. The findings of this research and the subsequent multivariate analysis, including PCA and PLS-SEM, yielded novel insights into the interrelationships among traits.

Freezing resistance can be conceptualized as a multifaceted quantitative trait that involves the activation of numerous cold-inducible genes in response to cold. These genes play a crucial role in helping plants survive under extreme cold conditions [45]. Several mechanisms, including changes in morphological patterns and physiological and biochemical processes, have been documented to help plants adapt to stressful environments [46]. Adaptation to freezing tolerance is associated with the accumulation of metabolically compatible compounds, such as the soluble sugar proline, and with maintaining osmotic homeostasis through metabolic adjustments. Modification of cell membrane stability and associated enzyme activity is also included [19; 20; 47]. Therefore, in the present study, these

seven genotypes were compared with the KAKA-sensitive genotype for conductivity, soluble sugar content, proline and chlorophyll content, and antioxidant enzyme activity under various freezing-temperature stress conditions. PCA is regarded as a powerful technique for data analysis and interpretation when multiple parameters are involved [48, 49]. In the present study, the primary objective was to use PCA to identify and demonstrate the significance of measured variables that the result exhibited: Chla, TP, OP, Fv′/Fm′, DW, ELL, ELC, APX, CAT, and APX in the first and second dimensions of the component analysis in relation to freezing stress (Fig. 7).

Membrane stability is a key factor in determining freezing tolerance because low temperatures induce lipid phase transitions, increasing membrane rigidity and disrupting cellular homeostasis [17]. This study found that EL increased sharply with freezing stress, especially in the sensitive KAKA genotype, which indicates compromised membranes (Fig. 2a and b). In contrast, tolerant genotypes maintained membrane integrity more effectively, which is consistent with their lower $LT_{50}$ values (A1). Such damage is likely attributable to the formation of ice crystals and subsequent cellular dehydration, both attributes of freezing stress [50]. This observation suggests high membrane stability in these genotypes, a phenomenon that could be driven by elevated cryoprotectant and antioxidant enzyme levels [19, 20]. The present findings are consistent with recent observations indicating that legumes' ability to resist freezing is contingent upon preserving cellular integrity under these conditions [51, 52]. Recent studies on chickpeas and their wild relatives have underscored the pivotal role of the ICE-CBF-COR signaling cascade in membrane stabilization. This process is facilitated by the upregulation of COR genes (e.g., COR47 and LTI), which enhance the production of osmoprotectants and maintain membrane fluidity under freezing stress [52]. This study did not assess direct lipid remodeling; however, the reduced EL in tolerant MCC genotypes likely involves alterations in membrane lipid composition, such as increased unsaturation or sterol content, as observed in cold-acclimated legumes and other plants where lipid remodeling prevents phase transitions and leakage during freeze-induced

dehydration [54]. The strong negative relationship between membrane damage and plant dry mass, as revealed by the PLS-SEM model, underscores the pivotal role of membrane integrity in integrating physiological performance and molecular adaptation mechanisms (Fig. 9; $p < 0.001$).

It has been demonstrated that the osmotic potential can be modified through the accumulation of osmoregulatory substances, such as PC and TSC, within the cell cytoplasm without compromising metabolic processes [55]. PC and TSC have been identified as critical osmoprotectants, capable of counteracting ROS and maintaining structural integrity, thereby facilitating the identification of freezing-tolerant cultivars [23; 56]. PC and TSC concentrations increased under freezing stress in all genotypes compared with the control; however, KAKA showed a moderate response, indicating effective osmoprotectant mechanisms against freezing stress (Fig. 3c and d). These results align with recent observations, further emphasizing the importance of PC and TSC in the context of leguminous plant response to cold stress [44].

Under extreme cold stress, it impedes photosynthesis by altering chlorophyll concentration and damaging chlorophyll and the photosynthetic apparatus [17]. The observed drop in chlorophyll content can be attributed to the impact of cold stress on chlorophyll synthesis, which has been shown to reduce it and accelerate chlorophyll decomposition [56]. Furthermore, Arslan et al. [58] posited that plants exhibiting elevated levels of cold tolerance maintain constant chlorophyll content, while those exhibiting diminished cold tolerance undergo a decline in chlorophyll content. In line with these findings, the present study demonstrates a decrease in Chla and TP content in all chickpea genotypes under freezing stress, but the reduction is lower in tolerant genotypes than in sensitive genotypes (Fig. 3c and d).

The reaction to freezing is characterized by increased oxidative stress, exacerbated by excessive accumulation of antioxidant systems in plants. Freezing-tolerant genotypes have been shown to have superior abilities in safeguarding against oxidative stress by activating

the antioxidant system [59]. The present study demonstrated that, under freezing stress, the activities of CAT, POD, and APX were enhanced in the genotypes. As stated in the reports by Heidarvand and Maali-Amiri [60], Arslan et al. [58], and Karimzadeh Soureshjani et al. [14], freezing-tolerant genotypes had higher levels of CAT, POD, and APX compared to freezing-sensitive genotypes when exposed to freezing stress. The results of this study correspond with these findings, indicating that CAT, POD, and APX activities were significantly lower in KAKA (a sensitive genotype) compared to the other genotypes evaluated (Fig. 4a, b, and c ).

Also, parallel increases in transcript levels of *caCAT*, *caPOD*, and *caAPX*, with higher fold-changes in tolerant genotypes (Fig. 6a, b, and c), indicate transcriptional upregulation as a primary regulatory mechanism under moderate freezing stress. In a similar manner, the CAT, POD, and APX genes respond to abiotic stresses, including salt, drought, and cold, across diverse plant species [61, 56; 62, 63]. These genes are essential for improving plant abiotic resistance by modulating antioxidant defense systems and osmotic regulation [56, 64, 65, 66]. Recent transcriptomic analyses in chickpea confirm that the CBF pathway indirectly strengthens antioxidant responses by increasing the expression of genes for peroxidases, glutathione S-transferases, and other ROS scavengers, thereby reducing oxidative stress during freezing [53]. Overexpression of CBFs has also been demonstrated to elevate CAT and POD activities, thereby establishing a link between the CBF regulon and enhanced ROS detoxification in cold-tolerant chickpea [67].

The comprehensive membership function ordered MCC911 highest (D=0.737) and KAKA lowest (D=0.222), validating our physiological and biochemical findings (Table 2). These findings indicate that MCC911 and MCC901 may be promising candidates for breeding cold-tolerant chickpea cultivars. Nevertheless, further field studies under real-world conditions are essential to confirm these results.

## 5. Conclusions

In this study, applying multivariate data analysis with PCA, PLS-SEM, correlation matrix, and comprehensive membership function techniques enabled effective discrimination among the various treatments, thereby substantiating the impact of freezing stress on the targeted traits and ranking the genotypes. The results underscore the importance of enzyme activation in autumn chickpeas' freezing tolerance. Discovering important traits (ELL, ELC, Chla, TP, PC, TPC, TSC, CAT, POD, APX, DW, OP, Fv′/Fm′, and the antioxidant genes *caAPX*, *caCAT*, and *caPOD*) and recognizing chickpea types that can handle freezing conditions will support researchers in developing chickpea varieties better suited for planting in the fall and winter. Furthermore, this study helps us understand how guided and unguided statistical analysis can provide more information about chickpea plants' physiological and chemical responses and how freezing temperatures affect them.

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**Declarations**

**Conflicts of Interest**

The authors have no conflicts of interest to declare.

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**Data availability**

The data provided in this study are available to the corresponding author and can be presented on request.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Authors' contributions**

Jafar Nabati contributed to the conception of the research; Jafar Nabati and Zahra Nemati carried out the experiments, reviewed data, collated the data; performed the data analyses and manuscript preparation; Amin Mirshamsi Kakhki, Ahmad Nezami and Alireza Hasanfard helped perform the analysis with constructive discussions. All authors read and approved the final manuscript



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