

Freezing tolerance of chickpea: biochemical and molecular changes at vegetative stage

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Abstract

The aim of this study was to find a correlation between the freezing tolerance of three chickpea (*Cicer arietinum* L.) cultivars (İnci, Işık-05, and Sarı-98) and their wild relative *C. echinospermum* and physiological responses. Chickpea plants (15-d-old) were subjected to cold acclimation (CA) (10 °C for 7 d), freezing (-3 or -5 °C for 2 h), and subsequent rewarming (25 °C for 7 d). In two separate experiments with three replications, we determined growth, water status, photosystem 2 photochemical activity, photosynthetic pigments, H₂O₂, malondialdehyde, and proline content, relative leakage ratio, antioxidant enzyme activities, and gene expressions in cultivars different in freezing tolerance. Freezing temperatures adversely affected all the physiological parameters of all cultivars. Rewarming did not lead to complete recovery. The cultivar İnci was more tolerant to the freezing temperatures than others.

Additional key words: antioxidant enzymes, chlorophyll, *Cicer arietinum*, *C. echinospermum*, gene expression, malondialdehyde, photochemical activity.

Introduction

Cold stress, including both chilling and freezing, is a major cause of reduced crop productivity and quality in many crops such as cotton, soybean, maize, chickpea, and rice (Thakur *et al.* 2010). At low temperatures, the degree of adverse affection of physiological, cellular, metabolic, and molecular processes may be so severe that it leads to death. However, exposure of plants to gradually increasing stress might initiate physiological and biochemical adjustment that protects them from injury when environmental stresses abruptly occur (Thomashow 1999). Cold acclimation (CA) is an enhanced tolerance to freezing stress. Low temperature negatively affects plant growth and development (Janská *et al.* 2010), membrane structure (Ruelland *et al.* 2009) and photosynthetic activity (Borawska-Jarmulowicz *et al.* 2014, Kalaji *et al.* 2016). Low temperature also imposes a dehydration and in order to protect the cells from dehydration, the plants accumulate a high amounts of osmoprotectants (Kaur *et al.* 2011). Alterations in structure and function of cell

membranes are early effects of stress injury and often have been attributed to oxidative damage. In order to detoxify stress-induced reactive oxygen species (ROS), plants develop a complex antioxidant system, in which numerous enzymes play essential roles.

Chickpea (*Cicer arietinum* L.) is a chilling sensitive legume and low temperature is one of the most important barrier for the production of this crop. However, wild relatives of chickpea, *Cicer reticulatum* and *Cicer echinospermum*, are a promising source of genes for tolerance to environmental stresses and these two wild relatives have been utilized for genetic improvement in breeding programs (Croser *et al.* 2003). In this study, *C. echinospermum*, the cold tolerant wild relative of chickpea (Croser *et al.* 2003), was used as a positive control in molecular analyses to understand the freezing tolerance ability of the local chickpea cultivars. The aim of this study was 1) to elucidate possible physiological mechanisms in chickpea under freezing temperatures and

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Abbreviations: APX - ascorbate peroxidase; CA - cold acclimation; CAT - catalase; Chl - chlorophyll; RLR - relative leakage ratio; GR - glutathione reductase; MDA - malondialdehyde; RT-qPCR - reverse-transcription quantitative polymerase chain reaction; PI_{tot} - total performance index; POD - peroxidase; PS - photosystem; ROS - reactive oxygen species; RWC - relative water content; SOD - superoxide dismutase.

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subsequent rewarming; 2) to understand the recovery capacity of cultivars after freezing of different severity; 3) to compare the freezing tolerance of studied cultivars;

and 4) to compare it with cold tolerant wild species *C. echinospermum*.

Materials and methods

The seeds of three local chickpea (*Cicer arietinum* L.) cultivars (İnci, Işık-05, and Sarı-98) were obtained from Ankara Central Research Institute for Field Crops in Turkey and the seeds of cold tolerant *Cicer echinospermum* P.H. Davis from ICARDA (Beirut, Lebanon). Before seeding, pesticides *Benomil* and *Thiam* were applied (3 g pesticide to each 1 kg seeds) against the fungal infections that chickpea often faces to. Some characteristics of the soil were as follows: water-holding capacity of 19.5 %; pH 7.52; electrical conductivity of 264 $\mu\text{S cm}^{-1}$; 1.54 g(N) kg^{-1} ; 16.42 mg(P) kg^{-1} , and 475 mg(K) kg^{-1} . Since, N, P, and K content of the soil was not sufficient for chickpea growth, 100 $\mu\text{g g}^{-1}$ NH_4NO_3 and 100 $\mu\text{g g}^{-1}$ KH_2PO_4 were added to the soil. Plants were grown in a controlled growth chamber at a constant temperature of $25 \pm 1^\circ\text{C}$, a relative humidity of $40 \pm 5\%$, a 16 h photoperiod, and an irradiance of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ under sufficient water supply for 15 d and then randomly divided into following groups: C₀, C₁, C₂: 15-, 22-, and 29-d-old control plants, respectively; CA: 22-d-old cold-acclimated plants (15 d at control conditions, then 7 d at $10 \pm 1^\circ\text{C}$ and an irradiance of $150 \mu\text{mol m}^{-2} \text{s}^{-1}$); S₁ and S₂: 22-d-old freezing-stressed plants that were cold acclimated and then subjected to -3 or -5°C for 2 h; R₁ and R₂: 29-d-old rewarming plants that were cold acclimated, then subjected to -3 or -5°C for 2 h and then returned to the control conditions for 7 d. For the freezing treatments, the temperature of the chamber was decreased at a rate of 1°C h^{-1} starting from 1°C and held for 2 h at each of the experimental temperatures (-3 and -5°C) and then raised at the same rate. The central leaves of 15-, 22- and 29-d-old plants were used in the experiments. The temperature -7°C was also tried, but plants were not able to survive.

Chlorophyll (Chl) *a* fluorescence measurements were performed in a growth chamber at 25°C using a portable, modulated fluorescence monitoring system (FMS-2, Hansatech Instruments, Norfolk, UK) on randomly selected leaves (6 replicates) and parameters were calculated as published earlier (Turan and Ekmekçi 2014). Total performance index (PI_{tot}) of the plants were calculated using the polyphasic OJIP fluorescence transient parameters measured with Handy PEA (Hansatech Instruments) fluorimeter on selected leaves of the plants. Control and rewarming plants were measured at room temperature, while freezing plants were measured at 1°C . Following a 30 min dark adaptation, samples were treated with light flash (650 nm peak wavelength; $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 1 s) provided by three LEDs, and Chl *a* fluorescence signals were recorded according to Strasser and Strasser (1995). Performance index (PI_{tot}) = $[\gamma\text{RC}/(1-\gamma\text{RC})]X [\phi\text{Po}/(1-\phi\text{Po})]X[\psi\text{o}/$

$(1-\psi\text{o})]*[(\delta\text{Ro}/(1-\delta\text{Ro}))]$ was calculated using Biolyzer software package and also JIP test (Strasser *et al.* 2004).

The content of Chl (*a+b*) and carotenoids (*x+c*) was determined spectrophotometrically (at wavelenghts of 470, 644.8, and 661.6 nm) in acetone extract and calculated according to Lichtenthaler (1987). To determine the anthocyanin content, fresh leaf tissues were grinded in acidified methanol and calculated according to Mancinelli *et al.* (1975). Flavonoid content was determined spectrophotometrically at 300 nm using the method of Mirecki and Teramura (1984) and expressed as the percentage of content of 15-d-old control plants (C₀).

The content of H₂O₂ was determined according to Esterbauer and Cheeseman (1990). H₂O₂ content was calculated according to the standard curve. Malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction as described by Hodges *et al.* (1999). MDA content was estimated by using a coefficient of absorbance (ϵ) of $157 \text{ mM}^{-1} \text{ cm}^{-1}$. Relative leakage ratio (RLR) was measured using Redmann *et al.* (1986) method with minor modifications. The RLR was measured indirectly as leakage of UV-absorbing substances at 280 nm, 24 h before (C₁) and after (C₂) incubation of leaf segments ($\varnothing 0.5 \text{ cm}$) in liquid nitrogen for 20 min and calculated as C₁/C₂. Free proline content of leaves was determined using the method of Bates *et al.* (1973). Proline was extracted from fresh leaf samples (0.5 g) based on the method of Weimberg (1987).

To determine the enzyme activities, fresh leaf samples (0.5 g) were ground with liquid nitrogen and then extracted in respective extraction buffer. The protein content in the extracts was determined according to Bradford (1976). APX (EC 1.11.1.1) activity was determined by the method of Wang *et al.* (1991) according to the decrease in the ascorbate concentration recorded at 290 nm against the assay solution free of enzyme extract and calculated using $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. GR (EC 1.6.4.2) activity was determined by the method of Rao *et al.* (1995) and calculated using $\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ at 340 nm. POD (EC 1.11.1.7) activity determination was based on the measurement of guaiacol oxidation by H₂O₂ ($\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 470 nm; Bergmeyer 1974). CAT (EC 1.11.1.6) activity was determined according to the decomposition of H₂O₂ measured spectrophotometrically by following the decrease in absorbance at 240 nm (Chance and Maehly 1955). The enzyme activity was calculated from the initial rate of the reaction using $\epsilon = 40 \text{ mM}^{-1} \text{ cm}^{-1}$ at 240 nm. Cu/Zn SOD activity was determined according to the photochemical reduction of nitroblue tetrazolium using the method of Beyer and Fridovich (1987). Further, 0.1 g leaf samples were ground with liquid nitrogen and suspended in a buffer containing

9 mM Tris-HCl (pH 6.8) and 13.6 % (v/v) glycerol (Burke and Oliver 1992). After centrifugation at 18 000 g and 4 °C for 5 min, the supernatant was loaded on acrylamide gel (12 %, m/v) as in the Laemmli (1970) method. Quantitative analysis of Cu/Zn SOD activity was performed by using the *Bio-ProfileV99* programme and *Vilber Gel Imager Lourmart* imaging system. Cu/Zn SOD unit activity was calculated by using a SOD standard (*Sigma*, St. Louis, USA). To distinguish SOD isoforms, Cu/Zn-SOD, Fe-SOD and Mn-SOD, the sensitivities of Cu/Zn-SOD to cyanide (3 mM), and Cu/Zn-SOD and Fe-SOD to H_2O_2 (5 mM) were used, whereas Mn-SOD was unaffected.

RNA was extracted from 0.3 g leaf samples according to Chomczynski and Sacchi (1987). RNA was purified using RNase-Free DNase Set (*Qiagen*, Hilden, Germany) and the first strand cDNA was synthesized using *QuantiTect* reverse transcription kit (*Qiagen*) according to suggested procedure. Primers were designed using *Vector NTI* and synthesized (Table 1 Suppl.). Expression of *Cu/Zn SOD* gene (GenBankID: AJ012691.1) and

uncharacterized protein LOC101496790 (GeneBankID: XP_004506718.1) gene containing a phosphatidyl-ethanolamine-binding protein (PEBP) domain (referred to as *PEBP-like* gene in this study) were monitored by real-time quantitative PCR (*Qiagen*) using *SYBR Green* (*Qiagen*). Coding sequence of LOC101496790 showed homology to drought upregulated mRNA (GenBankID: EG359333.1) and *Triticum aestivum* Wcor18, a cold regulated protein. *GAPDH* was used as housekeeping gene and data were normalized using the control samples (C_0).

Each experiment included 54 pots with 162 plants. The experiments were performed in a completely random design with 3 replicates, and differences in measured variables between cultivars and treatments were analyzed by *ANOVA* and according to the least significant difference (LSD) test at the 5 % level. To determine the correlation of the parameters, data were subjected to Z-score transformation and analyzed by the principal component analysis (PCA). All the analyses were performed using the *SPSS v. 20.0* (Chicago, IL, USA).

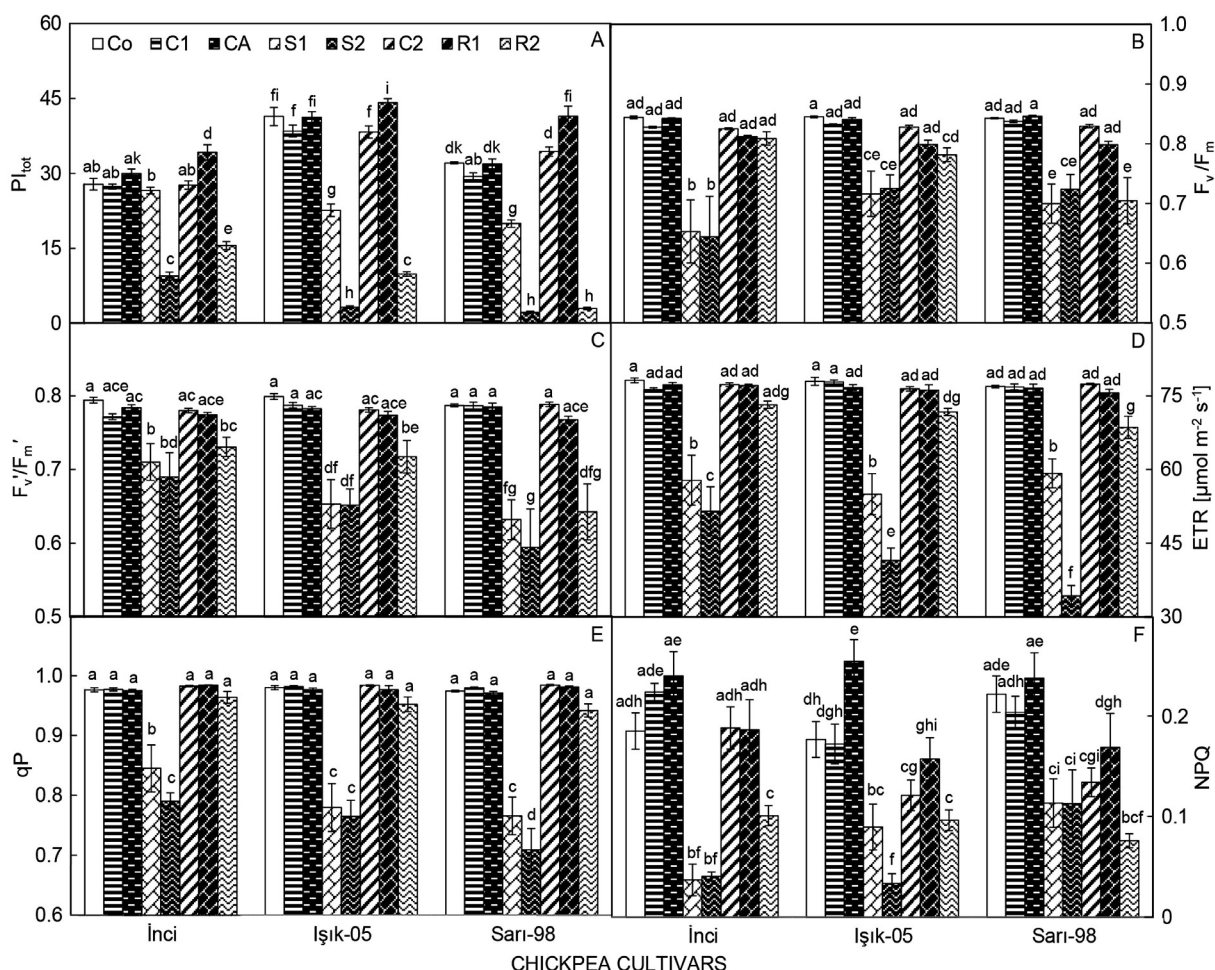


Fig. 1. The effect of freezing and rewarming on some Chl *a* fluorescence parameters: *A* - total performance index (PI_{tot}); *B* - quantum efficiency of PS 2 (F_v/F_m); *C* - quantum efficiency of excitation energy trapping by PS 2 (F_v'/F_m'); *D* - electron transport rate (ETR); *E* - photochemical quenching (qP); and *F* - non-photochemical quenching (NPQ). Means $\pm$ SEs, $n = 6$. Different letters indicate significant difference at $P < 0.05$ according to LSD 5 %. For different treatments see Table 1.

Results

Freezing temperatures (-3 and -5 °C) decreased shoot length, leaf number, fresh and dry mass and relative water content (RWC) of all cultivars (Table 2 Suppl.) and after rewarming period, growth parameters and water status of the cultivars did not recover.

Cold acclimation did not lead to any significant differences in Chl *a* fluorescence parameters (Fig. 1). Freezing temperatures resulted in a remarkable decrease in PI_{tot} , except in S_1 treatment of İnci (Fig. 1A). PI_{tot} of all cultivars significantly increased after R_1 treatment. Both freezing temperatures (-3 and -5 °C) induced a decrease in maximal quantum efficiency of PS 2 of dark-adapted plants (F_v/F_m) and quantum efficiency of excitation energy trapping by PS 2 (F_v'/F_m') of all cultivars (Fig. 1B,C). Since similar tendencies were detected in actual PS 2 photochemical efficiency of light-adapted leaves, $\phi_{PS II}$ data are not shown. The highest enhancement of F_v/F_m of İnci was determined after R_1 treatment when compared to S_1 (25 %). The F_v'/F_m' of Işık-05 and

Sarı-98 decreased significantly after rewarming only in R_2 treatment. Significantly declined values of electron transport rate (ETR), photochemical quenching (qP), and nonphotochemical quenching (NPQ) were determined after S_1 and S_2 treatments in all cultivars, whereas ETR, qP, and NPQ increased approximately to same extent at rewarming compared to freezing, except the R_2 treatment of Sarı-98 (Figs. 1D-F).

Freezing and rewarming reduced the Chl *a+b* content of all cultivars to a remarkably greater extent at S_2 and markedly in Sarı-98 (Table 1). Similarly, the carotenoid content of the cultivars decreased progressively with decreasing temperature (Table 1). In İnci and Işık-05, the content of anthocyanin was notably higher in rewarming plants, whereas the anthocyanin content of Sarı-98 after rewarming remained unchanged (R_1) or decreased (R_2) (Table 1). However, freezing temperatures and subsequent rewarming resulted in progressive enhancement of flavonoid content of all cultivars (Table 1).

Table 1. The content of chlorophyll (Chl) *a+b*, carotenoids, anthocyanins, and flavonoids of the leaves of chickpea cultivars exposed to freezing and rewarming. Means $\pm$ SEs, $n = 6$ (for Chl and carotenoids) or 3 (for anthocyanins and flavonoids). Different letters indicate significant difference at $P < 0.05$. Treatments: C_0 , C_1 , C_2 : 15-, 22-, and 29-d-old control plants, respectively; CA: 22-d-old cold-acclimated plants (15 d at control conditions, then 7 d at 10 ± 1 °C); S_1 : 22-d-old plants that were cold acclimated and then subjected to -3 °C for 2 h; S_2 : 22-d-old plants that were cold acclimated and then subjected to -5 °C for 2 h; R_1 : 29-d-old plants that were cold acclimated, then subjected to -3 °C for 2 h, and then returned to the control conditions for 7 d; R_2 : 29-d-old plants that were cold acclimated, then subjected to -5 °C for 2 h, and then returned to the control conditions for 7 d.

Cultivars	Treatments	Chl <i>a+b</i> [$\mu\text{g cm}^{-2}$]	Carotenoids [$\mu\text{g cm}^{-2}$]	Anthocyanins [$\mu\text{g g}^{-1}$ (f.m.)]	Flavonoids [% of control]
İnci	C_0	65 $\pm$ 2 af	13 $\pm$ 0.3 a	0.05 $\pm$ 0.001 ah	100
	C_1	59 $\pm$ 1 ab	13 $\pm$ 0.1 a	0.05 $\pm$ 0.001 ah	114
	CA	53 $\pm$ 1 b	10 $\pm$ 0.1 b	0.09 $\pm$ 0.001 aei	159
	S_1	41 $\pm$ 2 cd	8 $\pm$ 0.2 c	0.09 $\pm$ 0.001 aei	227
	S_2	35 $\pm$ 1 ch	5 $\pm$ 0.4 d	0.06 $\pm$ 0.001 ah	240
	C_2	65 $\pm$ 2 af	13 $\pm$ 0.4 a	0.39 $\pm$ 0.001 bj	179
	R_1	58 $\pm$ 4 bg	12 $\pm$ 0.8 e	0.47 $\pm$ 0.001 c	341
	R_2	42 $\pm$ 3 d	8 $\pm$ 0.1 c	0.60 $\pm$ 0.001 d	338
Işık-05	C_0	73 $\pm$ 1 ej	16 $\pm$ 0.1 f	0.06 $\pm$ 0.001 ah	100
	C_1	67 $\pm$ 1 ef	16 $\pm$ 0.1 f	0.06 $\pm$ 0.001 ah	87
	CA	61 $\pm$ 1 afg	11 $\pm$ 0.2 g	0.08 $\pm$ 0.001 aeh	163
	S_1	39 $\pm$ 1 cd	8 $\pm$ 0.2 c	0.12 $\pm$ 0.001 ei	249
	S_2	31 $\pm$ 1 h	4 $\pm$ 0.3 h	0.12 $\pm$ 0.001 ei	273
	C_2	72 $\pm$ 2 ej	13 $\pm$ 1.0 a	0.33 $\pm$ 0.001 f	140
	R_1	60 $\pm$ 1 ag	12 $\pm$ 0.5 e	0.67 $\pm$ 0.001 g	343
	R_2	42 $\pm$ 1 d	7 $\pm$ 0.1 i	0.59 $\pm$ 0.001 d	319
Sarı-98	C_0	67 $\pm$ 2 ef	14 $\pm$ 0.3 j	0.04 $\pm$ 0.001 h	100
	C_1	62 $\pm$ 2 afg	13 $\pm$ 0.3 a	0.06 $\pm$ 0.001 ah	92
	CA	56 $\pm$ 5 bg	10 $\pm$ 0.3 b	0.08 $\pm$ 0.001 aeh	159
	S_1	30 $\pm$ 2 h	8 $\pm$ 0.4 c	0.08 $\pm$ 0.001 aeh	177
	S_2	20 $\pm$ 2 i	2 $\pm$ 0.1 k	0.13 $\pm$ 0.001 i	234
	C_2	76 $\pm$ 2 j	15 $\pm$ 0.4 l	0.43 $\pm$ 0.001 bc	212
	R_1	59 $\pm$ 1 ab	12 $\pm$ 1.2 e	0.44 $\pm$ 0.001 c	326
	R_2	39 $\pm$ 3 cd	6 $\pm$ 0.1 m	0.35 $\pm$ 0.001 fj	287
LSD 5 %		7	1	0.05	

The H_2O_2 content increased in all cultivars at $-3\text{ }^{\circ}\text{C}$ (Fig. 2A), especially in İnci and Işık-05 (58.6 and 64 %, respectively). The MDA content of all cultivars increased after both freezing treatments and R_2 (Fig. 2B) and R_2 resulted in the highest MDA accumulation, especially in Işık-05 and Sarı-98 (1- and 2.2-fold of controls, respectively). Similarly to MDA results, RLR was higher than corresponding controls at both freezing and

rewarming treatments in all cultivars (Fig. 2C). Exposure to CA and rewarming resulted in enhancement of proline content of all cultivars (Fig. 2D).

APX activities increased significantly after CA and freezing treatments (Fig. 3A). The effects of freezing on GR activities of the cultivars were variable (Fig. 3B). Freezing and rewarming resulted in enhancement of POD activities of cultivars, with remarkable extent in İnci

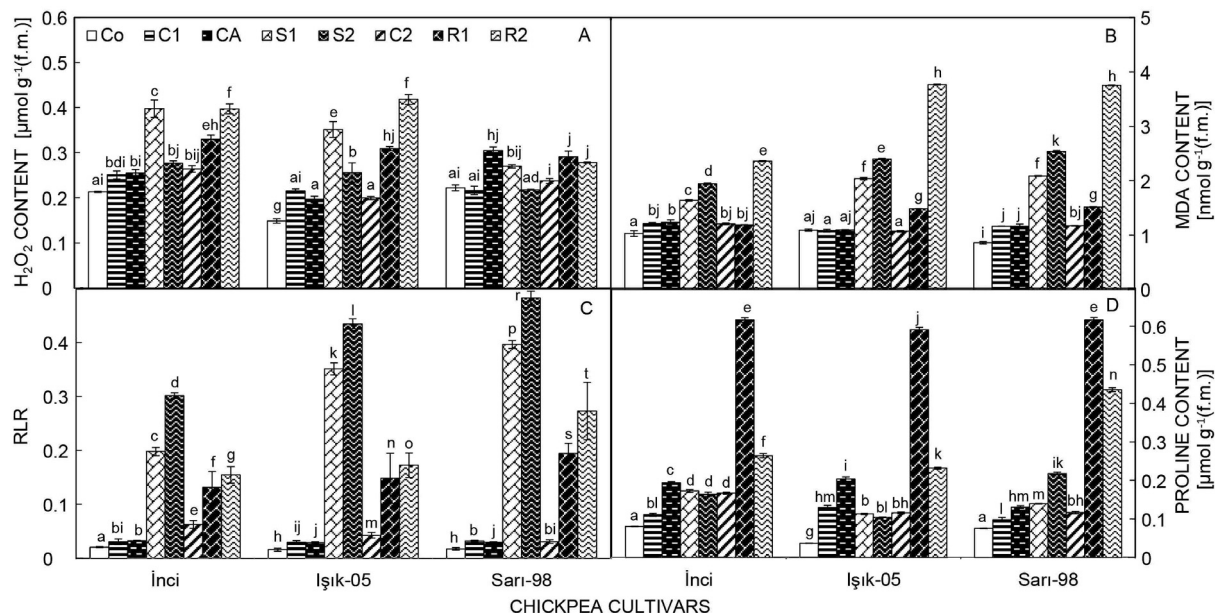


Fig. 2. The H_2O_2 content (A), malondialdehyde (MDA) content (B), relative leakage ratio (RLR; C), and proline content (D) of chickpea cultivars exposed to freezing and rewarming. Means $\pm$ SEs, $n = 3$ (H_2O_2 , MDA and proline) and 6 (RLR). Different letters indicate significant difference at $P < 0.05$ according to LSD 5%. For different treatments see Table 1.

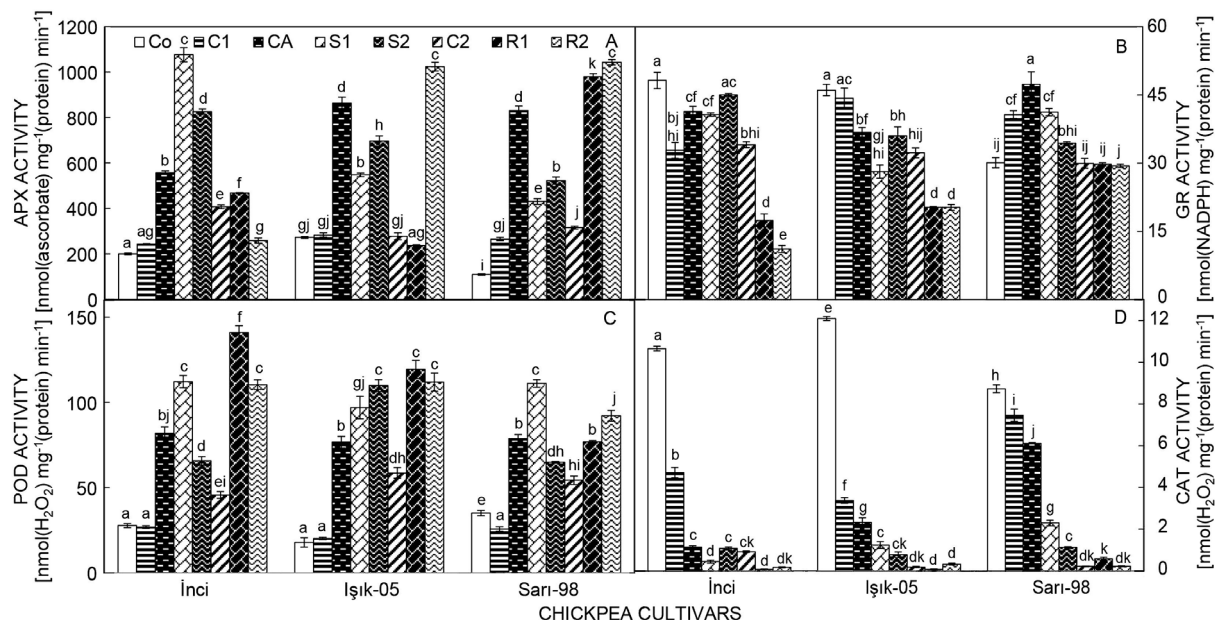


Fig. 3. The responses of antioxidant enzyme ascorbate peroxidase (APX; A), glutathione reductase (GR; B), peroxidase (POD; C), and catalase (CAT; D) activities in chickpea cultivars exposed to freezing and rewarming. Means $\pm$ SEs, $n = 3$. Different letters indicate significant difference at $P < 0.05$ according to LSD 5%. For different treatments see Table 1.

(Fig. 3C). At R₁ and R₂, this marked enhancement of POD activity of İnci was determined as 2- and 1.4-fold of control, respectively. On the contrary, CAT activity declined sharply in all treatments (Fig. 3D). The Cu/Zn-SOD activity was determined in all cultivars and *C. echinospermum* (Table 2). The Cu/Zn SOD activity progressively increased in İnci and *C. echinospermum* at freezing temperatures and the increase was highest in

C. echinospermum. Also *Cu/Zn-SOD* gene expression of İnci and *C. echinospermum* raised with decreasing temperature starting from 10 °C and *C. echinospermum* exhibited the highest Cu/Zn SOD gene expression (Table 2). CA and freezing treatments led to an increase of *PEBP-like* gene expression of all genotypes, especially in *C. echinospermum* (Table 2).

Table 2. The leaf Cu/Zn-SOD activity [U mg⁻¹ (protein)], the normalized data of *Cu/Zn-SOD* and *PEBP-like* gene expressions in chickpea cultivars and *C. echinospermum* subjected to freezing and rewarming. Means ± SEs, *n* = 3. Different letters indicate significant difference at *P* < 0.05. For treatment abbreviations see Table 1.

Chickpea cultivars	Treatments	Cu/Zn-SOD activity	<i>Cu/Zn-SOD</i> gene expression	<i>PEBP-like</i> gene expression
İnci	C ₁	5 ±0.02 a	1.0 ±0.0 a	1.0 ±0.0 a
	CA	12 ±0.5 bde	1.8 ±0.1 b	2.1 ±0.2 bd
	S ₁	12 ±0.8 bde	2.0 ±0.2 b	35.9 ±1.0 c
	S ₂	14 ±1.1 bd	1.7 ±0.4 b	3.9 ±0.3 be
	C ₂	11 ±0.8 bce	1.0 ±0.0 a	1.0 ±0.0 a
	R ₁	14 ±0.7 bd	1.1 ±0.2 a	3.0 ±0.2 bde
	R ₂	16 ±0.9 d	0.8 ±0.0 ac	1.3 ±0.0 ad
Işık-05	C ₁	6 ±0.3 ac	1.0 ±0.0 a	1.0 ±0.0 a
	CA	6 ±0.3 ac	0.8 ±0.1 ac	4.5 ±0.5 eh
	S ₁	5 ±0.4 a	0.7 ±0.1 ac	10.0 ±2.6 f
	S ₂	9 ±0.1 acd	0.7 ±0.0 ac	2.4 ±0.4 bd
	C ₂	7 ±0.5 ae	1.0 ±0.0 a	1.0 ±0.0 a
	R ₁	10 ±0.7 acd	0.7 ±0.2 ac	0.4 ±0.1 a
	R ₂	11 ±0.2 bce	0.4 ±0.1 c	3.8 ±0.2 eg
Sarı-98	C ₁	8 ±1.7 ae	1.0 ±0.0 a	1.0 ±0.0 a
	CA	8 ±0.5 ae	1.0 ±0.0 a	2.9 ±0.3 bde
	S ₁	5 ±0.5 a	0.5 ±0.1 c	2.5 ±0.5 bdg
	S ₂	8 ±0.4 ae	1.0 ±0.2 a	6.2 ±0.4 h
	C ₂	10 ±0.6 acd	1.0 ±0.0 a	1.0 ±0.0 a
	R ₁	12 ±0.8 bde	0.7 ±0.1 ac	0.1 ±0.0 a
	R ₂	10 ±0.4 acd	0.4 ±0.0 c	0.4 ±0.0 a
<i>C. echinospermum</i>	C ₁	16 ±2.8 b	1.0 ±0.0 a	1.0 ±0.0 a
	CA	24 ±5.0 f	5.3 ±0.3 d	12.4 ±1.1 f
	S ₁	46 ±2.8 g	4.7 ±0.2 e	4.2 ±2.4 eg
	S ₂	58 ±1.8 h	5.1 ±0.2 de	4.5 ±0.2 eh
	C ₂	35 ±1.3 i	1.0 ±0.0 a	1.0 ±0.0 a
	R ₁	22 ±1.9 f	0.7 ±0.1 ac	2.5 ±0.5 bdg
	R ₂	32 ±2.7 i	0.9 ±0.3 ac	2.9 ±0.6 bde
LSD 5 %		6	0.6	1.9

Discussion

Environmental variables, especially those affecting water availability and temperature, are the major determinants of plant growth and development. Decreased vegetative growth, including fresh and dry mass, at freezing and rewarming could occur as a result of decreased photosynthesis and water uptake and increased lipid peroxidation and membrane damage. The Chl *a* fluorescence is one of the most widely used methods to

probe photosystem activities (Kalaji *et al.* 2012, 2016). The Chl *a* fluorescence measurements showed that CA did not cause photoinhibition of PS 2 in all the cultivars, whereas freezing temperatures led to significant alterations that reflect the freeze-induced photoinhibition. In our experiment, the reason of declined PI_{tot}, especially at S₂, could be decreased Chl *a+b* content and ETR. On the contrary, R₁ resulted in significant rises of PI_{tot} which

demonstrate the recovered ability of energy transformation. The F_v/F_m alleviations are correlated with degradation of membrane integrity (Cavender-Bares 2007), which is in accordance with our findings. The decrease of F_v/F_m' that indicates excess energy dissipation was marked in Sari-98 at freezing treatments, whereas at rewarming, cultivars reached the control levels, except Sari-98 after R_2 . Photoinhibition of PS 2 is closely related to oxidation or reduction state of plastoquinone Q_A which can be determined by qP that expresses the photochemical use of excitation energy (Li *et al.* 2004). Freezing decreased qP values of all the cultivars, notably Sari-98, whereas qP reached control values after rewarming. Total Chl ($a+b$) content did not change at CA, while during freezing decreased sharply. It is well known that thylakoid membranes are the primary site of chloroplast freezing injury and subsequently Chl degradation occurs. Decreased Chl ($a+b$) content was correlated with decreased PS 2 photochemical efficiency and increased MDA accumulation and RLR ratios of all cultivars at S_2 . Among the cultivars, Sari-98 had the most decreased Chl ($a+b$) content. Recovery of the cultivars after rewarming could be observed from the increased photosystem activities. However, the Chl ($a+b$) content showed that subsequent rewarming period was not enough for the complete recovery of it from freezing injury. Total carotenoid content of all cultivars progressively decreased at CA and freezing treatments. The reductions of both total carotenoid content and NPQ values indicate that excess excitation energy could not be enough dissipated as heat, hence these protective mechanisms could not protect photosystems from injury. Anthocyanins prevent Chl from photoinhibition, and, at low temperatures, plays an antioxidant role by keeping the amount of ROS at low levels (Hao *et al.* 2009). In the present study, freezing and rewarming led to enhancement of anthocyanin content of cultivars, except Sari-98. Flavonoid content of cultivars progressively increased at all treatments. Although increased flavonoid content is often related to low temperature tolerance (Toda *et al.* 2011), MDA and RLR values at freezing showed that the flavonoid content was insufficient to prevent the chickpea plants from lipid peroxidation and membrane injuries.

Freezing temperatures induce H_2O_2 accumulation and increases ROS scavenging enzymes activities (Dai *et al.* 2009). The CA period resulted in an increased amount of H_2O_2 only in Sari-98, and increased activities of APX, GR, and POD of this cultivars were not sufficient to scavenge H_2O_2 . Freezing at $-3\text{ }^\circ\text{C}$ enhanced H_2O_2 amount in all cultivars and a marked decrease of PI_{tot} at R_1 treatment could be an indicator that elevated content of H_2O_2 inhibited the regeneration of PS 2 proteins. CA period did not lead to any significant change of MDA content and RLR ratio which are determinants of membrane injury. These findings suggest that cultivars maintained their membrane integrity at $10\text{ }^\circ\text{C}$ for 7 d by scavenging the free radicals with increased antioxidant enzyme activities. Plasma membrane is the primary

structure where freezing injury occurs (Ruelland *et al.* 2009). As the freezing temperature decreased, MDA content and RLR increased in all cultivars, notably in Sari-98, suggesting evident disruption of membrane integrity. After rewarming, cultivars restored membrane integrity, except MDA content of İnci at R_1 . Proline accumulated in all cultivars during CA and rewarming treatments, mostly in Sari-98. Although, increased proline content of plants during cold stress is usually correlated with cold tolerance (John *et al.* 2016), in Sari-98, the lowest RWC indicates that its proline was insufficient to increase water-binding capacity and increased proline content rather may occur as a result of cold injury-induced protein degradation.

It is well known that oxidative stress, which occurs as a result of elevated ROS accumulation, is a major component of low temperature stress in chilling sensitive plants. In cereals, the activities of most antioxidant enzymes (APX, GR, and POD) increase to alleviate the damaging effects of ROS, whereas CAT activity decreases (Janda *et al.* 2003). In chickpea cultivars, the CA induced significant increase in APX, GR, and POD activities suggesting the enhanced tolerance. Our result is in accordance with Kaur *et al.* (2011) who demonstrate that low temperature exposed chickpea genotypes increases their APX activity at various extent. APX and GR activities differences among our cultivars during rewarming treatments showed that cultivars probably detoxified H_2O_2 using POD instead of ascorbate-glutathione cycle. However, after both freezing and rewarming treatments, increased H_2O_2 , MDA, and RLR indicated that enhanced APX and POD activities were not sufficient to scavenge H_2O_2 and protect membranes from peroxidation and deterioration. In our study, exposure of chickpea cultivars to all treatments resulted in a significant decrease of CAT activity. High H_2O_2 content may lead to a decrease in CAT activity by substrate inactivation. Environmental stresses lead to expressions of SODs and Cu/Zn-SOD is the type of SOD which has critical roles in plant survival at stress conditions (Gill and Tuteja 2010). The CA and freezing resulted in raised Cu/Zn-SOD activity as well as *Cu/Zn-SOD* gene expression of both the cold tolerant wild species *C. echinospermum* and cv. İnci. *C. echinospermum* as cold tolerant positive control, was more tolerant to freezing temperatures than cultivars.

The low temperature tolerance of plants is mostly dependent on induction of the genes related to stress. Elevated freezing tolerance of İnci may be contributed with increased *Cu/Zn-SOD* gene expression. On the contrary, at rewarming treatments, Cu/Zn-SOD activities and *Cu/Zn-SOD* gene expressions in our cultivars remained unchanged. *PEBP-like* gene expression raised in genotypes exposed to CA and freezing. Predicted amino acid sequence of the *PEBP-like* gene showed homology to a cold regulated protein TaWcor18 (BAC41494.1) from wheat (Medini *et al.* 2009). It has been shown previously that cold-regulated/late embryogenesis abundant (COR/LEA) genes are induced by both

freezing and drought (Seki *et al.* 2002, Kume *et al.* 2005). Gene expression analyses showed that İnci exhibited the closest similarity to *C. echinospermum*.

In conclusion, freezing adversely affected the photosynthetic ability of genotypes and according to results, water content, photosynthetic pigments, and Chl *a* fluorescence parameters played a primary role to determine freezing tolerance or susceptibility of chickpea cultivars. The POD and flavonoids were efficient in detoxifying H₂O₂. The results of rewarming treatments, especially R₂, were effective to determine the tolerance of cultivars. Among the cultivars, İnci responded almost

similarly to freezing treatments as cold tolerant *C. echinospermum*. The molecular responses indicate that İnci may be used in breeding programmes and may be used as autumn sown cultivar. Moreover, at rewarming treatments, cultivars could maintain their vitality and mostly recovered from freezing injury occurred at -3 °C. These consequences show that chickpea may not be considered as completely chilling sensitive as it is often mentioned in the literature (Croser *et al.* 2003, Nayyar *et al.* 2005). In the future, productivity of these cultivars may be tested after exposure to freezing.

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