BIOLOGIA PLANTARUM 62 (1): 140-148, 2018 DOI: 10.1007/s10535-017-0760-5

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

Ö. ARSLAN[1+], F. EYİDOĞAN[2], and Y. EKMEKÇİ[1]*

Department of Biology, Faculty of Science, University of Hacettepe, 06800 Ankara, Turkey[1]
Department of Elementary Education, Education Faculty, Başkent University, 06810 Ankara, Turkey[2]

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, H_2O_2, 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-Jarmułowicz *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

Submitted 4 July 2015, *last revision* 18 May 2017, *accepted* 25 May 2017.
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.
Acknowledgements: The authors gratefully acknowledge the financial support of the Scientific Research Unit (Project No. 801601007) of the Hacettepe University.
[+]Current address: Department of Food Processing, University College of Espiye, University of Giresun, 28600 Giresun, Turkey
* Corresponding author: e-mail: yase@hacettepe.edu.tr

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 *Thriam* 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 μS 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 μg g^{-1} NH_4NO_3 and 100 μg g^{-1} KH_2PO_4 were added to the soil. Plants were grown in a controlled growth chamber at a constant temperature of 25 ± 1 °C, a relative humidity of 40 ± 5 %, a 16 h photoperiod, and an irradiance of 200 μmol m^2 s^{-1} under sufficient water supply for 15 d and then randomly divided into following groups: 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 and an irradiance of 150 μmol m^{-2} s^1); S_1 and S2: 22-d-old freezing-stressed plants that were cold acclimated and then subjected to -3 or -5 °C for 2 h; R_1 and R_2: 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 μmol m^{-2} 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}) = [γRC/(1-γRC)]X [φPo/(1-φPo)]X[ψo/(1-ψo)]*[(δRo/(1−δRo)] 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 wavelenths 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 (C_0).

The content of H_2O_2 was determined according to Esterbauer and Cheeseman (1990). H_2O_2 content was calculated according to the standard curve. Malon-dialdehyde (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 mM^{-1} 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 (C_1) and after (C_2) incubation of leaf segments (∅ 0.5 cm) in liquid nitrogen for 20 min and calculated as C_1/C_2. 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 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 ε = 2.8 mM^{-1} cm^{-1}. GR (EC 1.6.4.2) activity was determined by the method of Rao *et al*. (1995) and calculated using ε = 6.2 mM^{-1} cm^{-1} at 340 nm. POD (EC 1.11.1.7) activity determination was based on the measurement of guaiacol oxidation by H_2O_2 (ε = 26.6 mM^{-1} cm^{-1} at 470 nm; Bergmeyer 1974). CAT (EC 1.11.1.6) activity was determined according to the decomposition of H_2O_2 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 ε = 40 mM^{-1} 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).

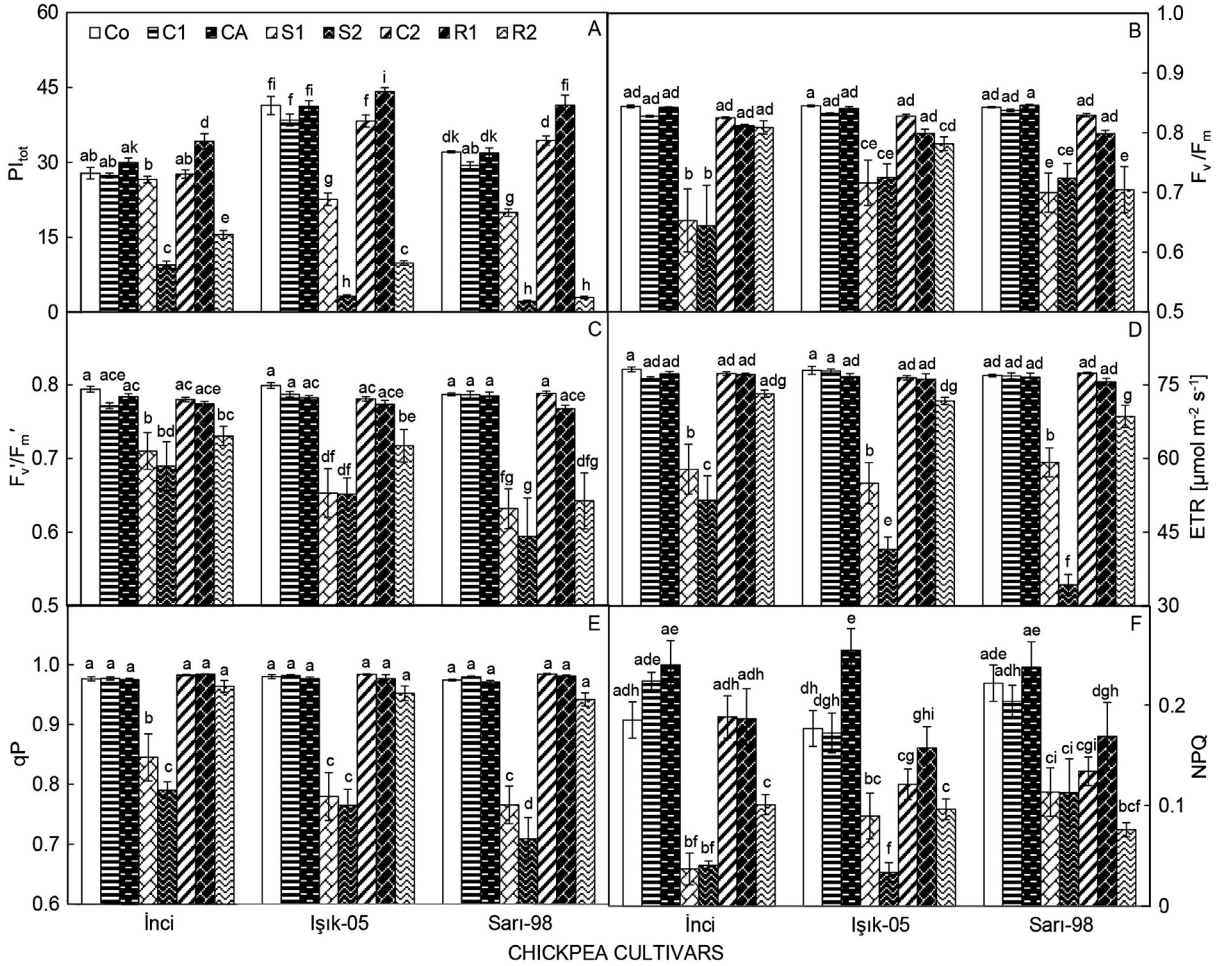

Fig. 1. The effect of freezing and rewarming on some Chl *a* fluorescence parameters: *A* - total performance index (PI_{tot}); *B* - quantum efficiency of PS 2 (F_v/F_m); *C* - quantum efficiency of excitation energy trapping by PS 2 (F_v'/F_m'); *D* - electron transport rate (ETR); *E* - photochemical quenching (qP); and *F* - non-photochemical quenching (NPQ). Means ± 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. 1*A*). 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. 1*B*,*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. 1*D*-*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 ± 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 *a*+*b* [μg cm^{-2}]	Carotenoids [μg cm^{-2}]	Anthocyanins [μg g^{-1}(f.m.)]	Flavonoids [% of control]
İnci	C_0	65±2 af	13 ±0.3 a	0.05 ±0.001 ah	100
	C_1	59 ±1 ab	13 ±0.1 a	0.05 ±0.001 ah	114
	CA	53 ±1 b	10 ±0.1 b	0.09 ±0.001 aei	159
	S_1	41 ±2 cd	8 ±0.2 c	0.09 ±0.001 aei	227
	S_2	35 ±1 ch	5 ±0.4 d	0.06 ±0.001 ah	240
	C_2	65 ±2 af	13 ±0.4 a	0.39 ±0.001 bj	179
	R_1	58 ±4 bg	12 ±0.8 e	0.47 ±0.001 c	341
	R_2	42 ±3 d	8 ±0.1 c	0.60 ±0.001 d	338
Işık-05	C_0	73 ±1 ej	16 ±0.1 f	0.06 ±0.001 ah	100
	C_1	67 ±1 ef	16 ±0.1 f	0.06 ±0.001 ah	87
	CA	61 ±1 afg	11 ±0.2 g	0.08 ±0.001 aeh	163
	S_1	39 ±1 cd	8 ±0.2 c	0.12 ±0.001 ei	249
	S_2	31 ±1 h	4 ±0.3 h	0.12 ±0.001 ei	273
	C_2	72 ±2 ej	13 ±1.0 a	0.33 ±0.001 f	140
	R_1	60 ±1 ag	12 ±0.5 e	0.67 ±0.001 g	343
	R_2	42 ±1 d	7 ±0.1 i	0.59 ±0.001 d	319
Sarı-98	C_0	67 ±2 ef	14 ±0.3 j	0.04 ±0.001 h	100
	C_1	62 ±2 afg	13 ±0.3 a	0.06 ±0.001 ah	92
	CA	56 ±5 bg	10 ±0.3 b	0.08 ±0.001 aeh	159
	S_1	30 ±2 h	8 ±0.4 c	0.08 ±0.001 aeh	177
	S_2	20 ±2 i	2 ±0.1 k	0.13 ±0.001 i	234
	C_2	76 ±2 j	15 ±0.4 l	0.43 ±0.001 bc	212
	R_1	59 ±1 ab	12 ±1.2 e	0.44 ±0.001 c	326
	R_2	39 ±3 cd	6 ±0.1 m	0.35 ±0.001 fj	287
LSD 5 %		7	1	0.05	

The H_2O_2 content increased in all cultivars at -3 °C (Fig. 2*A*), 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. 2*B*) 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. 2*C*). Exposure to CA and rewarming resulted in enhancement of proline content of all cultivars (Fig. 2*D*).

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

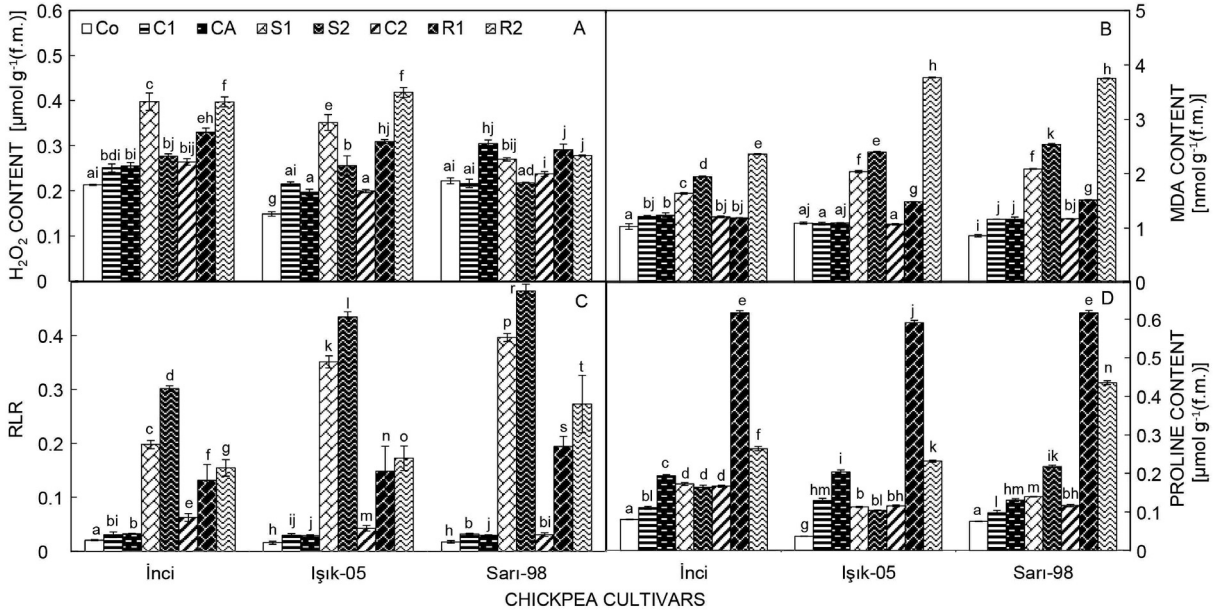

Fig. 2. The H_2O_2 content (*A*), malondialdehyde (MDA) content (*B*), relative leakage ratio (RLR; *C*), and proline content (D) of chickpea cultivars exposed to freezing and rewarming. Means ± SEs, $n = 3$ (H_2O_2, MDA and proline) and 6 (RLR). Different letters indicate significant difference at $P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

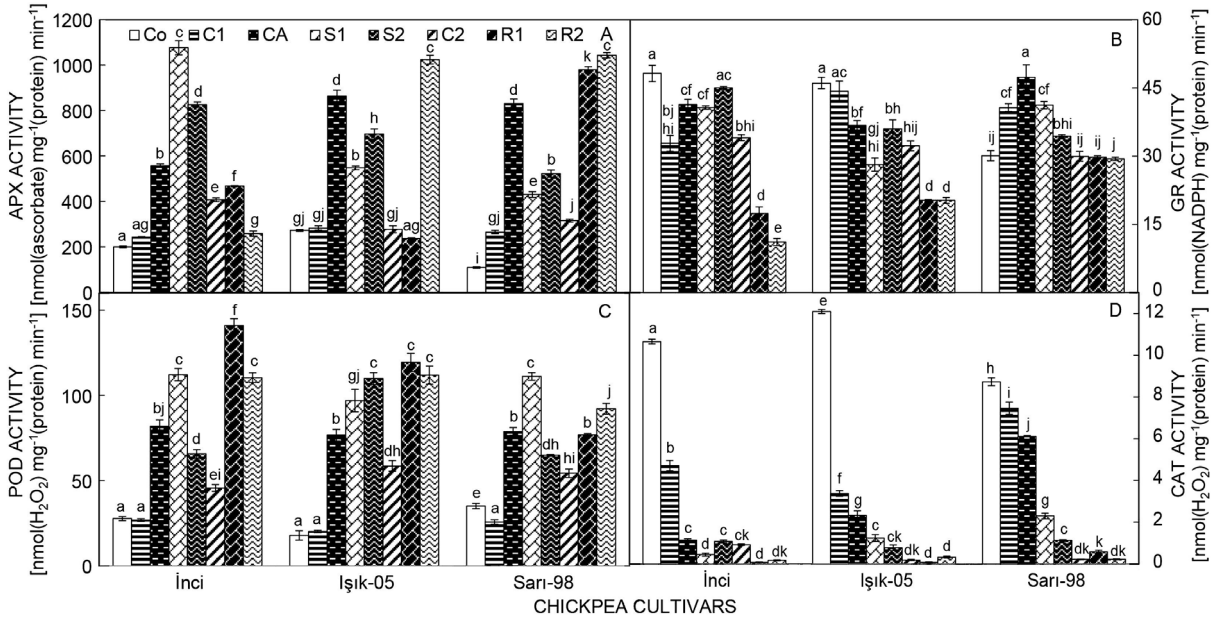

Fig. 3. The responses of antioxidant enzyme ascorbate peroxidase (APX; *A*), glutathione reductase (GR; *B*), peroxidase (POD; *C*) , and catalase (CAT; *D*) activities in chickpea cultivars exposed to freezing and rewarming. Means ± SEs, $n = 3$. Different letters indicate significant difference at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

(Fig. 3*C*). At R_1 and R_2, 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. 3*D*). 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	*Cu/Zn-SOD* gene expression	*PEBP-like* 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
C. echinospermum	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 S_2, could be decreased Chl *a*+*b* content and ETR. On the contrary, R_1 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 excessed energy dissipation was marked in Sarı-98 at freezing temperatures. The ETR showed decrease at freezing treatments, whereas at rewarming, cultivars reached the control levels, except Sarı-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 Sarı-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, Sarı-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 exceed 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 Sarı-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 Sarı-98, and increased activities of APX, GR, and POD of this cultivars were not sufficient to scavenge H_2O_2. Freezing at -3 °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 °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 Sarı-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 Sarı-98. Although, increased proline content of plants during cold stress is usually correlated with cold tolerance (John *et al*. 2016), in Sarı-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 H_2O_2. The results of rewarming treatments, especially R_2, were effective to determine the tolerance of cultivars. Among the cultivars, İnci responded almost similarly to 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.

References

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Bergmeyer, H.U.: Methods of Enzymatic Analysis, Vol. II. Section C: Methods for Determination of Enzyme Activity. - Academic Press, New York 1974.

Beyer, W.F., Fridovich, I.: Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. - Anal Biochem. **161**: 559-566, 1987.

Borawska-Jarmułowicz, B., Mastalerczuk, G., Kalaji, H.M., Carpentier, R., Pietkiewicz, S., Allakhverdiev, S.I.: Photosynthetic efficiency and survival of *Dactylis glomerata* and *Lolium perenne* following low temperature stress. - Russ. J. Plant Physiol. **61**: 281-288, 2014.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Burke, J.J., Oliver, M.J.: Differential temperature sensitivity of pea superoxide dismutase. - Plant Physiol. **100**: 1595-1598, 1992.

Cavender-Bares, J.: Chilling and freezing stress in live oaks (*Quercus* section *Virentes*): intra- and inter-specific variation in PS II sensitivity corresponds to latitude of origin. - Photosynth. Res. **94**: 437-453, 2007.

Chance, B., Maehly, A.C.: Assay of catalases and peroxidases. - Methods Enzymol. **2**: 764-817, 1955.

Chomczynski, P., Sacchi, N.: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. - Anal. Biochem. **162**: 156-159, 1987.

Croser, J., Clarke, H., Siddique, K., Khan, T.: Low-temperature stress: implications for chickpea (*Cicer arietinum* L.) improvement. - Crit. Rev. Plant Sci. **22**: 185-219, 2003.

Dai, F., Huang, Y., Zhou, M., Zhang, G.: The influence of cold acclimation on antioxidative enzymes and antioxidants in sensitive and tolerant barley cultivars. - Biol. Plant. **53**: 257-262, 2009.

Esterbauer, H., Cheeseman, K.H.: Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. - Methods Enzymol. **186**: 407-431, 1990.

Gill, S.S., Tuteja, N.: Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. - Plant Physiol. Biochem. **48**: 909-930, 2010.

Hao, W., Arora, R., Yadav, A.K., Joshee, N.: Freezing tolerance and cold acclimation in guava (*Psidium guajava* L.). - HortScience **44**: 1258-1266, 2009.

Hodges, D.M., DeLong, J.M., Forney, C.F., Prange, R.K.: Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. - Planta **207**: 604-611, 1999.

Janda, T., Szalai, G., Rios-Gonzalez, K., Veisz, O., Páldi, E.: Comparative study of frost tolerance and antioxidant activity in cereals. - Plant Sci. **164**: 301-306, 2003.

Janská, A., Maršík, P., Zelenková, S., Ovesná, J.: Cold stress and acclimation-what is important for metabolic adjustment?. - Plant Biol. **12**: 395-405, 2010.

John, R., Anjum, N.A., Sopory, S.K., Akram, N.A., Ashraf, M.: Some key physiological and molecular processes of cold acclimation. - Biol. Plant. **60**: 603-618, 2016.

Kalaji, H.M.., Goltsev, V., Bosa, K., Allakverdiev, S.I., Strasser, R.J., Govindjee.: Experimental *in vivo* measurements of light emission in plants: a perspective dedicated to David Walker. - Photosynth. Res., **114**: 69-96, 2012.

Kalaji, H.M., Jajoo, A., Oukarroum, A., Brestic, M., Zivcak, M., Samborska, I.A., Center, M.D., Łukasik, I., Goltsev, V., Ladle, R.: Chlorophyll *a* fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. - Acta Physiol. Plant. **38**: 102, 2016.

Kaur, G., Kumar, S., Thakur, P., Malik, J.A., Bhandhari, K., Sharma, K.D., Nayyar, H.: Involvement of proline in response of chickpea (*Cicer arietinum* L.) to chilling stress at reproductive stage. - Sci. Hort. **128**: 174-181, 2011.

Kume, S., Kobayashi, F., Ishibashi, M., Ohno, R., Nakamura, C., Takumi, S.: Differential and coordinated expression of *Cbf* and *Cor/Lea* genes during long-term cold acclimation in two wheat cultivars showing distinct levels of freezing tolerance. - Genes Genet. Syst. **80**: 185-197, 2005.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T(4). - Nature **227**: 680-685, 1970.

Li, X.G., Duan, W., Meng, Q.W., Zou, Q., Zhao, S.J.: The function of chloroplastic NAD(P)H dehydrogenase in tobacco during chilling stress under low irradiance. - Plant Cell Physiol. **45**: 103-108, 2004.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. - Method. Enzymol. **148**: 350-382, 1987.

Mancinelli, A.L., Yang, C.P.H., Lindquist, P., Anderson, O.R., Rabino, I.: Photocontrol of anthocyanin synthesis III. The action of streptomycin on the synthesis of chlorophyll and anthocyanin. - Plant Physiol. **55**: 251-257, 1975.

Medini, M., Baum, M., Hamza, S.: Transcript accumulation of putative drought responsive genes in drought-stressed chickpea seedlings. - Afr. J. Biotechnol. **8**: 4441-4449,

2009.

Mirecki, R.M., Teramura, A.H.: Effects of ultraviolet-B irradiance on soybean. V. The dependence of plant sensitivity on the photosynthetic photon flux density during and after leaf expansion. - Plant Physiol. **74**: 475-480, 1984.

Nayyar, H., Bains, T.S., Kumar, S.: Chilling stressed chickpea: effect of cold acclimation, calcium and abscisic acid on cryoprotective solutes and oxidative damage. - Environ. exp. Bot. **54**: 275-285, 2005.

Rao, V.M., Hale, B.A., Omrod, D.P.: Amelioration of ozone induced oxidative damage in wheat plants grown under high carbon dioxide. - Plant Physiol. **109**: 421-432, 1995.

Redmann, R.E., Haraldson, J., Gusta, L.V.: Leakage of UV-absorbing substances as a measure of salt injury in leaf tissue of woody species. - Physiol. Plant. **67**: 87-91, 1986.

Ruelland, E., Vaultier, M.N., Zachowski, A., Hurry, V.: Cold signalling and cold acclimation in plants. - Adv. bot. Res. **49**: 35-150, 2009.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., Satou, M., Akiyama, K., Taji, T., Yamaguchi-Shinozaki, K., Carninci, P., Kawai, J., Hayashizaki, Y., Shinozaki, K.: Monitoring the expression of 7 000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full length cDNA microarray. - Plant J. **31**: 279-292, 2002.

Strasser, B.J., Strasser, R.J.: Measuring fast fluorescence transients to address environmental questions: the JIP-test. - In: Mathis, P. (ed.): Photosynthesis: From Light to Biosphere. Vol. 5. Pp. 977-980. Kluwer Academic Publisher, Dordrecht 1995.

Strasser, R.J., Tsimilli-Michael, M., Srivastava, A.: Analysis of the fluorescence transient. - In: George, C., Papageorgiou, C., Govindjee. (ed.): Chlorophyll Fluorescence: A Signature of Photosynthesis. Pp. 321-362. Advances in Photosynthesis and Respiration. Springer, Dordrecht 2004.

Thakur, P., Kumar, S., Malik, J.A., Berger, J.D., Nayyar, H.: Cold stress effects on reproductive development in grain crops: an overview. - Environ. exp. Bot. **67**: 429-443, 2010.

Thomashow, M.F.: Plant cold acclimation. - Annu. Rev. Plant Physiol. Plant mol. Biol. **50**: 571-599, 1999.

Toda, K., Takahashi, R., Iwashina, T., Hajika, M.: Difference in chilling-induced flavonoid profiles, antioxidant activity and chilling tolerance between soybean near-isogenic lines for the pubescence color gene. - J. Plant Res. **124**: 173-182, 2011.

Turan, Ö., Ekmekçi, Y.: Chilling tolerance of *Cicer arietinum* lines evaluated by photosystem II and antioxidant activities. - Turk. J. Bot. **38**: 499-510, 2014.

Wang, S.Y., Jiao, H.J., Faust, M.: Changes in ascorbate, glutathione, and related enzyme activities during thidiazuron-induced bud break of apple. - Physiol. Plant. **82**: 231-236, 1991.

Weimberg, R.: Solute adjustments in leaves of two species of wheat at two different stages of growth in response to salinity. - Physiol. Plant. **70**: 381-388, 1987.