TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2014) 38: 499-510
© TÜBİTAK
doi:10.3906/bot-1309-7

Chilling tolerance of *Cicer arietinum* lines evaluated by photosystem II and antioxidant activities

Özlem TURAN[1], Yasemin EKMEKÇİ[2,*]

[1]Department of Food Processing, Espiye Vocational School, Giresun University, Giresun, Turkey
[2]Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey

Received: 05.09.2013 • **Accepted:** 20.12.2013 • **Published Online:** 31.03.2014 • **Printed:** 30.04.2014

Abstract: Two chickpea (*Cicer arietinum* L.) lines (AKN 87 and AKN 290) that have different chilling susceptibilities were exposed to 2 chilling temperatures (4 and 2 °C), either cold-acclimated (10 °C) or nonacclimated (25 °C), in order to understand and compare physiological and biochemical changes at the vegetative stage. Chilling temperatures resulted in reduced growth parameters, particularly in cold-acclimated lines, whereas nonacclimated plants exhibited the lowest water contents. Cold acclimation treatment led to protective changes of increased flavonoid, proline, and antioxidant enzyme activities, mostly superoxide dismutase (SOD) and ascorbate peroxidase (APX), in the lines. However, the 10 °C treatment did not significantly influence photosystem II (PSII) activity in chickpea plants. In chilling treatments, cold-acclimated plants exhibited better tolerance; of the 2 lines, AKN 87 had the higher PSII photochemical activity. The chlorophyll contents of lines decreased, while the anthocyanin and flavonoid contents of lines increased at decreasing temperatures. MDA and proline accumulation increased with the severity of the chilling stress. In conclusion, when the plants were exposed to cold acclimation, chilling induced the enhancement of antioxidant enzyme activities, particularly SOD and APX. The results demonstrated that cold acclimation reduced the deteriorative effects of chilling and provided better tolerance, specifically in AKN 87. The line AKN 87 has greater potential for cultivation as a chilling-tolerant cultivar.

Key words: Chilling, cold acclimation, photochemical, antioxidative response

1. Introduction

Suboptimal chilling temperatures can adversely affect crop production, mainly through the inhibition of key processes such as growth and photosynthesis. Physiological and biochemical damage occurs at temperatures below 10 °C, especially in chilling-sensitive plants of tropical or subtropical origin (Wang et al., 1997). Chilling causes reduced enzymatic activities, rigid membranes, destabilization of protein complexes, stabilized RNA secondary structure, accumulation of reactive oxygen species (ROS), impairment of photosynthesis, and leakage across membranes (Ruelland and Zachowski, 2010). Dysfunction caused by chilling was reported in previous research on various chilling-sensitive species, such as chickpea (Kaur et al., 2009), soybean (Strauss and van Heerden, 2011), and canola (Qaderi et al., 2012).

For chilling-sensitive species, exposure of the plant body to chilling temperatures results in a drastic decrease in photosynthetic activity. A major reason for disorder in photosynthetic function is the decreased activity of Calvin-cycle enzymes as a result of decreased temperature. Reduced activity of these enzymes leads to a decrease in utilized photons and an increase in photoinhibition (Sonoike, 1999). Therefore, chlorophyll antenna complexes trap more energy than can be processed biochemically (Ensminger et al., 2006) and thylakoid membranes become over-energized, which results in increased levels of formation of ROS, including singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) (Ruelland et al., 2009). As a result of the overproduction of ROS, oxidative stress responses such as membrane lipid peroxidation, protein denaturation and aggregation, DNA damage, and enzyme inactivation occur in plants. Plants have evolved many protective defense mechanisms including ROS scavenging systems such as enzymatic antioxidants [superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione peroxidase (GPX)] and nonenzymatic antioxidants [ascorbic acid (vitamin C), glutathione (GSH), α-tocopherols (vitamin E), carotenoids, and flavonoids] to mitigate the effects of oxidative stress (Gill and Tuteja, 2010; Baloğlu et al., 2012; Gharari et al., 2014; Verma et al., 2014). Cold acclimation, a phenomenon involving exposure to cool, nonchilling

* Correspondence: yase@hacettepe.edu.tr

temperatures before chilling, reduces chilling injury to chilling-sensitive plants. Cold acclimation is a cumulative process, and there is an inverse relationship between temperature and acclimation rate (Fowler, 2008). Cold acclimation decreases the susceptibility of cells to chilling temperatures by causing several metabolic alterations such as changes in gene expression and membrane composition, accumulation of substantial amounts of compatible solutes and antioxidants, and increases in ABA and calcium (Yuanyuan et al., 2009).

Chickpea (*Cicer arietinum* L.) is an important legume crop, and Turkey ranks third in production worldwide after India and Pakistan (http://www.fao.org, 2009). Croser et al. (2003) suggested that temperatures within the chilling range can limit the growth and viability of chickpea at all phenological stages, but chilling temperatures mostly damage the yield at the reproductive stage. Therefore, most studies on chickpea at chilling temperatures focused on reproductive development and yield (Clarke and Siddique, 2004; Nayyar et al., 2005b; Kumar et al., 2011). There are limited studies on the physiological approach to the vegetative stage of chilling-stressed chickpea cultivars and lines (Nayyar et al., 2005a; Turan and Ekmekçi, 2011). Moreover, chickpea vegetative-stage studies at chilling temperatures have recently become important in Turkey. Despite decreasing yields due to high temperature and drought, chickpea is still grown mostly in summer in Turkey. Hence, in order to eliminate the effects of drought and high temperature during its development period, the idea of planting chickpea in autumn instead of summer has been considered. In accordance with the information above, the aim of this study was as follows: 1) to compare physiological and biochemical changes in 2 recently developed chickpea breeding lines (AKN 87 and AKN 290) through exposure to low chilling temperatures (4 and 2 °C), either cold acclimated (10 °C) or not (25 °C); 2) to observe the contribution of cold acclimation to tolerance levels of the lines; and 3) to find a tolerant line that can be cultivated as a breed cultivar.

2. Materials and methods

The seeds of the chickpea (*Cicer arietinum* L.) lines (AKN 87 and AKN 290) that were selected from pretreatments were obtained from the Ankara Central Research Institute for Field Crops in Turkey, surface sterilized with 2% sodium hypochlorite (NaOCl) solution for 20 min, and soaked in distilled water for 12 h. After incubation, 8 seeds were planted in PVC pots holding 1000 g of air-dried soil with 38% water-holding capacity (Richards, 1954) and thinned to 5 after emergence. Some characteristics of the soil were as follows: texture, clay loam (Bouyoucos, 1951); pH 8.3 (Jackson, 1958); EC, 0.145 dS m^{-1} (Richards, 1954); total N, 0.19% (Bremner, 1965); P, 40.3 mg kg^{-1} (Pratt, 1965); and K, 525 mg kg^{-1} (Olsen et al., 1954). N, P, and K levels of the soil were sufficient for chickpea growth. Plants were grown in well-watered conditions at a constant temperature regime of 25 ± 1 °C with a 16-h photoperiod at 40 ± 5% humidity and 250 μmol m^{-2} s^{-1} light intensity in a controlled growth chamber. Plants were grown under the conditions mentioned above for 12 days (until they reached the 4–5 leaf stage) and were then randomly divided into groups to obtain the following combinations:

- C_0, C_1, C_2: 12-, 19-, and 31-day-old control plants, respectively;
- CA: 19-day-old cold-acclimated plants (12 days at control temperature, then 7 days at 10 ± 1 °C);
- CA+S_1: 31-day-old chilling-treated plants that were cold acclimated and then subjected to 4 °C for 12 days;
- NA+S_1: 31-day-old chilling-treated plants that were grown at control conditions for 19 days and then subjected to 4 °C for 12 days;
- CA+S_2: 31-day-old chilling-treated plants that were cold acclimated and then subjected to 2 °C for 12 days;
- NA+S_2: 31-day-old chilling-treated plants that were grown at control conditions for 19 days and then subjected to 2 °C for 12 days.

Plants were harvested at 12, 19, and 31 days after sowing to perform proper analysis.

Shoot length (the distance from soil surface to the node of a newly emerging leaf) of chickpea seedlings was measured (mm $plant^{-1}$) and the leaves were counted. Three plants from each group were taken randomly to determine fresh weights (g FW $plant^{-1}$) and were kept at 80 °C in an oven for 48 h to measure their dry weights (g DW $plant^{-1}$).

The water status of the leaves (3 leaflets from each treatment, 6 replications) was evaluated by calculating the percentage of relative water content (RWC) (Farrant, 2000).

For each treatment, photosynthetic pigments were extracted from leaflets (2 leaflets from each, 6 replicates). The content of chlorophyll ($a + b$) and carotenoids ($x + c$) was calculated as mg mL^{-1} g FW^{-1}, using adjusted extinction coefficients (Lichtenthaler, 1987). The anthocyanin content was calculated according to Mancinelli et al. (1975) and reported as mg mL^{-1} g FW^{-1}. The flavonoid content of leaves (0.1 g of fresh leaf tissue, 3 replications) was determined using the method of Mirecki and Teramura (1984) and was calculated as the percentage of 12-day-old control plants (C_0).

Chlorophyll *a* fluorescence measurements were performed in a growth chamber at 25 °C using a portable, modulated fluorescence monitoring system (FMS-2, Hansatech Instruments, UK) on randomly selected leaves (6 replicates). Following 30 min of dark adaptation, the minimum chlorophyll *a* fluorescence (F_o) was determined using a measuring beam of 0.2 μmol m^{-2} s^{-1} intensity. A

saturation pulse (1 s of white light, 7500 μmol m^{-2} s^{-1}) was used to obtain the maximum fluorescence (F_M) after a dark-adapted state. The maximal quantum efficiency of photosystem II (PSII) of dark-adapted plants (F_V/F_M) was calculated using $(F_M - F_o)/F_M$. The efficiency of the water-splitting complex on the donor side of PSII (F_V/F_O) was also calculated to assess PSII activity. Light-induced changes in chlorophyll *a* fluorescence were recorded following actinic illumination (300 μmol m^{-2} s^{-1}) prior to measurement of F_o' (minimum chlorophyll *a* fluorescence in light-saturated state) and F_M' (maximum fluorescence in light-saturated state). The quantum efficiency of PSII open centers in the light-adapted state, referred to as ΦPSII ($F_M' - F_S/F_M'$), was determined from F_M' and F_S (steady-state fluorescence in the light-saturated state) values, and the quantum efficiency of PSII excitation energy trapping (F_V'/F_M') was calculated according to the method of Genty et al. (1989). The actinic light was then removed, and minimum fluorescence in the light-adapted state (F_o') was determined by illuminating the leaves with far-red light (7 μmol m^{-2} s^{-1}). The photochemical quenching, $q_P = (F_M' - F_S)/(F_M' - F_o v)$, and nonphotochemical quenching, NPQ = $(F_M - F_M')/F_M'$, were calculated (Bilger and Björkman, 1990), and electron transport rate (ETR) was also calculated by the following formula: ETR = $(F_M' - F_S)/(F_M') \times PAR \times 0.84 \times 0.5$ (Genty et al., 1989).

Lipid peroxidation in the leaves was determined by measuring the amount of malondialdehyde (MDA) according to the method of Heath and Packer (1968) with some modifications (extraction solution contained 0.5% trichloroacetic acid and the homogenate was centrifuged at 12,000 rpm for 10 min). The content of MDA was determined by using the extinction coefficient of 155 mM^{-1} cm^{-1} and was expressed as nmol g FW^{-1}.

Free proline content of control and treated leaves (μmol g DW^{-1}) was determined using the method of Bates et al. (1973). Proline was extracted from leaf samples (20 mg DW, 3 replications) according to Weimberg (1987), with minor modifications.

For determination of enzyme activities, fresh leaf samples (0.5 g) from each treatment were ground with liquid nitrogen and suspended in specific buffer for each enzyme extraction. The protein concentrations of leaf extracts were determined according to Bradford's (1976) method.

Total SOD (EC 1.15.1.1) activity was assayed as described by Beyer and Fridovich (1987). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT photoreduction. The activity was expressed in units per milligram of protein.

APX (EC 1.11.1.1) activity was determined according to the method of Wang et al. (1991). The enzyme activity was calculated from the initial rate of the reaction using the extinction coefficient (ε) of ascorbate (ε = 2.8 mM cm^{-1} at 290 nm).

GR (EC 1.6.4.2) activity was determined according to the method of Rao et al. (1995). The enzyme activity was calculated from the initial rate of the reaction after subtracting the nonenzymatic oxidation using the extinction coefficient of NADPH (ε = 6.2 mM cm^{-1} at 340 nm). Guaiacol POD (EC 1.11.1.7) activity was based on the determination of guaiacol oxidation (ε = 26.6 mM cm^{-1}) at 470 nm by H_2O_2 (Bergmeyer, 1974). A unit of peroxidase activity was defined as nmol H_2O_2 decomposed per minute per milligram of protein.

The experiments were performed in a randomized design with 3 replicates. Differences among the treatments were tested using SPSS. Counted data, such as plant leaf numbers, were transformed into square roots to obtain the continuous scale and provide the normality assumption (Sokal and Rohlf, 1997). Statistical variance analysis was performed using ANOVA and compared with least significant differences (LSDs) at the 5% level.

3. Results

Cold acclimation (CA) (10 °C, 7 days) led to a 15% decrease in the shoot length of line AKN 87, whereas line AKN 290 exhibited no significant decrease according to corresponding controls (C_1) (Table 1). Chilling at 4 °C decreased only the shoot length of cold-acclimated lines. However, severe chilling temperature (2 °C) led to a significant decline in shoot elongation of both lines in both cold-acclimated ($CA+S_2$) and nonacclimated ($NA+S_2$) seedlings. The leaf numbers of AKN 87 and AKN 290 in CA treatments were significantly lower than in their controls (4% and 11%, respectively) (Table 1). Similarly, at both chilling temperatures (4 and 2 °C), the leaf numbers of both cold-acclimated ($CA+S_1$, $CA+S_2$) and nonacclimated ($NA+S_1$, $NA+S_2$) plants were significantly lower than in their corresponding controls (C_2), and this reduction occurred to the same extent in both lines (Table 1). The shoot fresh and dry weights for both lines in the $CA+S_2$ treatment decreased almost to the same extent at the drastic chilling temperature (Table 1).

Chilling treatments reduced the water content in leaves of the lines, whether they were cold-acclimated or not (Table 1). The reduction in water content of lines was marked at 2 °C, but the lowest RWC was determined in the $NA+S_2$ treatment of AKN 290 (30%).

CA resulted in a decrease of chlorophyll pigment of AKN 290 (34%) (Figure 1A). Similarly, at 4 °C the chlorophyll content reduced only for AKN 290, and to an approximately similar extent even when it was cold-acclimated, as compared to C_2. At the highest stress intensity, total chlorophyll content of lines decreased, mainly in nonacclimated leaves and to a lesser extent in AKN 87 than in AKN 290. The carotenoid

Table 1. Growth parameters and water contents of chickpea lines exposed to chilling temperatures.

Lines	Treatments	Length of shoot (mm plant^{-1})	Number of leaves (√number plant^{-1})	Fresh weight of shoot (g plant^{-1})	Dry weight of shoot (g plant^{-1})	RWC (%)
AKN 87	C_0	135* ± 1	2.2* ± 0.02	0.8** ± 0.04	0.1** ± 0.010	75*** ± 1.3
	C_1	170 ± 1	2.8 ± 0.01	1.3 ± 0.10	0.2 ± 0.002	75 ± 0.5
	CA	146 ± 1	2.7 ± 0.03	1.0 ± 0.02	0.2 ± 0.004	74 ± 0.5
	C_2	184 ± 1	3.3 ± 0.01	1.5 ± 0.04	0.4 ± 0.005	75 ± 0.4
	CA+S_1	154 ± 1	2.8 ± 0.02	1.4 ± 0.05	0.3 ± 0.010	70 ± 0.7
	NA+S_1	179 ± 2	2.9 ± 0.02	1.5 ± 0.10	0.3 ± 0.020	64 ± 0.5
	CA+S_2	147 ± 1	2.7 ± 0.02	1.0 ± 0.06	0.2 ± 0.020	65 ± 0.7
	NA+S_2	175 ± 1	2.9 ± 0.02	1.2 ± 0.10	0.2 ± 0.030	60 ± 0.7
AKN 290	C_0	124 ± 2	2.3 ± 0.02	0.8 ± 0.03	0.1 ± 0.004	77 ± 0.4
	C_1	159 ± 1	2.8 ± 0.02	1.0 ± 0.04	0.2 ± 0.010	76 ± 0.8
	CA	155 ± 1	2.5 ± 0.03	0.8 ± 0.07	0.1 ± 0.020	75 ± 0.6
	C_2	175 ± 3	3.2 ± 0.03	1.5 ± 0.25	0.3 ± 0.060	76 ± 0.9
	CA+S_1	162 ± 2	2.7 ± 0.02	1.2 ± 0.11	0.3 ± 0.020	69 ± 0.6
	NA+S_1	172 ± 3	2.8 ± 0.05	1.2 ± 0.10	0.3 ± 0.010	62 ± 0.5
	CA+S_2	156 ± 1	2.6 ± 0.03	1.1 ± 0.06	0.2 ± 0.010	63 ± 0.3
	NA+S_2	165 ± 1	2.8 ± 0.02	1.2 ± 0.07	0.3 ± 0.010	53 ± 0.6
LSD 5%		5	0.1	0.4	0.1	2

*: Each value represents the mean of 3 replicates, 5 plants each (n = 15), and its standard error (±SE).
**: Each value represents the mean of 3 replicates (n = 3) and its standard error (±SE).
***: Each value represents the mean of 6 replicates (n = 6) and its standard error (±SE).

content of the NA+S_2 treatment of AKN 87 increased significantly (14%) at the severe chilling temperature (Figure 1B). In the leaves of both lines, content of anthocyanin was higher in cold-acclimated leaves than in nonacclimated leaves when both were exposed to chilling temperatures (Figure 1C). Higher anthocyanin contents of lines were always significant compared to related controls (C_2). Similar to anthocyanin content, the flavonoid content was higher in both lines in CA, and it was more remarkable in AKN 290 (84%) (Figure 1D). Chilling temperatures resulted in a progressive enhancement of the flavonoid contents of AKN 87 and AKN 290, especially in cold-acclimated treatments (CA+S_1, CA+S_2). Slight differences in flavonoid content enhancement were also recorded between the 2 lines at 2 °C.

The *Fo* values of dark-adapted leaves of nonacclimated chickpea lines at 4 °C and both chilling treatments at 2 °C increased significantly (Figure 2A). On the other hand, F_M, F_V/F_M, and F_V/F_o values of dark-adapted leaves declined significantly at chilling temperatures, with the exception of the CA+S_1 treatment of AKN 87 (Figures 2B–2D). Similar to F_M, F_V/F_M, and F_V/F_o values, chilling temperatures induced a decreased pattern in $\varphi PSII$, F_V'/F_M', and ETR values of the leaves of both lines (Figures 2B–2D and 3A–3C). The reduction of these parameters was most evident for AKN 290 (Figures 3A–3C). No significant differences in the q_P values of light-adapted leaves were recorded for 4 °C treatments, while only the NPQ value of cold-acclimated AKN 87 rose sharply (80% of related control) (Figures 3D and 3E).

Chilling treatments resulted in a remarkable increase in MDA accumulation in leaf tissues of both lines in comparison with their controls (C_2) (Table 2). The gradual significant increase in the MDA content of both lines was more marked in nonacclimated leaves than in acclimated ones. Furthermore, severe chilling resulted in the highest MDA accumulation in both lines, specifically the NA+S_2 treatment of AKN 290 (2.5-fold of control).

Proline content of both lines increased progressively when compared with the corresponding controls (Table 3). Among the 4 °C treatments, proline accumulation in

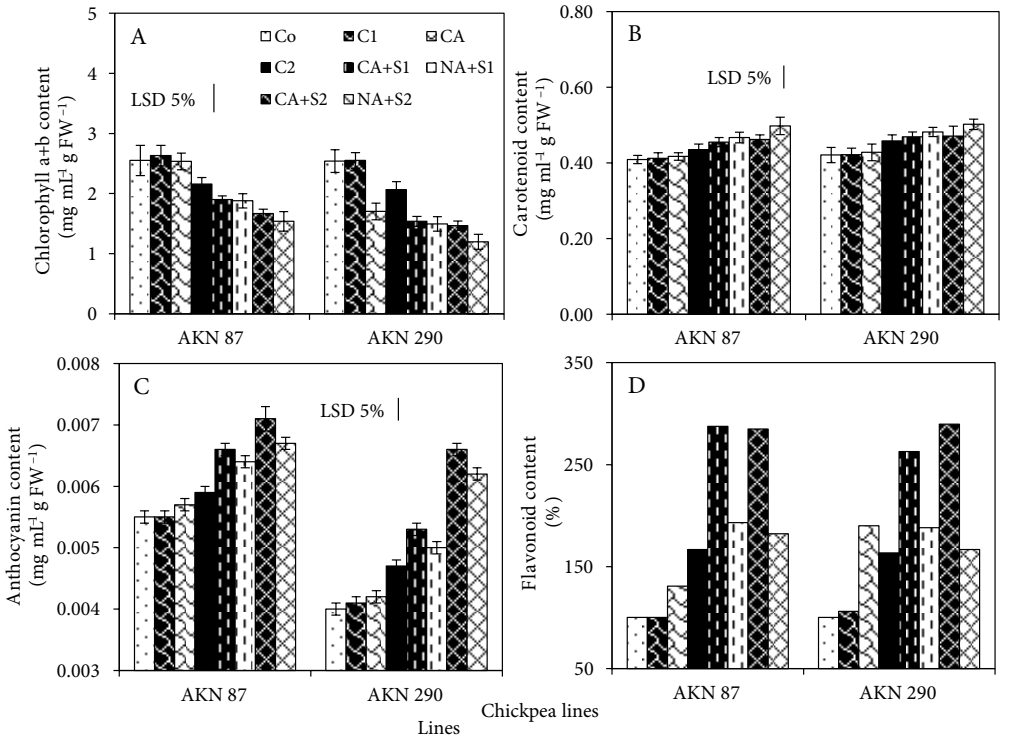

Figure 1. Effect of chilling on A) total chlorophyll, B) carotenoid, C) anthocyanin, and D) flavonoid contents of leaves of chickpea lines. The error bars represent the standard error (±SE) for 6 replicates.

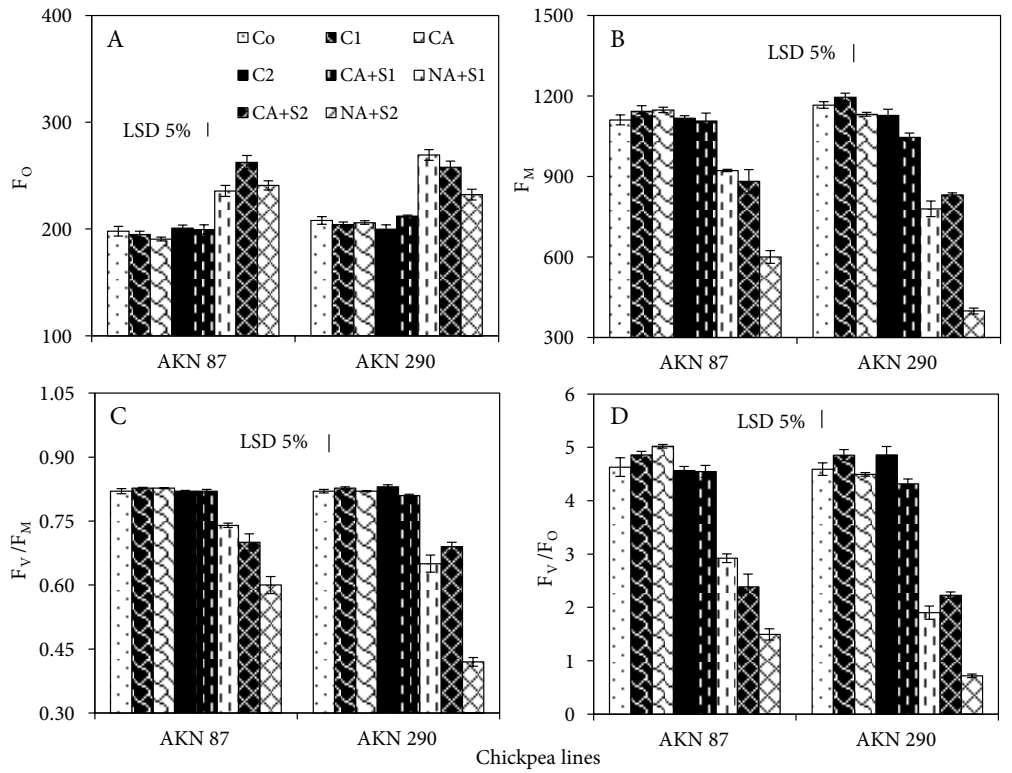

Figure 2. Chlorophyll *a* fluorescence parameters of the dark-adapted leaves of chickpea lines exposed to chilling temperatures: A) the minimum (F_o) and B) maximum (F_M) fluorescence, C) quantum efficiency of PSII (F_V/F_M), and D) efficiency of the water-splitting complex on the donor side of PSII (F_V/F_O). The error bars represent the standard error (±SE) for 6 replicates.

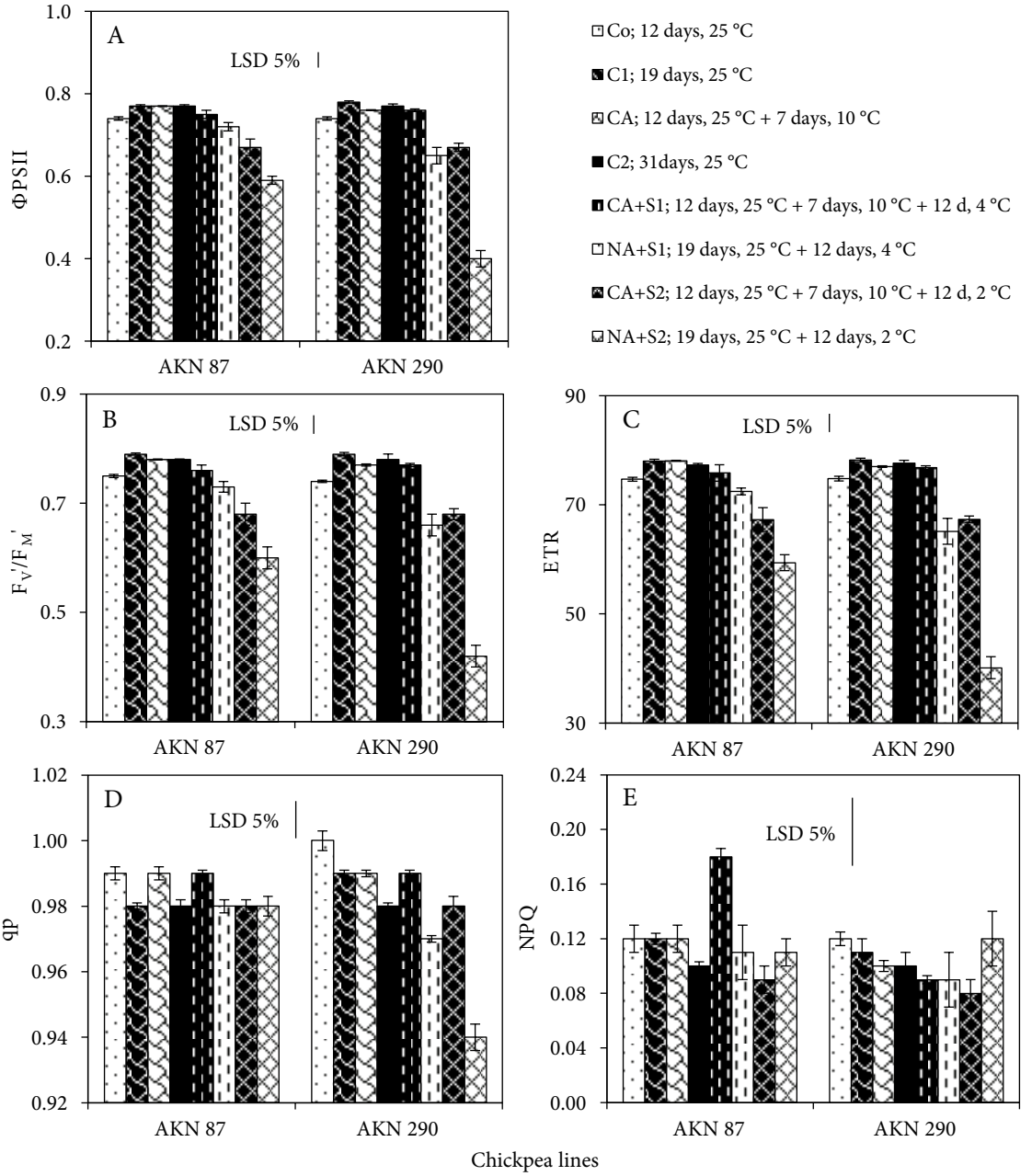

Figure 3. Chlorophyll *a* fluorescence parameters of the light-adapted leaves of chickpea lines exposed to chilling temperatures: A) the quantum efficiency of PSII open centers (ΦPSII), B) quantum efficiency of PSII excitation energy trapping (F_V'/F_M'), C) electron transport rate (ETR), D) photochemical quenching (*qp*), and E) nonphotochemical quenching (NPQ). The error bars represent the standard error (±SE) for 6 replicates.

AKN 87 and AKN 290 was 45% and 49%, respectively, when nonacclimated leaves (NA+S_1) were compared to cold-acclimated leaves (CA+S_1).

Both SOD and APX activities in leaves of the 2 lines increased significantly by CA treatment at 10 °C (Figures 4A and 4B). A remarkable increase in total SOD activity was determined in CA+S_1 as 5- and 7-fold of related controls (AKN 87 and AKN 290, respectively) (Figure 4A). Similar to 4 °C, severe chilling temperature resulted in highly significant increase in SOD activity in cold-acclimated leaves of AKN 87 and AKN 290 (2.5- and 5.8-fold of related controls, respectively). The APX activity of the cold-acclimated AKN 87 increased significantly at 4 °C, whereas the APX activity of the lines declined sharply

Table 2. The MDA content (nmol g FW^{-1}) of chickpea lines exposed to different chilling temperatures.

Lines	Treatments							
	C_0	C_1	CA	C_2	$CA+S_1$	$NA+S_1$	$CA+S_2$	$NA+S_2$
AKN 87	8* ± 0.3	12 ± 0.3	13 ± 0.1	15 ± 0.5	19 ± 0.1	20 ± 1.1	26 ± 0.4	31 ± 0.3
AKN 290	11 ± 0.3	14 ± 0.2	14 ± 0.1	15± 0.1	22 ± 0.2	27 ± 0.7	36 ± 0.8	38 ± 0.6
LSD 5%	2							

*: Each value represents the mean of 3 replicates (n = 3) and its standard error (±SE).

Table 3. The leaf proline contents (μmol g DW^{-1}) of chickpea lines exposed to different chilling temperatures.

Lines	Treatments							
	C_0	C_1	CA	C_2	$CA+S_1$	$NA+S_1$	$CA+S_2$	$NA+S_2$
AKN 87	1.8* ± 0.03	2.6 ± 0.02	3.9 ± 0.03	2.9 ± 0.05	4.2 ± 0.01	6.1 ± 0.02	5.7 ± 0.02	6.8 ± 0.04
AKN 290	1.8 ± 0.01	2.2 ± 0.03	3.6 ± 0.01	2.6 ± 0.03	3.9 ± 0.02	5.8 ± 0.06	4.9 ± 0.05	6.6 ± 0.05
LSD 5%	0.1							

*: Each value represents the mean of 3 replicates (n = 3) and its standard error (±SE).

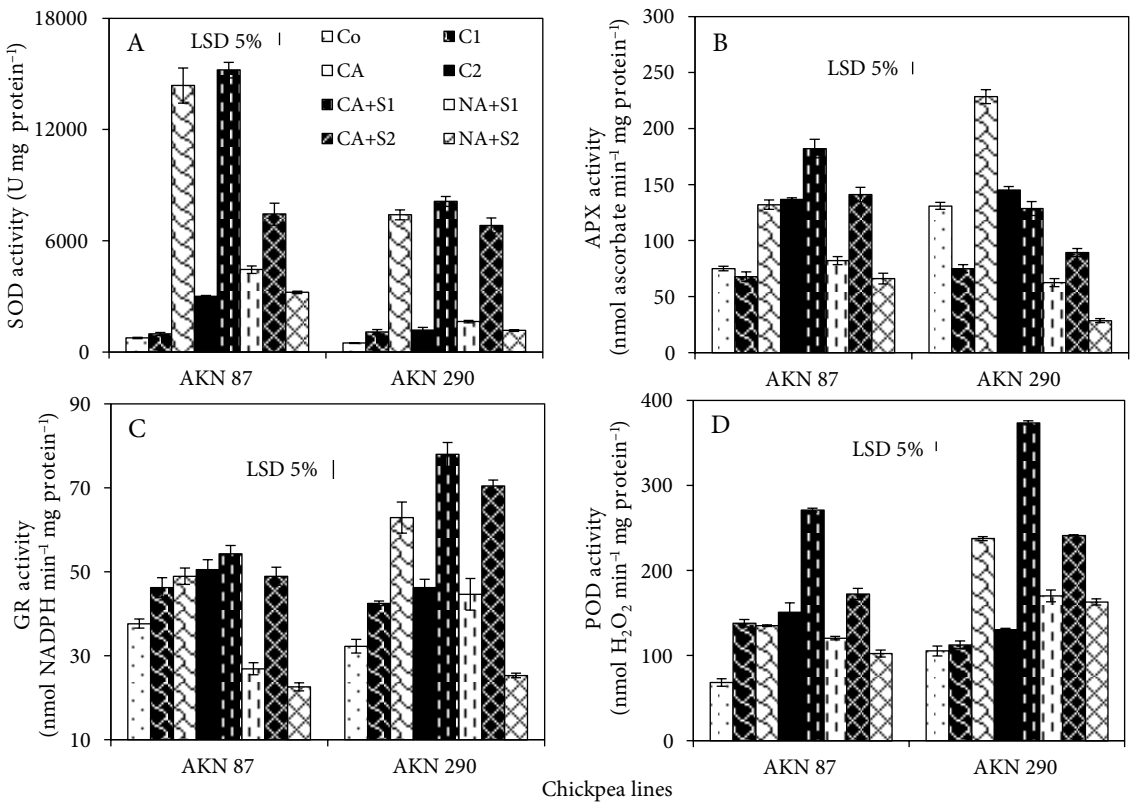

Figure 4. The responses of antioxidant enzyme activities of chickpea lines at different chilling temperatures: A) SOD activity, B) APX activity, C) GR activity, and D) POD activity. The error bars represent the standard error (±SE) for 3 replicates.

in the $NA+S_1$ treatment (Figure 4B). Similarly, drastic reduction of APX activity of lines was recorded at 2 °C, with the exception of AKN 87 at $CA+S_2$. The CA treatment of AKN 290 substantially increased GR and POD activities (1.5- and 2.1-fold, respectively) (Figures 4C and 4D). The effect of 4 °C on the GR activity of lines was variable (Figure 4C). The $CA+S_1$ treatment increased GR activity by 69% in AKN 290, while $NA+S_1$ decreased it by 47% of the control in AKN 87. At 2 °C, GR activity was raised only in the leaves of $CA+S_2$-treated AKN 290 (52%), while the lines exhibited significantly lower GR activities under $NA+S_2$. Chilling at 4 °C resulted in an enhancement of the POD activity of lines, with the exception of nonacclimated AKN 87, where 20% reduction was determined (Figure 4D). $CA+S_2$ treatment induced an enhancement of POD activity in the 2 lines, and AKN 290 had the highest activity (85% of the related control).

4. Discussion

The effects of cold acclimation on some growth and physiological aspects of 2 *Cicer arietinum* lines at 2 chilling temperatures were investigated. Under suboptimal but not yet freezing temperatures, plants of tropical or subtropical origin, such as chickpea, frequently suffer chilling injury. In our study, the 2 chilling temperatures (4 and 2 °C) had adverse effects on lines, even if the seedlings were cold-acclimated. The vegetative growth parameters of nonacclimated plants appeared to be higher than those of cold-acclimated ones, probably since the former continued vegetative growth under controlled conditions for 7 days more than the latter, which were exposed to 10 °C during this period. The shoot elongation and leaf production in both lines were reduced by chilling temperatures to nearly the same extent. Croser et al. (2003) mentioned that chilling temperatures negatively affect chickpea growth and dry matter production at the vegetative stage. The $CA+S_2$ treatment of chickpea lines, specifically AKN 87, resulted in a pronounced reduction in both fresh and dry weights of shoots, due to the lowest shoot elongation under this treatment (Table 1). The effects of chilling are compounded by its effect on plant water relations since chilling reduces the ability of roots to supply water (Aroca et al., 2003). Chilling led to drastic reductions in water content of both chickpea lines according to RWC values (Table 1). According to water content results, AKN 290 had the greatest dehydration. Treatments involving cold acclimation had significantly higher leaf water content compared to nonacclimation treatments. Elevated levels of cryoprotectants such as sugars and proline during the CA period may play a crucial role in maintaining water potential and thus the metabolic homeostasis of the chilled cells.

Chilling impairs all photosynthetic components by provoking reduction of stomatal conductance, changes in pigment complexes, and loss of photochemical efficiency (Fortunato et al., 2010). Total chlorophyll contents of lines were decreased dramatically by chilling temperature, regardless of severity, particularly in AKN 290 (Figure 1A). Mohanty et al. (2006) suggested that the reason for reduced chlorophyll content in chilled cells could be decreased chlorophyll synthesis, faster chlorophyll degradation, or both. CA resulted in a significant decline in total chlorophyll content of AKN 290, but, interestingly, that reduction did not lead to any photosynthetic activity disorders as determined by chlorophyll *a* fluorescence measurements (Figures 2 and 3). Contrary to total chlorophyll, the content of protective pigments, which prevent the photoinhibition of photosynthesis, increased at varying rates by chilling temperature. The gradual increase in carotenoid content was significant in $NA+S_2$ for AKN 87 (Figure 1B). Carotenoids protect the chlorophyll molecules from photooxidation by inhibiting the generation of singlet oxygen and scavenging the produced singlet oxygen. Chilling temperatures induce the enhancement of carotenoid content in leaves (Aroca et al., 2001). Accumulation of anthocyanin in the leaves of both lines was more pronounced at 2 °C (Figure 1C). Similar results regarding the increase in anthocyanin content at low temperatures were reported by Hasdai et al. (2006). Flavonoids have antioxidant roles such as scavenging ROS and preventing plants from chilling-induced photoinhibition and photooxidation under excess visible radiation (Havaux and Kloppstech, 2001). The flavonoid content of lines increased under all chilling treatments (Figure 1D). Moreover, CA enhanced the flavonoid content of the lines, and accumulation of flavonoids proceeded during treatments involving CA ($CA+S_1$, $CA+S_2$). It has been reported that soybean plants enhance chilling tolerance by enforcing defense mechanisms against oxidative stress via expression of genes for flavonoid biosynthesis (Toda et al., 2011). In chilling treatments, the increased anthocyanin and flavonoid contents of chickpea could act as a potential safeguard against the damage induced by chilling and, thereby, protect the photosynthetic apparatus against ROS.

Stress-induced alteration in the photosynthetic apparatus measured during chilling conditions by chlorophyll *a* fluorescence is a rapid screening method to assess chilling tolerance (Baker and Rosenquist, 2004; Strauss and van Heerden, 2011). In the present study, chlorophyll *a* fluorescence measurements showed that CA did not cause photoinhibition of PSII in the lines (Figures 2 and 3). However, exposure to chilling temperatures, cold acclimated or not, led to significant alterations of the chlorophyll *a* fluorescence parameters of the lines, reflecting the chilling-induced photoinhibition (Figures 2 and 3). There was a significant increase in the F_o values of dark-adapted leaves of lines subjected to chilling

temperatures, except in CA+S_1 treatment (Figure 2A). F_o is a measure for the initial distribution of energy to PSII, and the rise of F_o reflects reduced energy transport effectiveness from the antenna to reaction center of PSII (Georgieva and Lichtenthaler, 1999). The photochemical efficiency of dark-adapted leaves (F_V/F_M) of the lines declined in NA+S_1 and all 2 °C treatments (Figure 2C), which indicates that 4 °C seems to be the threshold chilling temperature at which the photosynthetic apparatus can protect itself from photoinhibition when the leaves are cold-acclimated. Loss of photochemical efficiency of PSII, as assessed by decreased F_V/F_M, indicates photochemically inactive reaction centers and reduced electron transport capacity in PSII (Genty et al., 1989). The efficiency of the water-splitting complex on the donor side of PSII (F_V/F_o) is the most sensitive component in the photosynthetic electron transport chain (Kalaji et al., 2011). In the present study, F_V/F_o values showed a marked decrease in both 2 °C and nonacclimated 4 °C treatments (Figure 2D). Pereira et al. (2000) suggested that decreasing F_V/F_o ratios result from photosynthetic electron transport impairment. Similarly, the actual PSII photochemical efficiency of light-adapted leaves (*φPSII*) was reduced by decreasing temperatures (Figure 3A). According to Colom and Vazzana (2003), reductions of *φPSII* are related to significant reductions of F_V'/F_M'. Hence, reductions of both *φPSII* and F_V'/F_M' occurred to the same extent at the chilling temperatures applied to the lines, and of the 2 lines, AKN 290 had the lowest values of these parameters (Figures 3A and B). F_V'/F_M' is the maximum efficiency of PSII when all PSII reaction centers are 'open' (Farage et al., 2006). Decreased levels of this parameter occur as a result of increased energy dissipation. Moreover, chilling temperatures reduced the electron transport rate (ETR) of the leaves of the lines (Figure 3C). The q_P value that shows the intensity of light energy consumption in photochemical processes decreased only in AKN 290 for nonacclimated treatments (Figure 3D). That result may demonstrate the chilling susceptibility of AKN 290 and the necessity of cold acclimation. Light energy dissipation as heat, which is indicated by increasing nonphotochemical quenching (NPQ) of chlorophyll fluorescence, is an important protective response mechanism to prevent over-reduction of quinone molecules (Demmig-Adams et al., 2005). NPQ is often used as an indicator of the excess energy dissipation to heat in PSII antenna complexes (Roháček, 2002). In the present study, only the CA+S_1 treatment of AKN 87 led to an increase in the NPQ parameter (Figure 3E). Consequently, chlorophyll *a* fluorescence results indicated that cold acclimation enhanced the protection of photoinhibition of PSII from subsequent chilling temperatures. Nonacclimated treatments of severe chilling temperature caused slowly developing photoinhibitory damage in both lines, but this damage was marked in AKN 290.

Lipoperoxidative activities, reported as MDA levels and leading to loss of membrane integrity, increased progressively in chilling temperature treatments (Table 2). During severe chilling, the increase was much higher than in control plants, specifically in AKN 290. This result indicates a distinct deterioration of membrane integrity and activation of lipid peroxidation in the leaves of the lines and is in accordance with previous findings that presented increased levels of MDA due to chilling injury (Ma et al., 2010). Lipid peroxidation triggers the action of free radicals. Carotenoids and flavonoids are physiologically important for protection against these radicals (Oliveira et al., 2009). However, in this study, the increase in lipid peroxidation occurred together with increased carotenoids and flavonoids at 2 °C (Figures 1B and 1D). This finding suggests a partial inability of these pigments to inhibit the peroxidation action of free radicals. The amino acid proline is thought to be a compatible solute and plays a role in protecting enzymes from denaturation, stabilizing the machinery of protein synthesis, regulating the cytosolic acidity, and increasing water-binding capacity (Ruelland et al., 2009). CA induced a remarkable accumulation of proline to the same extent in both lines (Table 3). Moreover, proline contents in nonacclimated treatments were always higher than in cold-acclimated treatments. Accumulation of proline during stress conditions was mostly correlated with tolerance (Ashraf and Foolad, 2007). However, our results indicate that enhanced levels of proline in nonacclimated leaves of lines may occur as a result of chilling injury-induced protein degradation.

The increased synthesis and accumulation of ROS have been associated with injury development through oxidative stress. Induction of oxidative stress in chilling-stressed plants has been described in previous studies (Pennycooke et al., 2005; Turan and Ekmekçi, 2011). These studies showed that the contents of enzymatic antioxidants played an important role in scavenging harmful oxygen species, and the activities of antioxidant enzymes were altered when plants were subjected to chilling stress. In the current study, we have shown that exposure to 10 °C led to a significant increase in the activities of tested antioxidant enzymes in both lines (Figure 4), with the exception of GR and POD activities of AKN 87 (Figures 4C and 4D). Among the activities of antioxidant enzymes, SOD activity reached the highest values during CA in both lines (Figure 4A). The ability of cold acclimation to enhance or preserve antioxidant enzyme activity in chickpea seedlings may contribute to the ability of this treatment to increase chilling tolerance. SOD provides the first line of defense against the toxic effects of elevated levels of ROS and removes superoxide ion ($O_2^{\cdot -}$) by catalyzing its dismutation

to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Gill and Tuteja, 2010). At both chilling temperatures, cold-acclimated chickpea lines enhanced their SOD activities (Figure 4A). APX is the first enzyme of the ascorbate-glutathione cycle and functions by reducing H_2O_2 to water (Asada, 1999). The APX activities of lines were variable (Figure 4B). While activity of APX increased with CA, it decreased dramatically by chilling temperature in both lines, with the exception of cold-acclimated treatments of AKN 87. GR is the last and rate-limiting enzyme of the ascorbate-glutathione cycle and it participates in the removal of H_2O_2 (Asada, 1999). In our study, the analysis of variance for GR activity indicated a highly significant difference between lines (Figure 4C). AKN 290 always had the highest GR activity and, among the treatments, CA and subsequent chilling temperatures resulted in increased GR activity, while nonacclimated treatments of this line remained unchanged ($NA+S_1$) or decreased ($NA+S_2$). To scavenge H_2O_2, plants evolved an antioxidant system, including POD, APX, GR, and CAT (Xu et al., 2008). Compared to optimum temperature, POD activity increases when plants are exposed to low temperatures (Song et al., 2006). Likewise, POD activities of leaves of chickpea lines increased in all chilling temperature treatments, except nonacclimated AKN 87 (Figure 4D). Enhancement in the leaves of AKN 290 was more marked.

These results showed that elevated levels of SOD in cold-acclimated lines appear to be correlated with increased protection against oxidative stress by cold acclimation, and the lines employ different pathways to cope with oxidative stress depending on their ability to scavenge ROS. The results of this study suggest that chilling temperature treatments lead to a serious level of injury in chickpea lines, especially when nonacclimated, and severe chilling temperature (2 °C) was more effective in representing the susceptibility of the lines. CA reduced the damage and enhanced the defense capacity of the lines for further chilling temperatures, as verified by data on protective mechanisms such as proline and antioxidant enzymes. Among the lines, AKN 87 had a better performance under chilling. In conclusion, AKN 87, a recently developed chickpea line, exhibited a higher potential for cultivation under conditions of chilling as a cultivar. When AKN 87 is cultivated, it can be successfully grown in the suboptimal temperatures of autumn at a vegetative stage, so that its reproductive developmental stage is not affected by the high temperature and drought of summer.

Acknowledgment

The authors would like to thank the Hacettepe University Scientific Research Unit (Project No.: 03026601001) for financial support.

References

Aroca R, Irigoyen JJ, Sánchez-Díaz M (2001). Photosynthetic characteristics and protective mechanisms against oxidative stress during chilling and subsequent recovery in two maize varieties differing in chilling sensitivity. Plant Sci 161: 719–726.

Aroca R, Vernieri P, Irigoyen JJ, Sánchez-Díaz M, Tognoni F, Pardossi A (2003). Involvement of abscisic acid in leaf and root of maize (*Zea mays* L.) in avoiding chilling-induced water stress. Plant Sci 165: 671–679.

Asada K (1999). The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 50: 601–639.

Ashraf M, Foolad MR (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59: 206–216.

Baker NR, Rosenquist E (2004). Applications of chlorophyll fluoresences can improve crop production strategies: an examination of future possibilities. J Exp Bot 55: 1607–1621.

Baloğlu MC, Kavas M, Aydın G, Öktem HA, Yücel AM (2012). Antioxidative and physiological responses of two sunflower (*Helianthus annuus*) cultivars under PEG-mediated drought stress. Turk J Bot 36: 707–714.

Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water-stress studies. Plant Soil 39: 205–207.

Bergmeyer HU (1974). Methods for determination of enzyme activity. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis, Vol. II. London, UK: Academic Press, pp. 685–690.

Beyer WF, Fridovich I (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 161: 559–566.

Bilger W, Björkman O (1990). Role of the xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in *Hedera canariensis*. Photosynth Res 25: 173–185.

Bouyoucos GJ (1951). A recalibration of hydrometer for marking mechanical analysis of soil. Agron J 43: 434–438.

Bradford MM (1976). 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.

Bremner JM (1965). Total nitrogen. In: Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. Madison, Wisconsin, USA: American Society of Agronomy, pp. 1149–1178.

Clarke HJ, Siddique KHM (2004). Response of chickpea genotypes to low temperature stress during reproductive development. Field Crop Res 90: 323–334.

Colom M, Vazzana C (2003). Photosynthesis and PSII functionality of drought-resistant and drought-sensitive weeping lovegrass. Environ Exp Bot 49: 135–144.

Croser JS, Clarke HJ, Siddique KHM, Khan TN (2003). Low temperature stress: implications for chickpea (*Cicer arietinum* L.) improvement. Critic Rev Pl Sci 22: 185–219.

Demmig-Adams B, Adams WW 3rd, Mattoo A (2005). Photoprotection, Photoinhibition, Gene Regulation, and Environment. Advances in Photosynthesis and Respiration, Vol. 21. Dordrecht, the Netherlands: Springer.

Ensminger I, Busch F, Huner NPA (2006). Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol Plant 126: 28–44.

Farage PK, Blowers D, Long SP, Baker NR (2006). Low growth temperatures modify the efficiency of light use by photosystem II for CO_2 assimilation in leaves of two chilling-tolerant C_4 species, *Cyperus longus* L. and *Miscanthus* × *giganteus*. Plant Cell Environ 29: 720–728.

Farrant JM (2000). A comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plant species. Plant Ecol 151: 29–39.

Fortunato AS, Lidon FC, Batista-Santos P, Leitão AE, Pais IP, Ribeiro AI, Ramalho JC (2010). Biochemical and molecular characterization of the antioxidative system of *Coffea* sp. under cold conditions in genotypes with contrasting tolerance. J Plant Physiol 167: 333–342.

Fowler DB (2008). Cold acclimation threshold induction temperatures in cereals. Crop Sci 48: 1147–1154.

Genty B, Briantais JM, Baker NR (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim Biophys Acta 990: 87–92.

Georgieva K, Lichtenthaler HK (1999). Photosynthetic activity and acclimation ability of pea plants to low and high temperature treatment as studied by means of chlorophyll fluorescence. J Plant Physiol 155: 416–423.

Gharari Z, Nejad RK, Band RS, Najafi F, Nabiuni M, Irian S (2014). The role of *Mn-SOD* and *Fe-SOD* genes in the response to low temperature in *chs* mutants of *Arabidopsis*. Turk J Bot 38: 80–88.

Gill SS, Tuteja N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48: 909–930.

Hasdai M, Weiss B, Levi A, Samac A, Porat R (2006). Differential responses of *Arabidopsis* ecotypes to cold, chilling and freezing temperatures. Ann Appl Biol 148: 113–120.

Havaux M, Kloppstech K (2001). The protective functions of carotenoid and flavonoid pigments. Planta 213: 953–966.

Heath RL, Packer L (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125: 189–198.

Jackson ML (1958). Soil Chemical Analysis. Englewood Cliffs, NJ, USA: Prentice Hall.

Kalaji HM, Govindjee, Bosa K, Kościelniak J, Żuk-Gołaszewska K (2011). Effects of salt stress on photosystem II efficiency and CO_2 assimilation of two Syrian barley landraces. Environ Exp Bot 73: 64–72.

Kaur S, Gupta AK, Kaur N, Sandhu JS, Gupta SK (2009). Antioxidative enzymes and sucrose synthase contribute to cold stress tolerance in chickpea. J Agron Crop Sci 195: 393–397.

Kumar S, Malik J, Thakur P, Kaistha S, Sharma KD, Upadhyaya HD, Berger JD, Nayyar H (2011). Growth and metabolic responses of contrasting chickpea (*Cicer arietinum* L.) genotypes to chilling stress at reproductive phase. Acta Physiol Plant 33: 779–787.

Lichtenthaler HK (1987). Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Method Enzymol 148: 350–382.

Ma YY, Zhang YL, Shao H, Lu J (2010). Differential physio-biochemical responses to cold stress of cold-tolerant and non-tolerant grapes (*Vitis* L.) from China. J Agron Crop Sci 196: 212–219.

Mancinelli AL, Yang CPH, Lindquist P, Anderson OR, Rabino I (1975). Photocontrol of anthocyanin synthesis III. The action of streptomycin on the synthesis of chlorophyll and anthocyanin. Plant Physiol 55: 251–257.

Mirecki RM, Teramura AH (1984). 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.

Mohanty S, Grimm B, Tripathy BC (2006). Light and dark modulation of chlorophyll biosynthetic genes in response to temperature. Planta 224: 692–699.

Nayyar H, Bains TS, Kumar S (2005a). Chilling stressed chickpea: effect of cold acclimation, calcium and abscisic acid on cryoprotective solutes and oxidative damage. Environ Exp Bot 54: 275–285.

Nayyar H, Bains TS, Kumar S (2005b). Low temperature induced floral abortion in chickpea: relationship to abscisic acid and cryoprotectants in reproductive organs. Environ Exp Bot 53: 39–47.

Oliveira JG, Alves PLCA, Vitória AP (2009). Alterations in chlorophyll *a* fluorescence, pigment concentrations and lipid peroxidation to chilling temperature in coffee seedlings. Environ Exp Bot 67: 71–76.

Olsen SR, Cole CV, Watanabe FS, Dean NC (1954). Estimation of Available Phosphorus in Soil by Extraction with Sodium Bicarbonate. Washington DC, USA: US Department of Agriculture.

Pennycooke JC, Cox S, Stushnoff C (2005). Relationship of cold acclimation, total phenolic content and antioxidant capacity with chilling tolerance in petunia (*Petunia* × *hybrida*). Environ Exp Bot 53: 225–232.

Pereira WE, de Siqueira DL, Martínez CA, Puiatti M (2000). Gas exchange and chlorophyll fluorescence in four citrus rootstocks under aluminium stress. J Plant Physiol 157: 513–520.

Pratt PF (1965). Potassium, sodium. In: Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. Madison, Wisconsin, USA: American Society of Agronomy, pp. 1022–1034.

Qaderi MM, Kurepin LV, Reid DM (2012). Effects of temperature and watering regime on growth, gas exchange and abscisic acid content of canola (*Brassica napus*) seedlings. Environ Exp Bot 75: 107–113.

Rao VM, Hale BA, Omrod DP (1995). Amelioration of ozone induced oxidative damage in wheat plants grown under high carbon dioxide. Plant Physiol 109: 421–432.

Richards LA (1954). Diagnosis and Improvement of Saline and Alkaline Soils. USDA Salinity Laboratory Agricultural Handbook, No. 60. Washington, DC, USA: US Department of Agriculture, pp. 110–118.

Roháček K (2002). Chlorophyll fluorescence parameters: the definitions, photosynthetic meaning, and mutual relationship. Photosynthetica 40: 13–29.

Ruelland E, Vaultier MN, Zachowski A, Hurry V (2009). Cold signalling and cold acclimation in plants. Adv Bot Res 49: 35–150.

Ruelland E, Zachowski A (2010). How plants sense temperature. Environ Exp Bot 69: 225–232.

Sokal RR, Rohlf FJ (1997). Biometry: The Principles and Practice of Statistics in Biological Research. 3rd ed. New York, NY, USA: W.H. Freeman and Company Publisher.

Song GL, Hou WH, Wang QH, Wang JL, Jin XC (2006). Effect of low temperature on eutrophicated waterbody restoration by *Spirodela polyrhiza*. Bioresour Technol 97: 1865–1869.

Sonoike K (1999). The different roles of chilling temperatures in the photoinhibition of photosystem I and photosystem II. J Photochem Photobiol B: Biol 48: 136–141.

Strauss AJ, van Heerden PDR (2011). Effects on both the roots and shoots of soybean during dark chilling determine the nature and extent of photosynthesis inhibition. Environ Exp Bot 74: 261–271.

Toda K, Takahashi R, Iwashina T, Hajika M (2011). 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.

Turan Ö, Ekmekçi Y (2011). Activities of photosystem II and antioxidant enzymes in chickpea (*Cicer arietinum* L.) cultivars exposed to chilling temperatures. Acta Physiol Plant 33: 67–78.

Wang SY, Jiao HJ, Faust M (1991). Changes in ascorbate, glutathione, and related enzyme activities during thidiazuron-induced bud break of apple. Physiol Plant 82: 231–236.

Wang Z, Reddy VR, Quebedeaux B (1997). Growth and photosynthetic responses of soybean to short-term cold temperature. Environ Exp Bot 37: 13–24.

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

Verma KK, Singh M, Gupta RK, Verma CL (2014). Photosynthetic gas exchange, chlorophyll fluorescence, antioxidant enzymes, and growth responses of *Jatropha curcas* during soil flooding. Turk J Bot 38: 130–140.

Xu PL, Guo YK, Bai JG, Shang L, Wang XJ (2008). Effects of long-term chilling on ultrastructure and antioxidant activity in leaves of two cucumber cultivars under low light. Physiol Plant 132: 467–478.

Yuanyuan M, Yali Z, Jiang L, Hongbo S (2009). Roles of plant soluble sugars and their responses to plant cold stress. Afr J Biotechnol 8: 2004–2010.