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GENOTYPIC VARIABILITY AND PHYSIO-BIOCHEMICAL CHARACTERISTICS OF IRANIAN BLACK CHICKPEA TO COLD STRESS

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ABSTRACT

The black chickpea (*Cicer arietinum* L.) is believed to be tolerant to environmental stresses, but no systematic study on its cold tolerance selection for breeding a cold-tolerant cultivar has been reported so far. In this study 28 black chickpea accessions were screened to identify genetic variability under field conditions and selected accessions, showing different performance, were compared for some physiological indexes, including indicators of injury (Electrolyte Leakage Index (ELI) and accumulation of malondialdehyde (MDA)) following cold-induced oxidative stress under short-term cold acclimation (STCA) and non-acclimation conditions. The cold-tolerant accession (4322) showed a lower membrane damage (ELI and MDA levels) compared to cold-sensitive accessions. CAT activity with cooperation of other antioxidant enzymes like GPX and PPO could play a significant role in defence against H_2O_2, especially after STCA in accession 4322 under cold stress (CS). The results suggested that accession 4322, as an appropriate cold tolerant candidate, showed an early higher tolerance to cold-induced oxidative stress compared to other accessions, as inferred from antioxidative activities of enzymes and higher yield (YLD) in field conditions. Thus, our results showed that, along with field studies, ELI and MDA under short-term cold treatments can be used to evaluate cold tolerance of chickpea profitably, in a short time.

Key words: black chickpea, cold responses, lipid peroxidation, membrane stability.

Abbreviations: CAT: catalase, CS: cold stress, DF: days to 50% flowering, ELI: electrolyte leakage Index, FM: fresh mass, GPX: guaiacol peroxidase, MDA: malondialdehyde, PBN: primary branches number, PHT: plant height, P/P: pods per plant, PPO: polyphenol oxidase, ROS: reactive oxygen species, SBN: secondary branches number, S/P: seeds per pod, STCA: short-term cold acclimation, SW: seed-weight, YLD: plant yield.

INTRODUCTION

Unlike animals, plants cannot move, but are forced to adapt to biotic and abiotic stresses by metabolic modifications. Response to cold stress (CS) is a characteristic of tolerant plant species, which is acquired after a period of cold acclimation (Mahajan and Tuteja, 2005; Apostolova et al., 2008). This process must involve the removal of limitations, which normally occur when plants grown at higher temperatures are suddenly exposed to CS. Chickpea (*Cicer arietinum* L.) has traditionally been grown worldwide, being adapted especially in dry areas of Middle East (Saxena, 1993; Singh, 1991). Chickpea growing in winter season has advantages over traditional spring-sowing season, such as approximately doubling the yield (YLD), increased water usage efficiency, and better moisture conditions (Millan et al., 2006; Heidarvand et al., 2011). The main disadvantage of chickpea winter-sowing season is the risk of winter-killing due to CS effects. Therefore, there is potential for expanding the range of chickpea winter-sowing by using conventional and molecular breeding for improved cold tolerance.

Although agronomic evaluations are an important prerequisite for using appropriate gene pools in crop improvement for specific plant attributes, the variability of field trials data makes it difficult to identify small but important differences among genotypes (Eugénia and Smith, 2006). Therefore, there has been a continuous search for more rapid,

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reliable and accurate screening methods to predict cold tolerance of genotypes.

Although the high level of Reactive Oxygen Species (ROS) is potentially harmful to plant cells, its production during CS could have a role in stress perception and protection (Suzuki and Mittler, 2006). If the equilibrium between the oxidative and antioxidative capacities is not established, the membrane lipids, proteins and nucleic acids may suffer damage which might lead to the death of the cells (Lee et al., 2003; Saibo et al., 2009). This balance is attained by a network of cellular responses in detoxification of ROS (Mittler et al., 2004), partly including antioxidant enzymes. Therefore study of antioxidative capacities under oxidative stress compared to indicators of cold injury may reflect some mechanisms underlying cold tolerance (Xing and Jian, 2011). In this study 28 black chickpea accessions were screened to identify genetic variability under field conditions and then were compared for some physiological factors, including indicators of cold injury [Electrolyte leakage Index (ELI) and accumulation of malondialdehyde (MDA)] following oxidative damage. Anti-oxidative capacities of leaves as expressed by catalase (CAT), guaiacol peroxidase (GPX) and polyphenol oxidase (PPO) for biochemical evaluation following thermal treatments were also examined in black chickpea.

MATERIAL AND METHODS

Field conditions

The materials consisted of 28 black chickpea (*Cicer arietinum* L.) accessions collected from various chickpea growing areas of Iran (Table 1), which were used for cold tolerance screening. The accessions were sown in November in a Randomized Complete Block Design (RCBD) with three replications at Tehran University Farm in Karaj city (51.06° E, 35.49° N and 1321 m above sea level) of Alborz province of Iran in 2010.

The accessions were grown in 4 rows of 3 m length with inter- and intra-row spacing of 25 and 10 cm, respectively. During growing season, CS was severe in January and February. Seedlings passed the lowest temperature -7.2°C without snow covering. For best comparison, we used selected accessions from previous experiments. Observations of five randomly selected plants from each plot were recorded on eight characters, namely days to 50% flowering (days from sowing to appearance of 50% flowering) (DF), number of pods per plant (P/P), number of seeds per pod (S/P), plant height (PHT) (cm), primary branches number (PBN), secondary branches number (SBN), 100 seeds weight (100SW) (g) and plant yield (YLD) [g $(4m^2)^{-1}$]. The recorded data were analyzed using simple statistics (i.e. mean) and for numerical taxonomic techniques, by the cluster analysis using computer software SPSS 18.0. Cluster analysis was conducted on the basis of average distance of k-means and the accessions in each cluster were analyzed for basic statistics.

*Table 1.*Original provinces of collection of 28 black chickpea accessions in Iran

Number	Accessions	Original provinces of collection
1	4322	Ardabil
2	5101	Azerbaijan1
3	5320	Azerbaijan 2
4	7050	Azerbaijan 3
5	7055	Azerbaijan 4
6	4268-1	Esfahan 1
7	4268-2	Esfahan 2
8	4488	Esfahan 3
9	4348-4	Karaj 1
10	4348-3	Karaj 2
11	4348-5	Karaj 3
12	4267-1	Kermanshah 1
13	4267-3	Kermanshah 2
14	4267-4	Kermanshah 3
15	4267-5	Kermanshah 4
16	4301	Khorasan 1
17	4307-1	Khorasan 2
17	4307-2	Khorasan 3
19	4307-3	Khorasan 4
20	4307-4	Khorasan 5
21	4307-5	Khorasan 6
22	4307-6	Khorasan 7
23	4321-2	Khorasan 8
24	Kaka	Kordestan
25	5436	Lorestan 2
26	4296	Mazandaran
27	4715	Lorestan 1
28	4269	Qazvin

Greenhouse conditions

Seeds of seven accessions of black chickpea, selected from the field experiment (Kaka, 5436, 4322, 4267-3, 4269, 4321-2, and 4348-3) were sterilized with 10% (v/v) sodium hypochlorite for 10 minutes, soaked in distilled water, and then germinated in Petri dishes on filter paper for 3 days in the dark at 25°C. Subsequently the seedlings were planted in pots containing soil, sand, and farmyard manure.

Plants were grown in a growth chamber (the luminescent lamps produce white light 200 μmol $m^{-2}s^{-1}$ irradiance, 16 h/8 h day/night regime, 25°C, and 75% relative humidity) for 21 days. One set of 21-days-old plants was placed into a climatic chamber (Grouc, Grouc, Tehran, Iran) chilled preliminary to 0°C. Temperature was lowered gradually to -10°C and plants were incubated at this temperature for 15 min (CS treatment). After removing plants from the climatic chamber, leaves were harvested immediately for physiological and biochemical analysis. These plants were defined as cold-stressed non-acclimated plants or shortly non-acclimated plants.

For the short-term cold acclimation (STCA) treatment, the other set of 21-days-old seedlings was exposed to 10^{o}C for 1 day and then were incubated under CS treatment. STCA is a common event in agricultural systems, due to the exposure of most plants to chilling temperatures before starting intense CS. The plants of this treatment were designated as cold-stressed acclimated plants or shortly acclimated plants. The seedlings grown at 25°C were considered as control plants. The cooling regime (the combination of temperature and incubation period) was established by preliminary experiments.

Cell membrane permeability

Cold tolerance was assessed by ELI in tissues damaged by treatments as previously described (Hepburn et al., 1986). The leaf samples (80 mg) were washed in distilled water for 5 min to remove electrolytes from the leaflets surface. Then, they were placed in glass tubes, poured with 10 ml of distilled water, and subjected to vacuum infiltration until the disappearance of regions not filled with water. Capped tubes containing samples were placed on a shaker (150 rpm) for 30 min. The water extract containing ions released from tissues, was placed in a cell with electrodes and the electrical conductivity of the extract was measured using a digital conductivity meter (WTW Tetra Con 325, InoLab Cond Level 1, Weilheim, Germany) at 25°C. The measurement was repeated after placing the tubes with leaf tissues in a boiling water bath for 10 min followed by shaking for 30 min. The ELI (I%) was calculated according to the formula: $I = [(L_t - L_0) / (L_b - L_0)] \times 100$, where L_t is electrical conductivity of the sample after temperature treatments, L_0 is electrical conductivity of the sample under control treatment, and L_b is an electrical conductivity of the same sample after boiling (Maali-Amiri et al., 2010).

Analysis of peroxidation of lipids in chickpea leaves

According to the method of Heath and Packer 1968, 300 mg samples of leaves without petioles were selected from the middle part of 3-5 plants. Leaves were homogenized in the extraction buffer (0.1 M Tris-HCl buffer, pH 7.6, containing 0.35 M NaCl). Solution of 2 ml of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid was added to 3 ml homogenate and incubated on a boiling water bath for 30 min. Leaf samples were centrifuged at 5,000 × *g* for 10 min and optical density of the supernatant was measured at the wavelength of 532 nm with a spectrophotometer (Shimadzu UV-160, Shimadzu Corporation, Kyoto, Japan). The extraction buffer with the reagent served as the control. MDA concentration was determined using: C= D/EL, where C is the concentration of MDA, D is the optical density, E is the coefficient of molar extinction (1.56×10^5 $M^{-1}cm^{-1}$); and L is the thickness of the layer of solution in the vessel (1 cm). Content of MDA was expressed in $\mu molg^{-1}FM$.

Estimation of the H_2O_2 content

H_2O_2 concentration was determined according to Loreto and Velikova 2001 method. Leaf fragments (0.35 g) were ground

in liquid nitrogen with a mortar and pestle and then homogenized in an ice bath with 5 ml of 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000 × *g* for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide. The absorbance of the supernatant was measured at 390 nm with a spectrophotometer (Shimadzu UV-160, Shimadzu Corporation, Kyoto, Japan). The content of H_2O_2 was calculated by comparison with a standard calibration curve previously made with different concentrations of H_2O_2 and expressed in $\mu molg^{-1}$ FM.

Soluble protein content and antioxidant enzymes activity

Samples (0.5 g FM) were ground in liquid nitrogen, homogenized by the extraction buffer (Tris-HCl, pH 7.8) containing 10% (v/v) glycerol. Extracts were centrifuged at 15,000 × *g*, 4ºC for 15 minutes. The supernatant was used for assaying CAT, GPX and PPO activities. Total soluble protein content was determined based on the Bradford method (Bradford, 1976).

CAT activity (EC-number: 1.11.1.6) was determined by monitoring the initial rate of disappearance of H_2O_2 (Scebba et al., 1998). The reaction mixture contained 3 ml phosphate buffer (pH 7.0), 5 µl of 30% (v/v) H_2O_2 (extinction coefficient, ε=39.4 mM^{-1} cm^{-1}) and 50 µl of crude enzyme extract and the decrease in absorbance was recorded at 240 nm. CAT activity was expressed in µmol of H_2O_2 decomposed $min^{-1}mg^{-1}$ protein.

GPX activity (EC-number: 1.11.1.7) was measured based on Dionisio-Sese and Tobita (1998) method. The reaction mixture consisted of 3 µl guaiacol and 10 µl H_2O_2 30% (v/v) in 3 ml sodium phosphate buffer, pH 7.0. The 50 µl crude enzyme preparation was added to 3 ml of the reaction mixture. Changes in absorbance at 470 nm were recorded in 20 s intervals and the activity of guaiacol peroxidase was expressed in µmol of guaiacol oxidized min^{-1} mg^{-1} protein assuming extinction coefficient of 26.6 $mM^{-1}cm^{-1}$.

PPO activity (EC-number: 1.14.18.1) was determined by the method of Kar and Mishra (1976). The standard reaction mixture consisted of 50 µl of 0.01 M pyrogallol, 3 ml of phosphate buffer (pH 7.0), and 50 µl of crude enzyme extract. PPO activity was determined by completely oxidizing a known amount of pyrogallol and measuring the absorbance of the reaction products at 420 nm at 20 s intervals. The results were expressed in µmol of purpurogallin formed min^{-1} mg^{-1} protein assuming extinction coefficient of 2.47 $mM^{-1}cm^{-1}$.

Statistical analysis

Field data were analyzed through analysis of variance (ANOVA) on the basis of Randomized Complete Block Design (RCBD). The Recorded data of physio-biochemical experiments were analyzed through analysis of variance (ANOVA) in a factorial experiment on the basis of Randomized Completely Randomized Design (RCRD) by using computer software MSTATC. Main and interaction effects of experimental factors were determined. We presented the results in the form of combination of treatments and not separately or individually. The treatment means were compared using Duncan's multiple range tests.

RESULTS

Field conditions

Field data showed that differences among accessions for characters were highly significant. The phenotypic correlations between characters showed significant positive correlations between YLD and P/P ($p<0.05$, r = 0.470), P/P and SBN ($p<0.05$, r = 0.407) and a significant negative correlation between S/P and 100SW ($p<0.01$, r = - 0.68) (Table 2).

The entries in this study were grouped into 3 clusters based on average linkage (Table 3). Cluster I consisted of 3 accessions, cluster II of 11 and cluster III of 14 accessions. The mean value and the standard deviation for each cluster revealed that accessions in cluster I had low 100SW with high S/P and P/P, therefore showed high YLD, whereas accessions grouped in cluster II had high 100SW with low S/P and P/P, and

were low YLD. Accessions in clusters III had medium 100SW, S/P and P/P, thus had medium YLD.

Table 2. Simple correlation (r) between values of the black chickpea accessions for different traits.

Trait	P/P	S/P	YLD [g (4 m^2)$^{-1}$]	100SW (g)	PHT (cm)	DF	PBN
S/P	0.132						
YLD [g (4 m^2)$^{-1}$]	0.47*	- 0.093					
100SW (g)	- 0.35	- 0.68**	0.177				
PHT (cm)	0.08	0.001	0.104	0.058			
DF	0.207	- 0.187	0.193	- 0.024	- 0.124		
PBN	- 0.043	- 0.144	- 0.068	0.23	0.258	- 0.009	
SBN	0.407*	- 0.185	0.275	0.051	0.1	0.141	0.349

Abbreviations: P/P = pods per plant, S/P = seeds per pod, YLD = plant yield, 100SW = 100 seeds weight, PHT = plant height, DF = days to 50% flowering, PBN = primary branches number, SBN = secondary branches number.

Note: *****and ** = significant at 5% and 1%, respectively.

Table 3. Mean and standard deviation for 3 clusters based on 8 characters in black chickpeas

Traits	Cluster 1	Cluster 2	Cluster 3
Number of accessions	3	11	14
DF	165.33±1.86	164.23±1.58	164.74±1.54
P/P	234.99±17.1	106.95±14.35	156.34±15.64
S/P	2±0	1.75±0.41	1.82±0.31
PHT (cm)	26.83±4.22	27.78±4.15	29.66±2.62
PBN	2.23±0.35	2.31±0.33	2.3±0.35
SBN	5.53±1.76	4.33±0.81	3.99±1.06
100SW (g)	11.01±0.97	12.59±1.78	11.88±1.33
YLD [g (4 m^2)$^{-1}$]	26.18±10.02	19.68±4.38	22.47±5.84

For abbreviations see Table 2.

Determination of the membrane status

The effect of temperature treatments on the plasma membrane intactness for selected accessions from field data, under acclimated and non-acclimated conditions, is shown in Figure 1a.

Compared to control plants, ELI increased under non-acclimated conditions in all accessions, however the least pronounced effect on plasma membrane leakage, belonged to the accession 4322 and the highest rate of ELI generally was observed for the accession 4321-2. In acclimated plants, the level of ELI showed dramatic decrease compared to non-acclimated plants.

Treating non-acclimated plants with CS provoked an increase in the generation of MDA in the leaves of all accessions, while the contents of MDA were not influenced significantly by inducing STCA.

Although accessions of 5436, 4322 and 4348-3 showed minimum changes under temperature treatments, the levels of MDA in the accession 4322 remained relatively stable during non-acclimated and acclimated conditions.

Although accessions of 5436, 4322 and 4348-3 showed minimum changes under temperature treatments, the levels of MDA in the accession 4322 remained relatively stable during non-acclimated and acclimated conditions. Therefore, this accession was not influenced by lipid peroxidation stress, as much as the others (Figure 1b).

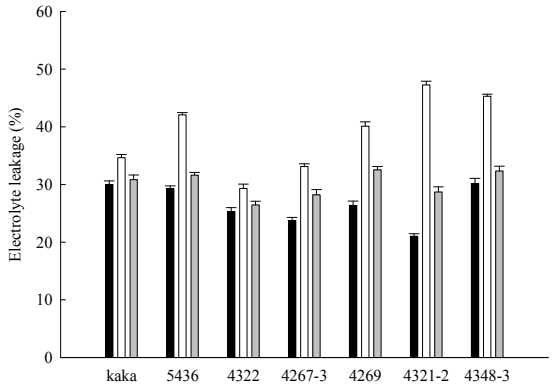

Figure 1a

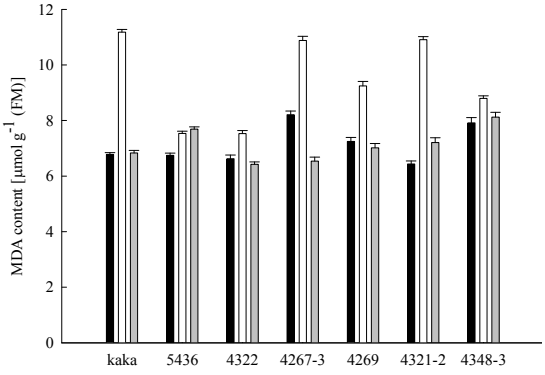

Figure 1b

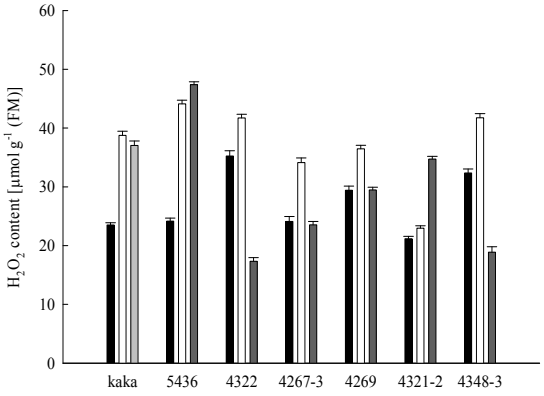

Figure 1c

Figure 1. Effects of temperature treatments on:
a) electrolyte leakage index (ELI);
b) malondialdehyde (MDA);
c) H_2O_2 content in the leaves of black chickpea accessions incubated under acclimation and non-acclimation conditions.
[The error bars represent the standard deviation (±SD) for replicates. Black, White and gray bars indicate control, non-acclimated and acclimated plants, respectively.]

Measurement of H_2O_2 content

The effects of temperature treatments on the contents of H_2O_2 in leaves of accessions did not give a similar trend response. Under non-acclimated conditions, H_2O_2 content of leaves significantly increased in all of accessions compared to control plants. According to Figure 1c, H_2O_2 content of leaves did not change in accessions 4267-3 and 4269 or decreased in accessions 4322 and 4348 under acclimated conditions.

The most pronounced effect on endogenous hydrogen peroxide level is shown in the accession of 5436 for acclimated plants and the least pronounced level of H_2O_2 belonged to accessions of 4322 and 4348.

CAT activity

In response to temperature treatments, a significant increase was shown in the activity of CAT in accession 4322 (Figure 2a). The accession 4322 had the highest inducement for CAT activity, though CS after STCA caused an increase almost five times from the control value (from 1.23 to 6.95), whereas under non-acclimated conditions, it changed from 1.23 to 4.31.

GPX activity

CS produced a significant increase of GPX activity in acclimated plants of accession 4322 compared to the control (from 0.32 to 0.34), whereas GPX activity did not change significantly under non-acclimated conditions (Figure 2b).

PPO activity

The acclimated plants of accession 4322 did not show significant changes ($p \leq 0.05$) in the activity of PPO; however data for this enzyme activity in response to CS revealed quite similar increase from value of 0.47 to 0.54 and 0.55 under acclimated and non-acclimated conditions, respectively. It seemed that PPO activity remained relatively stable during temperature treatments (Figure 2c).

DISCUSSION

In this study we compared field screening for CS and physio-biochemical profiles in black chickpea plants. Differences among accessions for all botanical traits were large, suggesting that selection for relevant traits

could be possible. The phenotypic correlations showed positive correlation between YLD and P/P and did not show any significant correlation of YLD with other morphological traits. This result suggested that P/P may be used as an indirect selection for YLD in black chickpea; however the lack of correlations between YLD and other traits under study indicate that those traits may not affect the performance of plant. But there might be some physio-biochemical studies that could be related to the CS, which need to be investigated because these assays can be applied as descriptors for facilitating indirect evaluations and to screen a large number of seedlings for cold tolerance. Our previous data showed that black chickpea plants in fall-sowing season had more successful performance compared to kabuli ones. For example, P/P in black chickpea accessions was 2-3 times greater than that of kabuli plants; therefore the data in this study confirmed our previous results (Heidarvand et al., 2011).

In our study we assumed that cold tolerant black chickpeas survive CS and produce more YLD under field conditions, therefore seven accessions, including Kaka, 5436, 4322, 4267-3, 4269, 4321-2 and 4348-3 (these accessions had different performances under field conditions and belonged to different clusters) were selected from field data analysis for physio-biochemical assays and to investigate the STCA process on cold tolerance under greenhouse conditions. On the other hand, the different YLD of accessions in field conditions could be caused by their more successful performance after finishing cold season (drought tolerance).

Therefore the study of more physio-biochemical responses of these accessions under controlled conditions should be used for precise evaluation of cold tolerance and understanding some mechanisms underlying CS. The exposure of seedlings to CS caused a dramatic increase in the ELI in most of the accessions under non-acclimated conditions compared to acclimated conditions. It was observed that the STCA could trigger necessary mechanisms for cold acclimation in chickpea plants and make membranes less leaky (Martin et al., 1987).

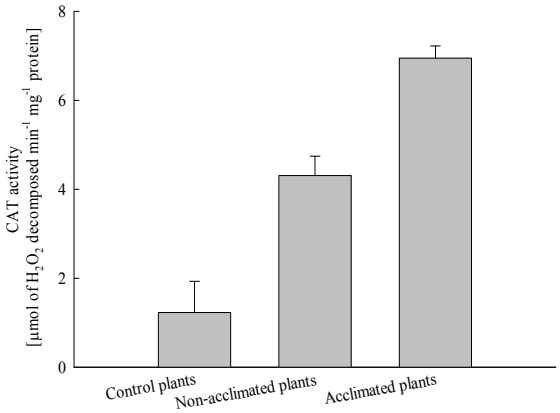

Figure 2a

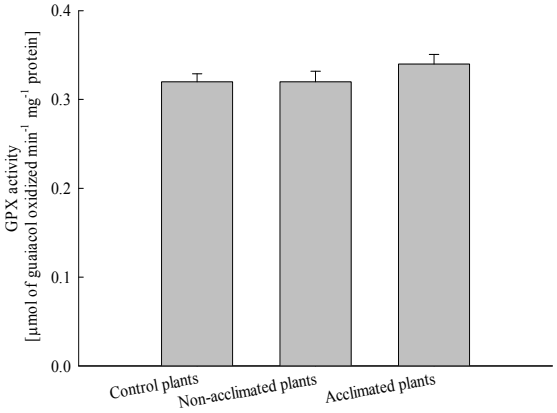

Figure 2b

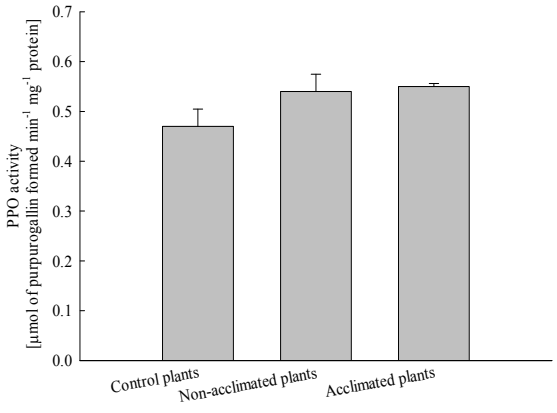

Figure 2c

Figure 2. Antioxidant enzyme activities of accession 4322 subjected to temperature treatments in acclimated or non-acclimated plants:
a) Catalase (CAT);
b) Guaiacol perpxidase (GPX);
c) Polyphenol oxidase (PPO)
[The error bars represent the standard deviation (±SD) for replicates.]

This may be a result of chilling requirement for an increased tolerance to CS in chickpea accessions. A low increase of ELI in the accession 4322 indicated activation of tolerance mechanisms after CS, compared to other accessions. Therefore STCA like long-term cold acclimations may be considered as a mechanism to improve black chickpea tolerance to CS (Heidarvand et al., 2011). A higher susceptibility of black chickpeas to CS was evidenced by the leakage increase during temperature treatments. Previous studies showed that this was related to MDA production and concentrations which commonly indicates the occurrence of lipid peroxidation (Grotto et al., 2009). Under non-acclimated conditions there was a slight increase in MDA content in accessions of 5436, 4322 and 4348-3 with a higher content in the accessions of Kaka, 4267-3, 4269 and 4321-2. After STCA, significant changes were not observed for the accessions 4322 and 4348-3, which probably means that the STCA may make these accessions tolerate subsequent CS.

However, the magnitude of the change in the content of MDA for accession 4322 was less than accession 4348-3 during temperature treatments. Thus, this accession may be candidate as more tolerant to CS. Surprisingly, MDA levels increased in acclimated plants of entry 5436 more than in non-acclimated plants. The results showed that STCA period was probably not sufficient to increase the tolerance of this accession against CS (Palliotti and Bongi, 1996; Dai et al., 2009), whereas it has been reported that some cold-sensitive plants acclimate if they are exposed to a chilling slightly above the threshold chilling temperature (Hao et al., 2009).

Thus, the higher tolerance of some accessions might be due to stabilization of the composition and physical properties of their membranes, which must be studied in details. However, because of the complexity of the stress response network, other hypotheses could be considered. Generally, accession 4322, showing higher YLD, low ELI and MDA, was identified as an appropriate cold tolerance candidate and could be used for later trials.

The lipid peroxidation can be caused due to the accumulation of the ROS which are the principal causes of oxidative stress-related membrane damage (Sairam et al., 2005; Zhou et al., 2005; Maaouia Houimli et al., 2010). H_2O_2 is the major ROS of the oxidative burst in plants, because it is the most long-lived and able to cross plant cell membranes and thereby act as a diffusible and relatively lasting signal (Karuppanapandian et al., 2011). In addition to the formation of MDA, induction of antioxidant mechanisms may be a sign of ROS overproduction and thereby of oxidative stress (Yang et al., 2008). Results showed that CS in non-acclimated plants only increased CAT and PPO, but did not change GPX activity. The lack of physiological responses to CS was perhaps indicative of insufficient time for increasing the activity of GPX under non-acclimated conditions, because in this study CS after STCA could induce more activity in this enzyme. Increase in CAT and PPO activities may not be enough for acclimating of plants to subsequent CS and therefore did not prevent the higher accumulation of H_2O_2 in these conditions. Different results have been reported about changes in activities of CAT and GPX under CS, depending on nature of CS (duration and severity), its interaction with other environmental stresses and the wide range of plant responses (Karuppanapandian et al., 2011). The results showed that CAT was not the only effective antioxidant enzyme and it could play a significant role in defence against H_2O_2 with cooperation of other antioxidant enzymes like GPX and PPO. As expected, the increase of activity for these enzymes after STCA was higher than in non-acclimated plants in accession 4322, suggesting that STCA may initiate defence mechanisms to decrease the level of H_2O_2 in chickpea plants during CS. Therefore, the level of H_2O_2 decreased after STCA and data on MDA and ELI also confirmed these results. The increase of H_2O_2 contents in non-acclimated plants of accession 4322 led to more damage in cell membranes, similar to data on MDA and ELI. The data suggested that gradual or sudden reductions in ambient temperature can produce important variations in antioxidative responses to CS, which may be the basis of

the diversity of cellular responses between plant genotypes or species. Thus, our data showed that there was a substantial genetic variation for cold tolerance and applied cold treatments could screen sensitive and tolerant chickpea accessions. It is important to note that the origin of accession 4322 belongs to Ardabil province, a cold region, located on Iranian plateau in the north-west of Iran, and its relative cold tolerance could have developed evolutionally, based on natural selection. This should be the subject of survey in detailed studies.

CONCLUSIONS

The black chickpea seedlings showed the capacity to increase cold tolerance after STCA. Accession 4322 showed an early higher tolerance to oxidative stress-related damages under direct and indirect CS, compared to other accessions. This could be inferred from a lower damage on membranes (ELI and MDA levels), antioxidative activities of enzymes and more successful performance under field conditions. Comparative study of this accession with kabuli local cultivars may help to understand plant strategies under CS. Accession 4322 may tolerate CS in winter-sowing in dryland areas and therefore could be used in extending the growing season and geographical range of black chickpea.

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