J. Agr. Sci. Tech. (2023) Vol. 25(1): 99-113

Cold and Drought Cross-Acclimation Enhance Freezing Tolerance of Chickpea (*Cicer arietinum* L.)

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ABSTRACT

Cross-acclimation of mild drought stress and cold acclimation may additionally increase the chickpea's cold tolerance due to transferring sowing date from spring to winter in Mediterranean high lands. Two weeks after sowing in greenhouse, chickpea seedlings were subjected to the following treatments in a controlled environment: (i) Well-Watered under an optimum temperature regime (WW); (ii) Well-Watered under a Cold temperature regime (WWC); (iii) Drought Preconditioned under an optimum temperature regime (DP); and (iv) Drought Preconditioned under a Cold temperature regime (DPC). After three-week acclimation period, plants were frozen on the thermogradient freezer, then, recovered for three weeks in a greenhouse. In the acclimation period, with decreasing temperatures, a clear decrease of the electrolyte leakage (EL) were observed for both genotypes: 51% for cold tolerant MCC252 and 36% for cold sensitive MCC505. Cold acclimation induced the greatest accumulation of proline and MDA contents (about 75% for both genotypes) and drought preconditioning most consistently induced an increase in soluble carbohydrate content (25% for MCC252 and 51.7% for MCC505) during the acclimation period. The survival percentage increased 9.3% for MCC252 and 21.25% for MCC505 by both cold and drought acclimation under freezing conditions. Generally, drought preconditioning had a synergistic effect on the cold acclimation period to improve freezing tolerance (as indicated by the lowest LT50el and LT50su) and leading to an increase in the freezing tolerance for the cold sensitive genotypes (MCC505). Thus, the greatest gains in freezing tolerance for both genotypes were associated with cross-acclimation treatment (DPC).

Keywords: Climate changes, Cold acclimation, Drought precondition, Physiological and biochemical changes, Shifting sowing date.

INTRODUCTION

Traditionally, chickpea (*Cicer arietinum* L.) is a spring-sown crop in the highlands of West Asia and North Africa (Singh *et al*., 1997), because of the harsh winters of these area. In this condition, the yield of crop is decreased, because the plant faces both high temperatures and soil moisture deficit in the late growing season (Mousavi et al., 2007). The published reports from International Center for Agriculture Research in the Dry Areas (ICARDA） showed that, in the Mediterranean low lands areas, seed yield of chickpea increased about 50-100% when sown in fall/winter (Hawtin and Singh, 1984). However, in the high lands, because of the lack of cold tolerant varieties, this cultivation system is impossible. Therefore, for autumn-winter chickpea planting in high lands, increasing crop freezing-tolerance is one of the most important prerequisites. Even in spring cultivation, cold tolerance at the early stages of plant growth is necessary (Marouf *et al.,* 2009).

Cool season plants increase their cold tolerance in cool and short-day conditions of

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autumn; this phenomenon is known as cold acclimation. Cold acclimation is a complex process that allows plants to develop freezing tolerance and survive through multiple levels of biochemical and morphophysiological changes (Yadav, 2010). In addition to cold temperatures, mild drought stress can induce cellular changes similar to those associated with cold acclimation. The overlap of abiotic stress signaling pathways, "cross-adaptation" may cause plants to improve their tolerance to multiple stress through exposure to a single type of abiotic stress (Chinnusamy *et al*., 2004).

By 2080, the global atmospheric temperature is predicted to rise by approximately 4°C. (FAO, 2019). Furthermore, Lelieveld *et al*. (2012) reported that annual precipitation is expected to decrease in the Eastern Mediterranean and the Middle East with a substantial increase in the number of days without rainfall in winter. Accordingly, prevention of acclimation prior to winter freezing temperatures may negatively affect winter survival.

Stresses are analogous mechanisms for both freezing and drought that allow plants to increase their cellular stability and decrease cell membrane injury. A significant overlap has been observed in gene regulation during exposure to both of these types of stress (Tommasini *et al*., 2008). As a result, in some plant species, freezing tolerance may be enhanced by exposing plants to drought preconditioning (Medeiros and Pockman, 2011). In a study on two perennial ryegrass (*Lolium perenne* L.) genotypes ('Buccaneer' and 'Sunkissed'), exposing them to moderate drought stress caused an improvement in cold tolerance for Buccaneer, but had no significant effect on freezing tolerance of Sunkissed. Furthermore, depending on genotype, tissue and temperature regime, drought preconditioning resulted in an increase in carbohydrate and proline contents (Hoffman *et al*., 2012). Rajashekar and Panda (2014) reported that low temperature and water stress significantly contributed to the induction of freezing tolerance. Water stress was a dominant factor in inducing freezing tolerance, contributing roughly to 56% of freezing tolerance acquired by natural cold acclimation in strawberry. The capacity to accumulate similar protective compounds, including carbohydrates and amino acids, that minimize the negative effects of desiccation, has been associated with increases in both drought and freezing tolerance (Hoekstra *et al*., 2001).

We presumed that drought preconditioning may improve the freezing tolerance of chickpeas in the absence of cold acclimation by increasing the production of protective compounds and also the combination of drought preconditioning and cold acclimation may increase the chickpea cold tolerance as much as each one alone. Therefore, the objectives of this research were to: (i) Evaluate the effects of cold acclimation, drought preconditioning, and cross-acclimation on freezing tolerance of two chickpea genotypes; and (ii) Assay some physiological and biochemical changes of chickpea genotypes in response to acclimation conditions.

MATERIALS AND METHODS

Plant Material and Growth Condition

The seeds of two chickpea genotypes (MCC252, cold tolerant; and MCC505, cold-sensitive) from the seed bank of the Research Center for Plant Science of Ferdowsi University of Mashhad were selected based on previous winter hardiness data (Nezami and Bagheri, 2005; Najibnia *et al*., 2008).

Crop Husbandry

The germinated seeds were sown in 1-L pots (15 cm in length and 12 cm in width) containing a soil mix (2:1 sand-farm soil) at 22/16˚C (day/night temperature) under

natural day light on 10 August, 2015. The planted pots were placed in the greenhouse for two weeks (4-6 leaves); then, a three-week acclimation period was imposed on the controlled environment of a growth chamber facility (Conviron model E8; Winnipeg, MB, Canada) at the Agricultural College of Ferdowsi University of Mashhad, Mashhad, Iran. The experiment was conducted using a completely randomized block design with three replications.

Cold Acclimation and Drought Preconditioning

To determine whether drought preconditioning could enhance freezing tolerance under the non-cold-acclimation and/or cold-acclimation condition, the following four treatments were designed:

I. Well-Watered under an optimum temperature regime (WW);
II. Well-Watered under a Cold temperature regime (WWC);
III. Drought Preconditioned under an optimum temperature regime (DP),
IV. Drought Preconditioned under a Cold temperature regime (DPC).

The experimental design is shown in Figure 1.

Drought preconditioning was conducted using wilt-based irrigation, which induced mild drought stress. Water was withheld until visual signs (withered state) in apical part of the shoot occurred. At that point, the plants were re-watered to the pot capacity. In addition, each wilt cycle in the top fully developed leaf of the plants was measured at the control and cold treatment temperatures to assess any detrimental effects of drought preconditioning on plant growth.

For cold acclimation treatments, the temperatures were 10/7˚C (day/night) with an 11-h day length (light intensity of ~400 µmol m^{-2} s^{-1} at top of the plants) in the first week, then 7/5˚C, 10 hours and 5/3˚C, 9 hours in the second and third weeks, respectively. Plants maintained under optimum temperature regime consisted of 22/16˚C day/night temperatures, 12-hour photoperiod, and photosynthetic photon flux density of 500 µmolm^{-2} s^{-1} at top of the plants.

Freezing Evaluations and Recovery

After the respective growth treatments, the plantlets were placed in a thermogradient freezer at 3 or 22˚C depending on control or treated plant. The freezer was programmed

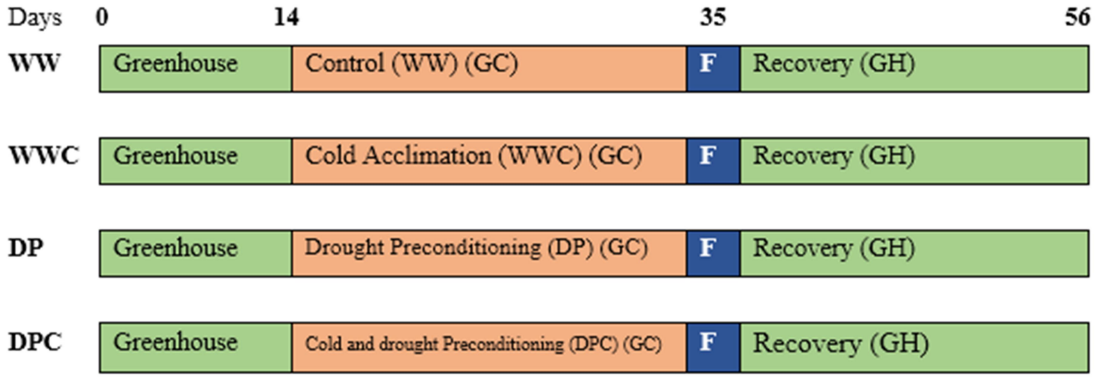

Figure 1. Experimental scheme: WW: Well-Watered under an optimum temperature regime; WWC: Well-Watered under a Cold temperature regime; DP: Drought Preconditioned under an optimum temperature regime; DPC: Drought Preconditioned under a Cold temperature regime; F: Freezing day; GC: Growth Chamber, GH: Greenhouse.

to reach a target freezing temperature at a rate of 2˚C h^{-1}. Plants were held at the target temperature for one hour and removed at various test temperatures (0, -4, -8, -12, and -16˚C) to evaluate the freezing injury. In all treatments, at -3˚C, the plants were sprayed with Ice Nucleation Active Bacteria (INAB) to promote nucleation and prevent supercooling. Samples were allowed to thaw overnight at 4˚C, and then were moved to the greenhouse for three weeks recovery.

Observations

Several parameters including: (i) The EL (Electrolyte Leakage) of seedling leaves and the related parameter [Lethal Temperature 50 according to the EL (LT_{50el})], samples were collected after freezing and thawing period; (ii) Biochemical estimation (lipid peroxidation, proline and soluble carbohydrates), samples were collected during three weeks of acclimation; and (iii) Survival (SU) and the related parameter [Lethal Temperature 50 according to the survival (LT_{50su})] were measured after recovery.

Electrolyte Leakage Evaluation:

Three of the top leaflets (4-5 cm in length) were excised and transferred to 100 mL vials containing 50 mL of double-distilled water. The samples were totally submersed and placed in room temperature for 24 hours before the primary EL was measured. The EL measurement was made using a JENWAY bench conductivity/TDS meter, Model (4510 JENWAY, UK). To evaluate the total EL of cells, the samples were placed in an autoclave for 20 min (1.2 bar – 120˚C). Subsequently, the samples were transferred to the laboratory and exposed to the room temperature. The second EL was measured after 24 hours. The EL percentage was calculated as follows:

$$EL\% = (EL1/EL2)\times 100 \quad (1)$$

Where, EL1= Primary reading of Electrolyte Leakage and EL2= Secondary reading of Electrolyte Leakage.

LT_{50el}: The temperature at which 50% of the electrolytes were leaked was determined using the method similar to that described elsewhere for this type of experiment developed by electrolyte leakage data (Equation 1), (Anderson *et al*., 1988).

$$EL_p = EL_1+[(EL_m-EL_1)/(1+e^{-B(T-Tm)})] \quad (1)$$

Where, ELp= Predicted EL value, EL1= Lower bound of EL value, Elm= Higher bound of EL value, e= 2.718, B= Rate of temperature increase, T is absolute value of the treatment Temperature, and Tm= Inflection point of the curve. The inflection point is defined as the midpoint between the lower and upper asymptote of the curve (Zhu and Liu, 1987).

Proline Assay: Leaf tissues were harvested, submerged in liquid nitrogen, and stored at −80˚C until the proline content was measured according to the method of Bates *et al*. (1973).

Lipid Peroxidation: The level of lipid peroxidation was measured in terms of Thiobarbituric Acid (TBA) reactive substance (TBARS) contents (Heath and Packer, 1968), which determined Malondialdehyde (MDA) as an end product of lipid peroxidation.

Determination of Soluble Carbohydrates: Soluble carbohydrates were determined based on the modified phenol sulfuric acid method (Dubois *et al*., 1956). Glucose was used as the standard.

Survival Evaluation: Survival (SU) evaluation was calculated according to the method of Nezami *et al*., (2012). The SU of seedling that had been frozen was evaluated based on remained plant's as determined three weeks after freezing. The number of plants that were alive were counted at the end of the recovery period, and the Survival Percentage Index (PSI) was calculated using Equation (2).

$$PSI\% = (A/B)\times 100 \quad (2)$$

Where, PSI= Survival Percentage Index, A= Number of alive plants at the end of the

recovery period, and B= Number of plants before freezing treatment.

LT_{50su}: The temperature at which plants showed 50% reduction at survival estimated based on the PSI by plotting these values against the freezing temperatures and probit analysis of survival percentage and the linear equations obtained for all treatments.

Dry Weight: For plant recovery assessments, all alive plants were clipped from the soil surface to measure plant dry weight after oven drying.

Statistical Analysis

The experiment was arranged as a factorial based on a completely randomized design with three replications. All data were subjected to Analysis Of Variance (ANOVA), and significant means were separated using the Least Significant Difference (LSD) test at P< 0.05. The percentage data were transformed to arcsine prior to analysis. Interrelationship among different traits was calculated using Pearson's correlation analysis. The path coefficient analysis was performed according to Dewey and Lu (1959) to record direct and indirect effects of different traits on survival and final dry weight.

RESULTS

MDA

In general, MCC252 exhibited a higher amount of MDA compared with MCC505 and the highest amount of MDA for both genotypes was observed in DPC treatment (Table 1). In MCC252, a gradual increase (2.73%) was observed during three weeks of acclimation for control (WW), while for MCC505, the rate of increase was significantly higher (47.7%). DP treatment exhibited a similar trend as control treatment for both MCC252 and MCC505 (13.7% and 60.5%, respectively). In WWC treatment, MDA content in the first and second week of acclimation showed the smallest amount for both genotypes (6.8 and 4.9 nm g^{-1} fw in average, respectively), but increased significantly at the third week (72 and 78% for MCC252 and MCC505, respectively) (Figure 2).

Proline

Cold temperatures caused a significant increase in proline content for both genotypes, being markedly higher in WWC

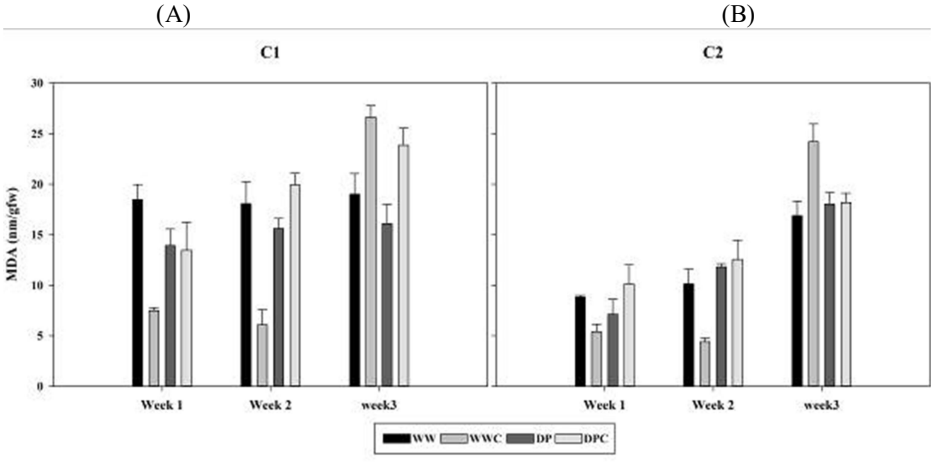

Figure 2. (A) Changes in Malondialdehyde (MDA) content of chickpea seedlings of MCC252, and (B) MCC505, following exposure to WW, WWC, DP, and DPC (as defined in Figure 1 and text) during three weeks of acclimation. Vertical bars represent SE (n= 3).

Table 1. Mean comparison of Malondialdehyde (MDA) (nm g^{-1} fw), proline (mg g^{-1} fw), and Soluble Carbohydrate content (SC) (mg g^{-1} fw) of chickpea seedling MCC252 (C1) and MCC505 (C2) exposure to WW, WWC, DP and DPC in three weeks of acclimation.[a]

	MDA		Proline		SC	
	MCC252	MCC505	MCC252	MCC505	MCC252	MCC505
WW	18.52[a]	11.95[a]	0.114[c]	0.110[c]	10.22[b]	13.33[b]
WWC	13.40[a]	11.33[a]	0.449[a]	0.448[a]	24.40[a]	15.95[b]
DP	15.22[a]	12.33[a]	0.228[b]	0.292[b]	22.36[a]	19.86[a]
DPC	19.08[a]	13.08[a]	0.459[a]	0.459[a]	23.72[a]	21.95[a]

[a] For each genotype, means followed by the same letter within a column are not significantly different based on Duncan values ($P \leq 0.01$)

and DPC (about 75% for both genotypes) compared to WW, while in DP it was significantly lower than WWC and DPC for both MCC252 and MCC505 (61 and 50%, respectively) (Table 1). This difference was observed during three weeks of acclimation. Finally, during the last week, the amount of proline was significantly higher in DPC than in other treatments. Proline contents were not significantly different between MCC252 and MCC505. Overall, exposure to low temperatures resulted in a four-fold increase in proline levels for both genotypes (Figures 3-A and -B).

Soluble Carbohydrate Content

Acclimation treatments markedly affected the carbohydrate content of both genotypes (Table 1). A noticeably decline was observed in WW for both MCC252 (32.3%) and MCC505 (33.0%) during acclimation period (Figures 4-A and -B). Genotypes exhibited different reactions in response to treatments. MCC252, WWC and DPC exhibited similar trends, whereby under WWC and DPC, the amount of carbohydrate increased conspicuously (46.3 and 43.4%, respectively) compared to WW in the first

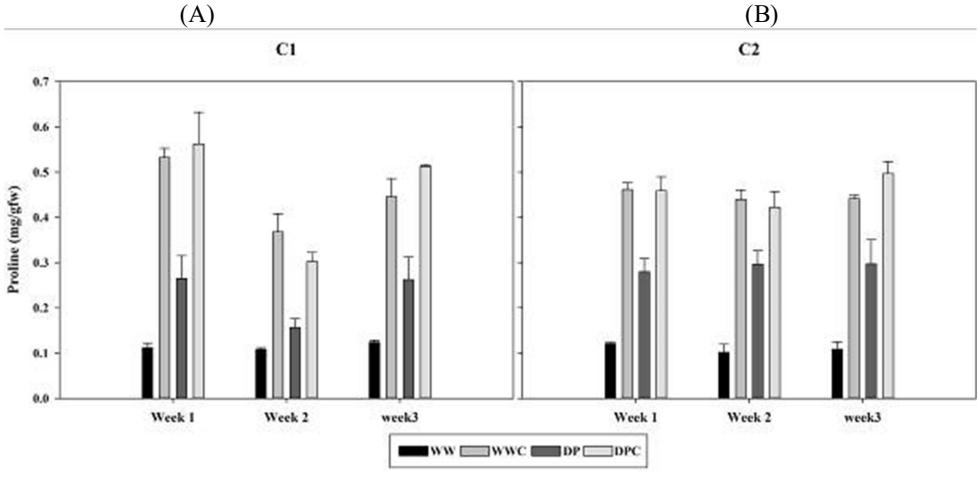

Figure 3. (A) Proline level of chickpea seedlings MCC252 (C1) and (B) MCC505 (C2) following exposure to WW, WWC, DP , and DPC (as defined in Figure 1 and text) during three weeks of acclimation. Vertical bars represent SE (n= 3).

week and stayed stable during the next two weeks of acclimation. In the same time, for MCC505, carbohydrate content decreased (22.7%) under WWC, and increased in DPC (27.0%). Under DP treatment, the amount of carbohydrate increased during three weeks of acclimation for both genotypes (25% for MCC252 and 51.7% for MCC505) (Figures 4-A and -B).

Electrolyte Leakage and Survival

The stress injury was evaluated in terms of the increase in EL and the loss of SU. In general, the percentage of membrane damage based on the EL showed significant differences in various treatments. In the treated plants, EL was observed to be significantly lower in DP and DPC compared with the WWC and the control (WW) plants (Figures 5-A and -B). In MCC252, the EL increased markedly at temperatures lower than -8 °C for WW plants, while at -12 and -16°C, the EL was 1.6 and 2.9 times higher than the control, respectively. At (as defined in Figure 1 and text), MCC252 and MCC505 showed about 60 and 37% less EL under DPC compared to WW, respectively.

The SU increased significantly by 23.9, 33.6, and 41.8%, respectively, when plants were under treatment WWC, DP, and DPC, compared with the control (WW). All plants survived completely from 0 to −4°C, and reducing the temperature to −8°C caused a significant reduction in the SU% of WW plants in both genotypes. Lowering the temperature to −12°C resulted in 33 and 38% plant mortality in MCC252 for WW and WWC, respectively; under the same conditions, the plant mortality for MCC505 were about 42 and 70%, respectively (Figures 5-C and -D). For MCC252 and MCC505 plants under DPC treatment, 0% and about 60% of plants mortality were observed at -16°C, respectively. In general, the SU was significantly increased by both cold and drought acclimation under freezing conditions.

Dry Weight

The seedlings grew more slowly under acclimation treatment (WWC, DP, DPC) compared to the control (WW). Excess dry weight decreased by 55.1, 52.7, and 57.8%, respectively, for MCC252 and 50.9, 66.3, and 71.2%, respectively, for MCC505. After freezing and three weeks of recovery, chickpea genotypes exhibited different

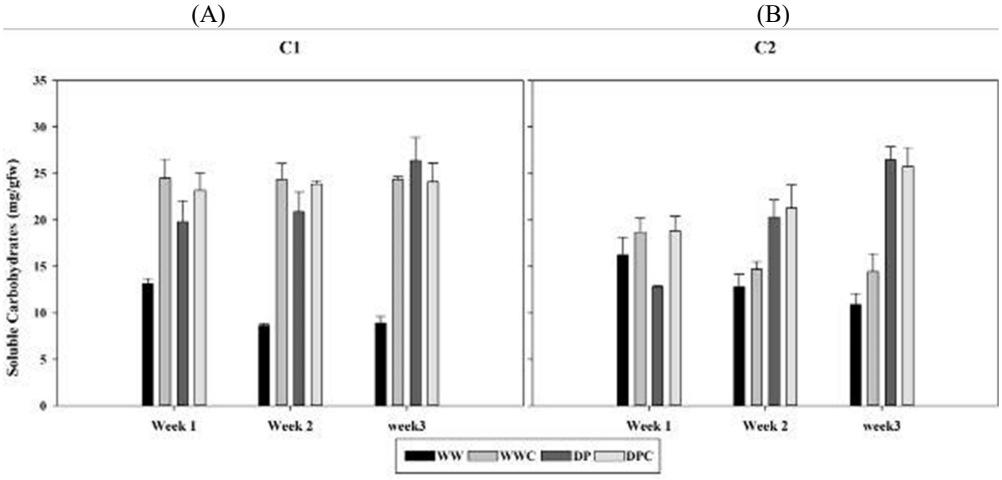

Figure 4. (A) Soluble carbohydrate content of chickpea seedlings MCC252 (C1), and (B) MCC505 (C2) following exposure to WW, WWC, DP, and DPC (as defined in Figure 1 and text) during three weeks of acclimation. Vertical bars represent SE (n= 3).

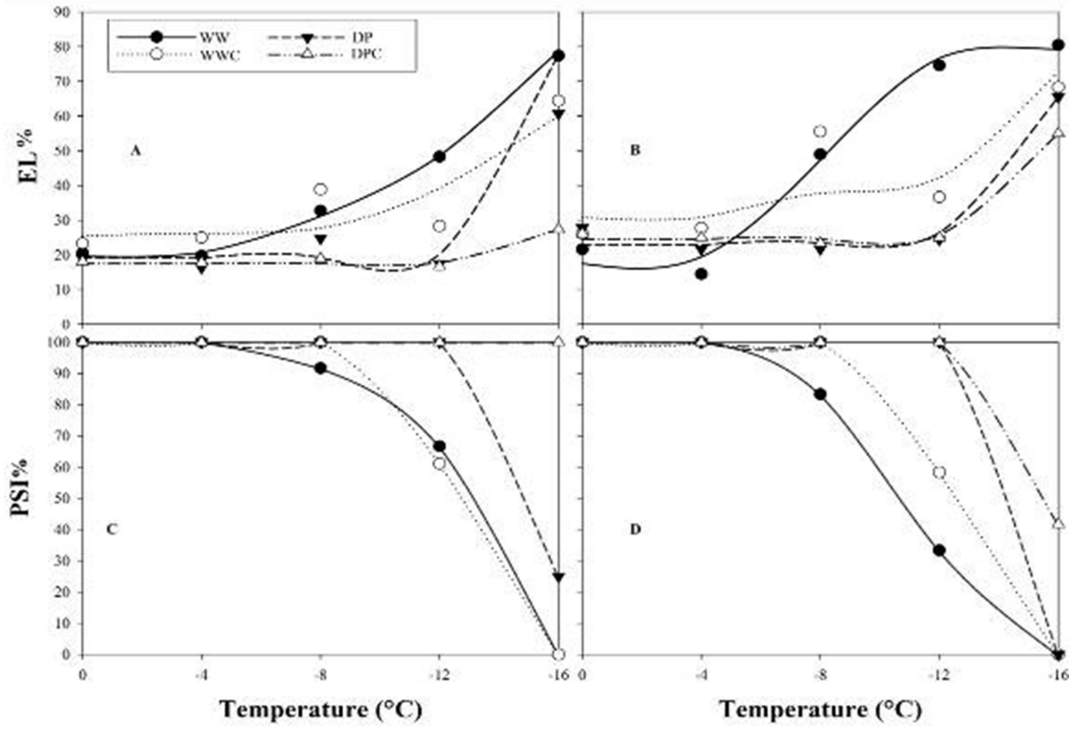

Figure 5. Electrolyte leakage of four treatments (WW, WWC, DP, and DPC) were determined after freezing temperatures in: (A) MCC252 and (B) MCC505. (C) Survival Percentage Index (PSI) of treated plants were based on regrowth after three weeks of freezing temperatures in MCC252 and, (D) MCC505.

reaction as consequence of treatments (Figures 6-A and -B). For MCC252, dry weight increased 54.5 and 56.6% under DP and DPC treatments, respectively, compared to acclimation period, while under WWC, increase rate was 26.8%. For MCC505, dry weight considerably increased (70.7%) under DP. WWC and DPC showed similar trend of increase (41.2 and 64.1%, respectively). Generally, WWC plants exhibited lower dry weight in acclimation and recovery period for both genotypes (Figures 6-A and -B).

LT_{50el} and LT_{50su}

Based on an estimation of LT_{50el} and LT_{50su}, in all treatments, MCC252 exhibited a higher baseline freezing tolerance compared with MCC505. The results for LT_{50el} and LT_{50su} showed that freezing tolerance varied among treatments and plants under DPC had the minimum amount of mortality in both genotypes. LT_{50el} significantly decreased from -11.5°C at WW to −14.1°C in response to DPC for MCC252 (18.8%) and from -7.9C to -14.1°C for MCC505 (43.9%). The positive effect of acclimation treatment on decreasing plant mortality was significantly higher for MCC505 compared to MCC252. Based on LT_{50su}, freezing tolerance achieved the greatest level in response to DPC (−16.5°C for MCC252 and -15.4°C for MCC505) (Table 2). A positive and significant ($P \leq 0.01$) correlation was recorded between LT_{50el} and LT_{50su} (Table 3).

The results of correlation coefficient of the two chickpea genotypes among 10 traits studied during acclimation and after freezing (recovery) period indicated that proline, MDA, and soluble carbohydrate content had negative and highly significant correlation

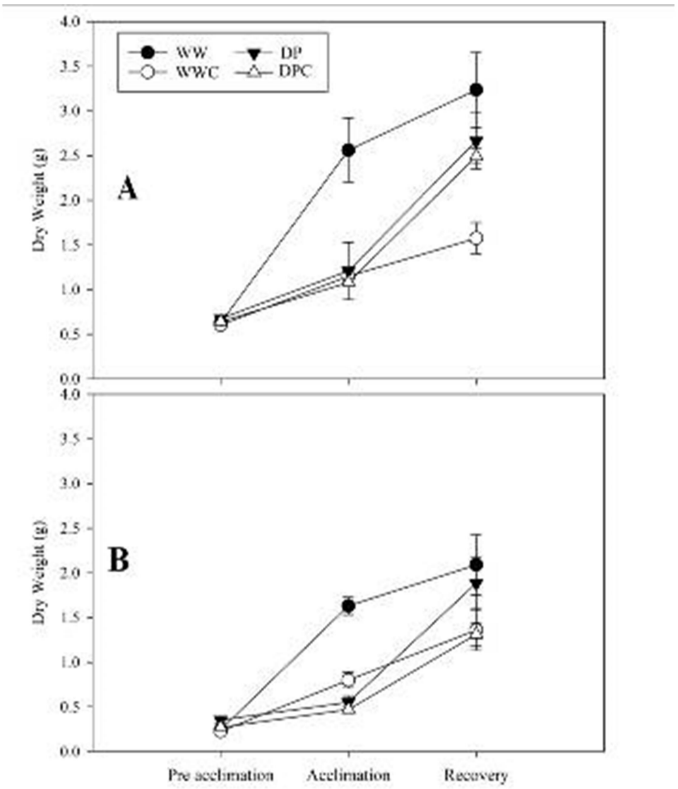

Figure 6. (A) Dry weight of chickpea seedlings MCC252 and (B) MCC505 following exposure to WW, WWC, DP and DPC before acclimation period (pre acclimation), during acclimation and after freezing treatment (recovery). Vertical bars represent SE (n= 3).

with electrolyte leakage (r^2= -0.44*, r^2= -0.48** and r^2= -0.69**, respectively). Also, the three traits had positive and strong significant correlation with LT_{50el} and LT_{50su} (r^2= 0.84**, r^2= 0.85** and r^2= 0.64**, respectively). The relationship between survival and electrolyte leakage was very significant and negative (r^2=-0.86**); also, SU showed a positive association with MDA, proline, and SC content (r^2= 0.46*, r^2= 0.44* and r^2= 0.59*, respectively). Great significant and negative correlation were found between survival and LT_{50el} and LT_{50su} (r^2= -0.71** and r^2= -0.98** respectively). A weak and negative relationship was found between final dry weight and electrolyte leakage, LT_{50el} and LT_{50su}. However, correlation between FDW and proline was highly strong and negative, but with PDW and MDA it was positive (Table 3).

DISCUSSION

It is well established that cold acclimation develops plants' freezing tolerance (Gusta *et al*., 1982). Generally, accepting the scenarios of climate change and increase in winter temperatures (Masson-Delmotte *et al*., 2018) will result in less optimal cold acclimation conditions and lead to decreases in freezing tolerance, and identifying traits that are necessary for winter survival is increasingly important. It has been shown that drought preconditioning affects the improvement of tolerance to various types of abiotic stress, including low temperature. Hoffman *et al*. (2012) reported that a five-week drought period in perennial ryegrass increased freezing tolerance. Rajashekar and Panda (2014) showed that inducing freezing

Table 2. Comparison of LT_{50el} and LT_{50su} of chickpea seedlings of MCC252 (C1) and MCC505 (C2) following exposure to WW, WWC, DP and DPC.[a]

	LT_{50el}		LT_{50su}	
Treatments	MCC252	MCC505	MCC252	MCC505
WW	-11.5[a]	-7.9[b]	-12.1[bc]	-10.9[c]
WWC	-12.5[a]	-8.0[b]	-12.7[a-c]	-12.1[bc]
DP	-14.1[a]	-13.9[a]	-14.8[a-c]	-13.9[a-c]
DPC	-14.1[a]	-14.0[a]	-16.5[a]	-15.4[ab]

[a] For each genotype, means followed by the same letter within a column are not significantly different based on Duncan values ($P \leq 0.01$).

Table **3**. Correlation coefficients among different traits of two chickpea genotypes affected by drought and temperature treatments in acclimation and recovery period.[a]

	1	2	3	4	5	6	7	8	9
1. El	1								
2. PDW	0.36ns	1							
3. MDA	-0.49**	0.41*	1						
4. P	-0.44*	-0.65**	-0.11ns	1					
5. SC	-0.69**	-0.60*	-0.03ns	0.68**	1				
6. LT_{50el}	0.84**	0.34ns	-0.42*	-0.27ns	-0.63**	1			
7. LT_{50su}	0.85**	0.44*	-0.43*	-0.43*	-0.61*	0.76**	1		
8. SU	-0.87**	-0.38*	0.46*	0.45*	0.59*	-0.71**	-0.98**	1	
9. FDW	-0.19ns	0.69**	0.72**	-0.62**	-0.34ns	-0.19ns	-0.13ns	0.15ns	1

[a] El= Electrolyte leakage, PDW= Primary Dry Weight (before freezing), MDA= Malondialdehyde, P= Proline, SC= Soluble Carbohydrate content, SU= Survival percentage and, FDW= Final Dry Weight (after recovery).

tolerance in strawberry plants was possible with low temperature and water stress.

Under stress conditions, membrane integrity and cell compartmentation are affected, and membrane permeability increases. Increasing the EL is a sign of the cell perception of stress. Confirming this hypothesis is possible by comparing the electrolyte leakage of acclimated and control plants during the acclimation period. In this study, in one hand, for both genotypes, EL decreased in response to DP and DPC, on the other hand, we found a highly significant negative correlation between EL and SU that resulted in increased survival of DP and DPC treatments. Moreover, with decreasing EL; LT_{50el}, LT_{50su} decreased too and led to higher freezing tolerance. Such significant correlation between EL and LT_{50el}, LT_{50su} was also observed in Viola (Viola wittrockiana) (Oraee *et al*., 2020) and Zoysiagrass (Zoysia spp.) ((Patton *et al*., 2007) and Centipede grass (Eremochloa ophiuroides) (Cai *et al*., 2004). This point was also confirmed by a very significant negative correlation observed between MDA and EL (Table 3), while the MDA increase was synchronized with the decreased EL in the treated plants. Kazemi Shahandashti *et al*. (2013) also reported a similar increase in freezing tolerance due to a five-day cold acclimation period followed by two-day severe cold stress in chickpea.

In the study of Nezami *et al*. (2007) and Cardona *et al*. (1997), for resistant genotypes, the slope of the EL curve versus freezing temperatures was lower than that of the susceptible ones. Under cold stress conditions, the resistant genotypes indicated a lower EL rate. They suggested that, for an index of cold tolerance in plant species, one can consider the slope of the EL curve. According to our results, the slope of EL for treated plants (WWC, DP and DPC) were lower than the control (WW), which could be deemed a sign of the acclimation effect

and cell defense activity that increase the freezing tolerance of our plant species (Figure 5A and 5B).

Over three weeks of acclimation period, MDA content of DP and DPC indicated a normal increasing trend, but for WWC a sudden emerge was found in the last week for both genotypes (Figure 2A and 2B). In general, DP and DPC exhibit the higher amount of MDA. In our study, we found that the plant with higher primary dry weight had higher amount of MDA, this followed by decreasing the EL and LT_{50el} and LT_{50su} that resulted in higher freezing tolerance and, in consequence, less mortality and more final dry weight. These findings are in agreement with results from Kazemi-Shahandashti *et al*. (2014).

Proline production is a well-known phenomenon among plants (Banu *et al*., 2009). Proline has been associated with cold tolerance (Zhang *et al*., 2011; Nabati *et al*., 2020), drought resistance (Moreno-Galván *et al*., 2020; Wang *et al*., 2006) and both cold and desiccation conditions (Hoffman *et al*., 2012; Oraee *et al*., 2020) to help in maintaining cell stability and form hydration barriers around proteins, nucleic acids, and cell membranes, all due to its hydrophilic nature (Hoekstra *et al*., 2001). In the current study, cold acclimation resulted in the larger increases in proline content for both genotypes. Reduction of PDW led to promotion of proline that associated with decrease in electrolyte leakage. The higher amount of proline significantly increased survival percentage. The results from other investigations are in agreement with our findings and support the importance of proline in response to low temperature (Patton *et al*., 2007) and drought (DaCosta and Huang, 2007) as a protective component. The cold-acclimated plants showed an initial rapid increase in the proline in the first week, thereafter, as the acclimation persisted, it remained elevated till the third week. Proline has diverse protective roles under stress conditions, so, it is conjecture that the effect of proline might be indirect in maintaining the homeostatic status of the cells (Hare and Cress, 1997).

Carbohydrates and proline were reported to delay freezing through the direct inhibition of ice crystal growth in the apoplast (Livingston *et al*., 2009), and improve membrane stability in response to dehydration-related stresses (Valluru and Van den Ende, 2008). Soluble carbohydrate may function as a typical osmoprotectant for maintaining turgor and protect cell from the effects of freezing and dehydration (Hartmann and Trumbore, 2016; Zhang *et al*., 2016). We found that genotypes demonstrate different reaction in consequence to treatments, which, for MCC252 cold treatment (WWC) resulted in higher SC content while for MCC505, the amount of SC increased in response to DP.

One of the major objectives for our study was to evaluate changes in the accumulation of specific compounds in response to cold and drought acclimation treatments, including soluble carbohydrates, proline and MDA. In general, we found that cold acclimation induced the greatest accumulation of MDA and proline contents (Figures 2 and 3) and drought preconditioning most consistently induced an increase in soluble carbohydrate content for both genotypes during the acclimation period (Figures 4-A and -B).

Both dehydration and low temperature affect metabolic activities and may decrease or completely abolish them (Beck *et al*., 2007). Low temperatures delay the dissipation of photosynthetic energy, as well as retard metabolic processes (Vogg *et al*., 1998). On the other hand, drought means water loss from cells, which causes the abolition of metabolic processes and membrane disintegration (Mahajan and Tuteja, 2005). However, when returns the plant to normal temperatures, these changes can be reversed rapidly (Sasaki *et al*., 1996).

In our study, survival was not directly involved in final dry weight. In other words, increasing plant survival was not along with strong growth activity in recovery period, but, generally, the lower number of dead

plants after freezing injury resulted in higher final dry weight.

CONCLUSIONS

Overall, we found that drought preconditioning had a synergistic effect on the cold acclimation period to improve freezing tolerance (as indicated by the lowest LT_{50el} and LT_{50su}) and the greatest gain in freezing tolerance for both genotypes was associated with DPC treatment. In the current study, when comparing the genotypes in control condition, MCC252 exhibited better freezing tolerance compared to MCC505, however, acclimation treatments seemed to have a greater effect on improving freezing tolerance of the less freezing-tolerant genotype. In fact, exposing MCC505 to WWC, DP, and DPC treatments allowed this genotype to achieve levels of freezing tolerance that were comparable to MCC252. Additional research is necessary to evaluate the effect of DP on the freezing tolerance of species and genotypes varying in their sensitivities to drought stress. In addition, future research studies are needed to explore the effects of repeated mild drought events on freezing tolerance, by using different drought preconditioning strategies, such as partial rootzone drying or deficit irrigation.

Abbreviations

DP: Drought Preconditioned under an optimum temperature regime; DPC: Drought Preconditioned under a Cold temperature regime; LT50: Lethal Temperature resulting in 50% mortality; MCC: Mashhad Chickpea Collection, MDA: Malondialdehyde, WW: Well-Watered under an optimum temperature regime; WWC: Well-Watered under a Cold temperature regime.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Research Center for Plant Sciences for worthful technical assistance on biochemical analysis. We are also grateful to Ferdowsi University of Mashhad, Iran, for providing financial support for the present research.

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اثر متقابل دمای پایین و خشکی بر بهبود تحمل به یخ زدگی گیاه نخود (*Cicer arietinum* L.)

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چکیده

اثر متقابل خشکی ملایم و دمای پایین که در اثر تغییر تاریخ کاشت از بهار به پاییز در مناطق مرتفع مدیترنه ای رخ می دهد، در گیاه نخود می تواند باعث افزایش تحمل به یخ زدگی در این گیاه شود. دو هفته بعد از کشت این گیاه در گلخانه، نهالبذر های نخود تحت تیمارهای سرماسازگاری به محیط کنترل شده منتقل شدند.این تیمار ها شامل: i) آبیاری مطلوب تحت دمای بهینه (WW)، ii) آبیاری بهینه تحت دمای پایین (WWC)، iii) شرایط خشکی تت دمای مطلوب (DP) و iv) شرایط خشکی تحت دمای پایین (DPC). بعد از سه هفته تیمار دهی، بوته ها در فریزر ترموگرادیان تت دمای یخ زدگی قرار گرفتند سپس سه هفته در گلخانه بازیابی شدند. در دوره ی سرماسازگاری، با کاهش دما، نشت الکترولیت در هر دو ژنوتیپ کاهش داشت (۵۱ درصد در MCC252 (مقاوم به سرما) و ۳۶ درصد در MCC505(حساس به سرما)). دمای پایین بیشترین تاثیر را در افزایش پرولین و محتوای مالون دی آلدهید داشت (تقریبا ۷۵ درصد برای هر دو ژنوتیپ) و خشکی باعث افزایش محتوای کربوهیدرات محلول گردید (۲۵ درصد برای ژنوتیپ MCC252 و ۵۱/۷ در MCC505). درصد بقاء در اثر اعمال تیمارهای سرماسازگاری به میزان ۹/۳ درصد در ژنوتیپ MCC252 و ۲۱/۵ درصد در ژنوتیپ MCC505 افزایش داشت. به طور کلی در دوره ی سرماسازگاری، خشکی و دمای پایین اثر هم افزایی داشته و باعث افزایش تحمل به یخ زدگی در هر دو ژنوتیپ مخصوصا ژنوتیپ حساس به سرما گردیدند (بر اساس داده های LT50el و LT50su). در حقیقت، بالاترین تاثیر بر تحمل به یخ زدگی در هر دو ژنوتیپ مر بوط به تیمار توام سرما و خشکی (DPC) بود.