

## Cold Acclimation Capacity and Freezing Tolerance in Some Iranian Barley Cultivars

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### ABSTRACT

Low temperature is one of the most widespread stress factors limiting productivity of cereals. Tolerance to low temperature was studied in 12 barley (*Hordeum vulgare* L.) cultivars with different growth habits (winter, spring, and facultative) subjected to low, non-freezing temperature (5°C) for cold acclimation, then exposed to freezing (-5°C) treatment. Different phenotypic assays, including biomass production and survival rate (%) were conducted to determine the range of genotypic variation for tolerance to low temperature. Although the most tolerant cultivar was a winter/facultative one ('Abidar') and the most susceptible one was a spring cultivar ('Nik'), little relationship existed between frost tolerance and growth habit when all 12 tested cultivars were compared. According to the data obtained, four cultivars with contrasting survival rate were selected and metabolic alterations were analyzed in these cultivars after cold acclimation and de-acclimation (25°C) treatments. Soluble carbohydrates and proline were accumulated in the leaves up to 20-70% ( $P < 0.05$ ) and 37 fold ( $P < 0.001$ ) during the acclimation period, respectively, and decreased upon de-acclimation by 9-42% ( $P < 0.05$ ). In contrast to the total protein, proteins in the leaf apoplast, along with carbohydrates, accumulated up to 2-4 fold ( $P < 0.05$ ) during the acclimation period. The frost tolerance of cultivars was correlated ( $r = 0.91$ ,  $P < 0.05$ ) with the concentrations of apoplastic carbohydrates but not with the total and apoplastic proteins or total carbohydrates. Our data revealed that barley cultivars were different in the metabolic adjustments upon acclimation treatment; some of these metabolic responses were directly associated with frost tolerance of cultivars.

**Keywords:** Apoplast, Cold stress, De-acclimation, Frost tolerance, *Hordeum vulgare*, Growth habit.

### INTRODUCTION

Production of cereals extends from the boreal regions of the world to the tropics in both high and low altitudes. Under these conditions, plant growth cycle is completed under a range of environmental stresses. Low temperature, including chilling (0–15°C) and freezing (< 0°C) temperatures, is one of the major abiotic stresses and is a significant limiting factor on world production and distribution of cereals (Rizza *et al.*, 2011). Sub-zero temperatures that

promote ice formation in plants cause damage to plant cells and reduce significantly the yield. Damage at vegetative stage is the most widespread type of frost damage and is a production constrain predominantly in cereal producing countries in the northern hemisphere (Liu, 2015).

To survive at freezing temperatures and to achieve maximum freezing tolerance, plants have developed an adaptation strategy, a process known as cold acclimation (Janská *et al.*, 2010). Cold acclimation process in cereals and other plants is associated with

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numerous physiological, biochemical and molecular alterations, including accumulation of compatible solutes, such as sugars and amino acids, alteration in membrane lipid composition, and synthesis of proteins responsible for inhibition of ice recrystallization and leaf accumulation of various heat-shock proteins (Janská *et al.*, 2010). Another challenge of overwintering plants is de-acclimation and the resulting freeze damage. Winter survival is not only related to cold acclimation, but de-acclimation resistance also plays an important role (Rapacz *et al.*, 2000). De-acclimation refers to the reduction/loss of freezing tolerance that occurs naturally in early spring with the rise of temperatures, but also results from midwinter warm periods (Arora and Rowland, 2011). Winter temperature fluctuation is one of the most obvious effects of climate change that causes significant freezing damage to plants (Arora and Rowland, 2011).

The apoplast, consisting of extracellular spaces outside the plasma membrane (cell walls, spaces between the cells, and xylem) is the first plant compartment encountering environmental signals (Clarkson, 2007). As temperatures drop below freezing, ice forms primarily in the intercellular spaces (Dietz, 2000). The apoplast of winter cereals accumulate soluble low-molecular weight carbohydrates, amino acids, and antifreeze substances of proteins and non-protein nature (Griffith and Yaish, 2004).

Barley (*Hordeum vulgare* L.) ranks fourth in the world cereal production and is one of the first domesticated cereals and a model experimental system because of its short life cycle (Meng *et al.*, 2016). Barley is known as a relatively drought and salt tolerant crop species, while exhibits less freezing tolerance in comparison to wheat cultivars with the same growth habits (Kosová *et al.*, 2014). Cold-induced responses differ among species and genotypes (Ruelland *et al.*, 2009). Information on these mechanisms can lead to breeding cold-tolerant crops, reduction of production loss, and expanding

crop cultivation areas (Fowler *et al.*, 2014; Daskalova and Spetsov, 2017).

Variations in response to low temperature have been investigated in different barley genotypes (Rizza *et al.*, 2011). However, relationship between growth habit (spring vs. winter) and tolerance to low temperatures and/or response to acclimation treatment are still poorly understood in barley genotypes. In the present work, physiological and biochemical responses to cold acclimation and de-acclimation treatments were studied in some Iranian barley cultivars with contrasting growth habit and cold tolerance.

## MATERIALS AND METHODS

### Plant Growth and Treatments

Barley (*Hordeum vulgare* L.) cultivars released by Dryland Agricultural Research Institute (DARI) (Maragheh, Iran) or Seed and Plant Improvement Institute (SPII) (Karaj, Iran) were used in this study (Table 1). The Zadoks decimal code (Zadoks *et al.*, 1974) was used to identify plants developmental stage at each treatment time. Seeds were surface-sterilized with 5% sodium hypochlorite and germinated on perlite in the dark (Zadoks stage 07). Four-day-old young seedlings (Zadoks stage 10) were transferred to light and irrigated with Hoagland nutrient solution at 300 mL week<sup>-1</sup> for three weeks. Plants were grown under growth chamber conditions with a day/night temperature regime of 25-28/15-17°C, a relative humidity of 70/80% and a photoperiod of 17/7 hours at a photon flux density of about 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by fluorescent lamps.

Experiment I: Selection of Cultivars: Twelve cultivars of barley plants, including three winter cultivars, 'Abidar' (winter/facultative), 'Radical' (obligate), 'Dikto' (obligate) and four facultative (intermediate) cultivars, 'Ansar', 'Sasaroud', 'Sahand' and 'Afzal', together with five spring cultivars, 'Torkaman', 'Yousof',

**Table 1.** The growth type, origin, releasing institute, vernalization requirement and the best climatic conditions for cultivation of 12 barley cultivars used in this work.

Barley cultivar	Growth type	Country of origin	Breeder/releasing institute	Vernalization requirement	Suitable climatic conditions for cultivation
'Dikto'	Winter	Russia	DARI	4 weeks below 2°C	Cold
'Radical'	Winter	Russia	DARI	4 weeks below 2°C	Cold
'Abidar'	Winter/facultative	ICARDA	DARI	2 weeks below 2°C	Cold
'Sahand'	facultative	ICARDA	DARI	2 weeks below 2°C	Cold
'Ansar'	facultative	ICARDA	DARI	2 weeks below 2°C	Cold
'Sraroud'	facultative	ICARDA	DARI	1 weeks below 2°C	Temperate
'Afzal'	facultative	Iranian native population	SPII	2 weeks below 2°C	Temperate
'Zehak'	Spring	CIMMYT	SPII	---	Warm/Dry
'Yousof'	Spring	Iranian native population	SPII	---	Warm/Temperate
'Torkaman'	Spring	ICARDA	SPII	---	Warm/Temperate
'Reyhan'	Spring	ICARDA	SPII	---	Warm/Temperate
'Nik'	Spring	ICARDA	SPII	---	Temperate

'Zehak', 'Reyhan' and 'Nik' were studied in this experiment. After three weeks growth under control conditions (Zadoks stage 14), half of the plants were randomly assigned to the low, non-freezing temperature (as cold Acclimation treatment), including without (-Ac) and with (+Ac, 5°C) acclimation that was provided as a gradual temperature decrease by 5 °C day<sup>-1</sup> from 25 °C to 5 °C. Acclimation temperature was applied under the same illumination conditions as the control treatment. Three weeks after starting Acclimation treatments (Zadoks stage 21 for -Ac plants and Zadoks stage 18 for +Ac plants), the control (-Ac) plants together with the first group of the +Ac plants were harvested for the determination of Dry Weight (DW). Before harvest, maximum quantum yield of photosystem II (PSII) ( $F_v/F_m$ ) was measured in the dark-adapted leaves, using a fluorometer (OSF1, ADC Bioscientific Ltd., UK). The second group of +Ac plants was subjected to the Freezing (Fr, -5°C) temperature in the dark. After 48 hours, plants were harvested and the survival

rate was determined according to the method described below.

Experiment II: Study of Acclimation and De-Acclimation Treatments: According to the data of survival rate obtained in Exp. I, Exp. II was conducted with four cultivars differing in the survival rate including 'Abidar', 'Torkaman', 'Reyhan' and 'Nik'. After three weeks pre-culture (Zadoks stage 14), one group of plants were randomly selected and harvested (Starting exp.) and the second group was subjected to -Ac and +Ac temperatures with two different periods, i.e. 7 (-Ac7d and +Ac7d) and 21 days (-Ac21d and +Ac21d), and one De-Acclimation (DeAc) treatment, i.e., transfer of +Ac21d plants to the control (25°C) conditions and subsequent growth for 4 days. At the end of each acclimation treatment, plants were harvested [at Zadoks stages 16 (-Ac7d plants) or 15 (+Ac7d plants), and at Zadoks stages 21 (-Ac21d plants) or 18 (+Ac21d plants)] for the determination of biochemical parameters and electrolyte leakage. The third group of



plants with different Ac treatments were subjected to the freezing treatments, ( $-5^{\circ}\text{C}$  in the dark) for 48 h. Survival rate, Lethal Temperature ( $\text{LT}_{50}$ , see below) and electrolyte leakage were determined in this group of plants.

#### Measurement of Electrolyte Leakage, Plants' Survival and Lethal Temperature ( $\text{LT}_{50}$ )

Electrolyte leakage was measured according to the method described by Campos *et al.* (2003) using an Electrical Conductivity (EC) meter (Hanna HI 9812, Hanna instruments, Italy). For determination of survival rate, plants were exposed to  $-5^{\circ}\text{C}$ . After 48 hours freezing treatment, plants were allowed to adapt for 12 hours. After adaptation, plants were returned to growth chamber conditions and grown for a further three days. Survival (%) was determined as the capacity of plants to resume growth after three days under control conditions. The freezing injury was quantified according to the method of Turhan *et al.* (2012) as the temperature causing death in 50% of plants ( $\text{LT}_{50}$ ) was determined.

#### Biochemical Determinations

Total soluble protein concentration was determined using Bradford reagent (Sigma, USA). For determination of non-structural carbohydrates, samples were homogenized in 100 mM phosphate buffer (pH 7.5) at  $4^{\circ}\text{C}$ , after centrifugation at  $12,000\times\text{g}$  for 15 minutes, supernatant was used for the determination of total soluble sugars whereas the pellets were kept for starch analysis (Yemm and Willis, 1954). For determination of proline, samples were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at  $3,000\times\text{g}$  for 20 minutes. The supernatant was treated with acetic acid and ninhydrin, boiled for 1 hour, and then absorbance at

520 nm was determined. Proline (Sigma, USA) was used for production of a standard curve (Bates *et al.*, 1973).

#### Extraction and Analysis of Leaf Apoplast

Apoplastic fluid was extracted, using the infiltration-centrifugation technique (Witzel *et al.*, 2011). Leaves were cut into 1.5 cm-long segments and washed with deionized water three times and were infiltrated by placing in plastic syringes (60 mL) filled with 40 mL of 25 mM Tris-HCl (pH 8.0) and were infiltrated by pulling the plunger. Thereafter, intact leaf segments were carefully blot dried and then placed in a 10 mL plastic vessel and centrifuged immediately at  $400\times\text{g}$  for 5 minutes at  $5^{\circ}\text{C}$ . The clear infiltrate, now referred to as apoplastic fluid, was collected at the bottom of the tube and stored in liquid nitrogen until analysis.

#### Statistical Analysis

These experiments were undertaken using Complete Randomized Block (CRD) design with two factors (temperature treatment and cultivar) and four independent replications. Statistical analyses were carried out using Sigma Stat 3.5 software (Systat Software Inc., USA). Two-way ANOVA was performed for all parameters determined in Exp. II (Table 3). Pairwise multiple comparison procedure was performed with Fisher's Least Significant Difference (LSD) test ( $P < 0.05$ ).

## RESULTS and DISCUSSION

### Effect of Low Temperature in 12 Cultivars

Three weeks growth at low temperature ( $5^{\circ}\text{C}$ ) applied as acclimation treatment expectedly declined shoot biomass.

According to the data of shoot DW reduction (% over the control plants), tolerance to non-freezing temperature was in the order of 'Dikto' > 'Abidar' > ('Nik' = 'Sahand' = 'Zehak' = 'Yousof' = 'Reyhan' = 'Afzal' = 'Radical') > ('Ansar' = 'Torkaman' = 'Sarasoud') (Table 2).

The maximum quantum yield of the photosystem II photochemistry measured as the variable (Fv) to maximal (Fm) Fluorescence ratio (Fv/Fm) was also affected by low non-freezing temperature (Table 2). Chilling-induced inhibition of photosynthesis results from reduced stomata aperture and reduced CO<sub>2</sub> fixation. This inhibition also involves reduced electron transport via PSII due to down-regulation of water splitting and degradation of the D1 protein of PSII reaction center and photo inhibition of PSII (Sayed, 2003). According to the Fv/Fm data, tolerance to non-freezing temperature was in the order of ('Sahand' = 'Ansar' = 'Abidar' = 'Sarasoud' = 'Dikto') > ('Radical' = 'Reyhan') > 'Zehak' > 'Torkaman' > 'Yousof' > ('Nik' = 'Afzal') (Table 2).

After the exposure of plants to the freezing temperature, survival rate (%) was greatly different among cultivars. Tolerance to freezing temperature indicated by the survival data was in the order of ('Abidar' = 'Ansar' = 'Sarasoud' = 'Radical') > 'Sahand' > ('Dikto' = 'Torkaman' = 'Yousof' = 'Zehak' =

'Reyhan') > 'Afzal' > 'Nik' (Table 2).

Comparison of the extent of adverse effect of low temperature on the shoot DW indicated that reduction of vegetative growth was not higher in the spring compared to the (obligate) winter cultivars and both low and high sensitivity to low temperature were found in both spring and winter cultivars. In the facultative cultivars, reduction of shoot DW was in the range of 40-61%, except for 'Abidar', a winter/facultative cultivar that showed the lowest reduction of DW (28%) after 'Dikto', an obligate winter cultivar. In contrast, an association seemed to exist between susceptibility of leaf photochemistry to low temperature and growth type, so that the highest reduction of Fv/Fm upon cold temperature was observed in the spring cultivars. Facultative cultivars indicated mainly a high tolerance to low temperature, except for 'Afzal' with up to 11% reduction (P < 0.05) of Fv/Fm under low temperature conditions.

Survival of plants after a period of freezing treatment was in the range of 8-100% depending on cultivars. The survival data indicated that even spring cultivars could acclimate by low, non-freezing temperatures. Rapacz *et al.* (2000) also reported that pre-hardening enables spring barley plants to reach similar tolerance levels seen during overwintering in winter cultivars. Although the highest survival rate

**Table 2.** Results of the experiment with twelve barley (*Hordeum vulgare* L.) cultivars.<sup>a</sup>

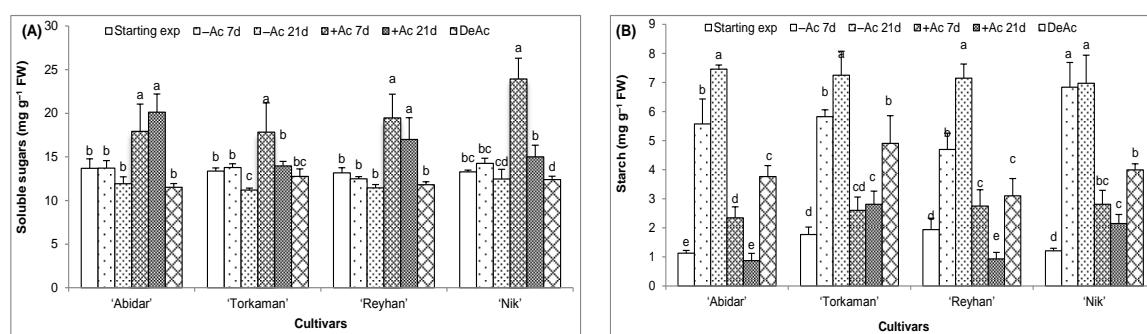
Barley cultivars	Growth habit	Reduction of DW (%)	Reduction of Fv/Fm (%)	Survival (%)
'Abidar'	Winter/Facultative	28±4 <sup>d</sup>	-1.13±0.06 <sup>f</sup>	100±0 <sup>a</sup>
'Ansar'	Facultative	61±9 <sup>ab</sup>	-1.64±0.09 <sup>f</sup>	97±2.9 <sup>ab</sup>
'Sarasoud'	Facultative	71±8 <sup>a</sup>	-1.13±0.07 <sup>f</sup>	96±3.2 <sup>ab</sup>
'Radical'	Winter	56±5 <sup>b</sup>	1.13±0.06 <sup>e</sup>	94±5.3 <sup>b</sup>
'Sahand'	Facultative	40±7 <sup>bc</sup>	-1.76±0.08 <sup>f</sup>	84±3.8 <sup>c</sup>
'Dikto'	Winter	8±2 <sup>e</sup>	-0.87±0.03 <sup>f</sup>	73±2.5 <sup>d</sup>
'Torkaman'	Spring	66±3 <sup>a</sup>	5.41±0.81 <sup>c</sup>	70±5.1 <sup>d</sup>
'Yousof'	Spring	43±3 <sup>bc</sup>	9.67±0.73 <sup>b</sup>	64±2.3 <sup>d</sup>
'Zehak'	Spring	40±7 <sup>bc</sup>	3.17±0.05 <sup>d</sup>	64±2.3 <sup>d</sup>
'Reyhan'	Spring	43±6 <sup>bc</sup>	1.50±0.05 <sup>e</sup>	64±4.0 <sup>d</sup>
'Afzal'	Facultative	51±4 <sup>b</sup>	10.9±0.98 <sup>a</sup>	43±2.7 <sup>e</sup>
'Nik'	Spring	39±5 <sup>c</sup>	10.5±1.12 <sup>a</sup>	8±2.7 <sup>f</sup>

<sup>a</sup> Data of each column indicated by the same letter are not significantly different (LSD test, P ≤ 0.05).

**Table 3.** Results of the ANOVA analysis (mean squares) for Exp. II with two factors including Temperature Treatment (TT) and Cultivar (C) and interactions between these factors.<sup>a</sup>

Parameters	TT	C	TT×C
Soluble sugars	183.19***	10.94**	14.08***
Starch	80.91***	4.07***	1.30***
Total protein	187.55***	6.61 <sup>ns</sup>	7.30 <sup>ns</sup>
Proline	12778.52***	2456.32***	990.61***
Apoplasmic proteins	0.78***	0.49***	0.052***
Apoplasmic sugars	27.45***	13.74***	2.48***
Electrolyte leakage (-Fr)	1872.25***	28.575 <sup>ns</sup>	23.29 <sup>ns</sup>
Electrolyte leakage (+Fr)	1499.99***	124.74***	28.98*
Survival	12532.28***	6075.29***	952.90***
LT <sub>50</sub>	227.41***	20.54***	4.93***

<sup>a</sup> Effects are presented as ns: Non-significant and Significant difference: \*\*\* P ≤ 0.001, \*\* P ≤ 0.01, \* P ≤ 0.05 (LSD test, Alpha= 0.05).



**Figure 1.** Leaf concentration of soluble sugars (A) and starch (B) in four barley (*Hordeum vulgare* L.) cultivars subjected to two Acclimation treatments including without (-Ac) and with (+Ac, 5°C) acclimation either for 7 or 21 days. For de-acclimation treatment (DeAc), plants that were acclimated for 21 days at 5°C, were transferred to 25°C and harvested after 4 days. Data of each cultivar indicated by the same letter are not significantly different (LSD test, P ≤ 0.05).

(100%) was found in a winter/facultative cultivar ('Abidar) and the lowest survival rate (8%) was detected in a spring cultivar ('Nik'), a relatively wide range of survival rate (43-97%) was found in the other 10 cultivars with different growth habits (winter, spring and facultative). Some authors have investigated a probable relationship between growth habit and cold stress tolerance in wheat and barley (Kosová *et al.*, 2008). Two groups of genes are found to be responsible for cold tolerance in these species; i.e., *Vernalization* genes (VRN) delaying flowering until the end of the winter and thus protecting the leaf primordium and a series of transcription factors considered to be regulators of the

*FR2* genes, conferring resistance to low temperatures and frost (Kosová *et al.*, 2008).

In wheat, the *VRN* and *FR* loci are not tightly linked to each other and a similar level of acquired frost tolerance is found in the winter and spring wheat cultivars (Ishibashi *et al.*, 2007). In barley, some authors have postulated the relationship between vernalization requirement and frost tolerance (Cuesta-Marcos *et al.*, 2015).

Similar to our work, however, high levels of freezing tolerance and winter hardiness were found in both winter and facultative growth habits in European barley cultivars (Rizza *et al.*, 2011). Likewise, a relationship was not observed between growth habit and cold

tolerance in barley cultivars from Algeria (Benkherbache *et al.*, 2016).

Comparison of three parameters used to evaluate tolerance to low temperature in this work, revealed that  $Fv/Fm$  and survival rate were more reliable phenotypic parameters than DW to evaluate the response of cultivars to low temperature and reflected their suitable climatic conditions for cultivation as indicated by the breeders (Table 1). In addition, a correlation existed between survival rate and response of  $Fv/Fm$  to low temperature ( $r= 0.82$ ,  $P< 0.001$ ), while such correlation was not found between the response of DW and survival rate ( $r= 0.18$ ) or between the response of DW and  $Fv/Fm$  ( $r=0.08$ ). The  $Fv/Fm$  ratio has been suggested as a reliable method of screening genetic diversity for frost tolerance in plants at early growth stages (Rizza *et al.*, 2011). The survival rate after freezing is an important criterion for plants productivity in cold climates (Rizza *et al.*, 2011).

#### Effect of Low Temperature in Four Cultivars

To select the most tolerant and susceptible cultivars to cold stress in this work, 'Abidar' and 'Nik' with the highest (100%) and the lowest (8%) survival rate, respectively, along with 'Torkaman' and 'Reyhan' with intermediate survival rates (64-70%) were selected and subjected to further studies on the effect of the duration of acclimation period and de-acclimation treatment.

Results of the ANOVA showed that temperature treatment significantly ( $P< 0.001$ ) affected all the analyzed parameters (Table 3). Effect of cultivar and the interaction between cultivar and temperature treatment were significant for all analyzed parameters, except for total protein and electrolyte leakage in the absence of Freezing (-Fr) treatment (Table 3).

#### Changes in the Concentration of Non-Structural Carbohydrates upon

#### Acclimation Treatment

Acclimation treatment significantly increased ( $P< 0.001$ ) soluble sugars concentration up to 20-70% in all analyzed cultivars (Figure 1-A). However, no relationship existed between DW reduction ( $r= 0.06$ ,  $P= 0.755$ ) or survival rate ( $r= 0.89$ ,  $P= 0.057$ ) and concentration of soluble sugars 21 days after the acclimation treatment. In contrast to our data, Gusta and Wisniewski (2013) stated that the main difference between winter and spring cereals is the ability of winter cereals to effectively accumulate sugars under cold acclimating conditions while spring cereals do not. It has been proposed that the accumulation of carbohydrates in the cell changes the osmotic potential and decreases the difference in water potential between inside the cell and ice forming outside the cell. Sugars also play a role in decreasing the ice nucleation temperatures and could suppress ice growth during freezing (Tarkowski and Van den Ende, 2015). Starch concentration increased during experimental time in plants in the absence of any treatment while acclimation treatment decreased it significantly in all tested cultivars irrespective of the acclimation period (Figure 1-B). Accumulation of soluble sugars upon cold acclimation concomitant with reduction of starch in the leaves observed here in four selected barley cultivars has also been found in other cereal species (Wang *et al.*, 2015). It has been observed that starch is broken down into soluble sugars after being exposed to cold conditions and mutants that are impaired in starch degradation show a reduced freezing tolerance (Krasensky and Jonak, 2012). Interestingly, spring cultivars were not able to maintain higher soluble sugar concentrations during longer acclimation period (21 days). Similar results were obtained by Murelli *et al.* (1995). They found that the concentration of monosaccharides and sucrose decreased 25-90% during prolonged cold acclimation (20



days) in barley. De-acclimation treatment, expectedly, returned the concentration of soluble sugars to the levels of -Ac plants (Figure 1-B).

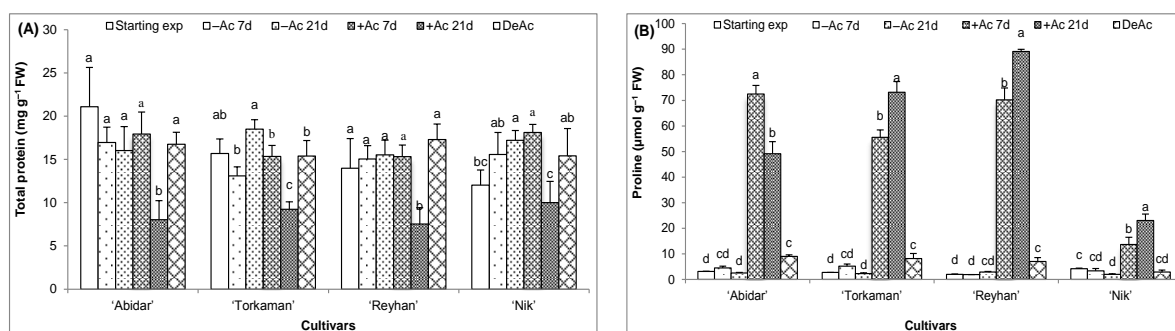
### Changes in the Concentration of Nitrogenous Compounds upon Acclimation Treatment

Total protein concentration remained mainly constant after 7 days acclimation treatment and decreased significantly after 21 days acclimation. De-acclimation treatment restored protein concentration to the levels of that in +Ac7d plants (Figure 2-A). Similar to the total soluble sugars, no relationship was found between soluble proteins concentration and DW reduction ( $r= 0.09$ ,  $P= 0.690$ ) or survival rate ( $r= 0.53$ ,  $P= 0.273$ ). Proline concentration increased up to 37-fold by acclimation treatment, a prolonged +Ac treatment (21 days) resulted in 30-68% higher ( $P< 0.01$ ) proline concentration, except for 'Abidar'. De-acclimation treatment decreased ( $P< 0.001$ ) proline accumulation by 31-88%; this effect was more pronounced than the effect on carbohydrates (Figure 2-B). It has been observed that during the process of cold acclimation, the amino acid proline begins to accumulate in the tissue (Ruelland *et al.*, 2009; Tarkowski and Van den Ende, 2015). Proline plays a multifunctional role in stress defense; it has a hydrophilic nature and provides a barrier around membranes, proteins, and nucleic acids during

dehydration stress. Proline is also considered to act as an osmolyte, a ROS scavenger, and a molecular chaperone stabilizing the structure of proteins, thereby protecting cells from damage caused by stress (Szabados and Savoure, 2010). Frost tolerance and winter survival is higher in the proline accumulating line of barley plant (Tantau *et al.*, 2004). The constitutive concentration of proline in this work was not different between analyzed cultivars, however, acclimation-induced proline accumulation was lower in 'Nik' compared to other three cultivars. It was likely one of the mechanisms for higher susceptibility of this cultivar to freezing temperature.

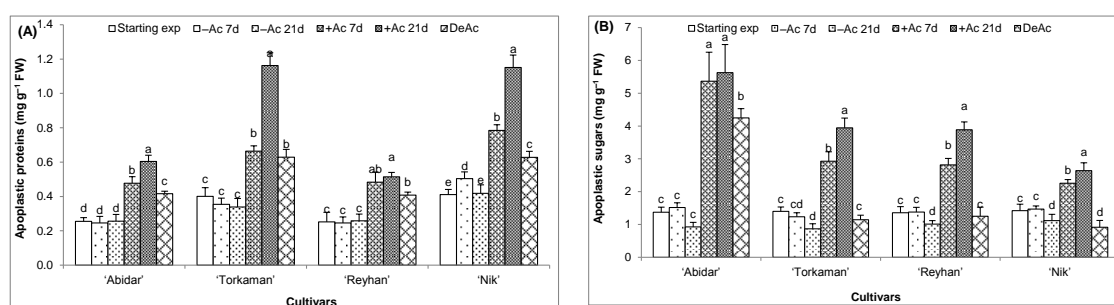
### Modifications in the Leaf Apoplast in Response to Acclimation Treatments

In contrast to the total soluble protein, proteins in the leaf apoplast accumulated in response to cold acclimation, particularly during a prolonged (21 days) acclimation period. De-acclimation treatment decreased the concentration of protein in the leaf apoplast (Figure 3-A). During cold acclimation, the metabolism of nitrogenous compounds is redirected towards synthesis of some cryoprotective proteins (Janská *et al.*, 2010; Tarkowski and Van den Ende, 2015). Our data suggested that cold acclimation considerably increased synthesis and release of soluble proteins to the apoplast (2-3 fold), being more pronounced



**Figure 2.** Leaf concentration of total soluble proteins (A) and proline (B) in four barley (*Hordeum vulgare* L.) cultivars subjected to two acclimation treatments. Treatments symbols are defined under Figure 1 and in the text. Data of each cultivar indicated by the same letter are not significantly different (LSD test,  $P \leq 0.05$ ).





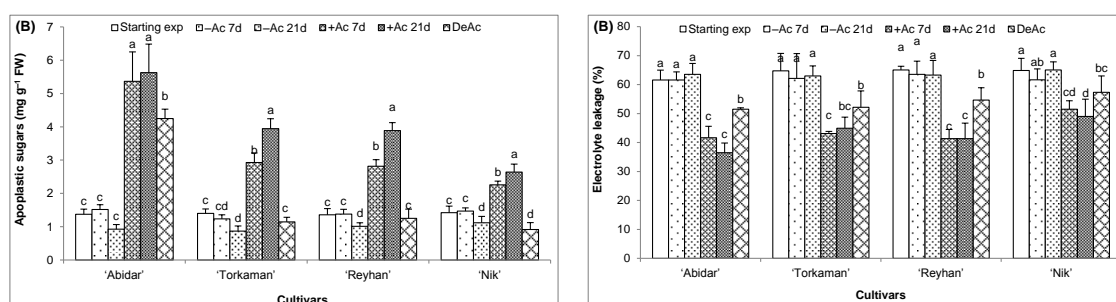
**Figure 3.** Concentrations of protein (A) and soluble carbohydrates (B) in the leaf apoplast of four barley (*Hordeum vulgare* L.) cultivars subjected to two acclimation treatments. Treatments symbols are defined under Figure 1 and in the text. Data of each cultivar indicated by the same letter are not significantly different (LSD test,  $P \leq 0.05$ ).

in 'Torkaman' and 'Nik' than the other two cultivars ( $P < 0.001$ ). Similarly, concentration of soluble sugars in the leaf apoplast was 1.8-3.6 fold higher in cold-acclimated plants and increased further by approximately 17-37% ( $P < 0.05$ ) after a longer (21 days) acclimation period that was significant only in three spring cultivars. De-acclimation treatment decreased soluble carbohydrates concentration in the leaf apoplast by 24-68% ( $P < 0.05$ ); however, in 'Abidar', it was still higher than -Ac plants (Figure 3-B). Cryoprotectant molecules such as soluble sugars and sugar alcohols are released from the symplast and serve to protect the plasma membrane from ice adhesion and subsequent cell disruption (Gusta and Wisniewski, 2013). Lethal intracellular freezing damage is avoided in cold acclimated cells through the blockage

of ice propagation from the apoplastic space to the intracellular region (Liu, 2015). Sugars also play a role in protecting plasma membranes from freeze-thaw cycles by generating protection from intercellular ice, preventing membrane damage and loss of osmotic reactivity (Ruelland *et al.*, 2009; Janská *et al.*, 2010). The survival rate of the analyzed cultivars was correlated ( $r = 0.91$ ,  $P = 0.046$ ) with the concentrations of apoplastic soluble sugars. Nevertheless, such correlation was not found between the survival rate and apoplastic proteins ( $r = 0.35$ ,  $P = 0.406$ ).

### Effect of Acclimation and De-Acclimation Treatments on Freezing Tolerance

In the absence of freezing temperature, +Ac



**Figure 4.** Electrolyte leakage (%) before (A) and after (B) freezing ( $-5^{\circ}\text{C}$ ) treatment in four barley (*Hordeum vulgare* L.) cultivars subjected to two acclimation treatments. Treatments symbols are defined under Figure 1 and in the text. Data of each cultivar indicated by the same letter are not significantly different (LSD test,  $P \leq 0.05$ ).

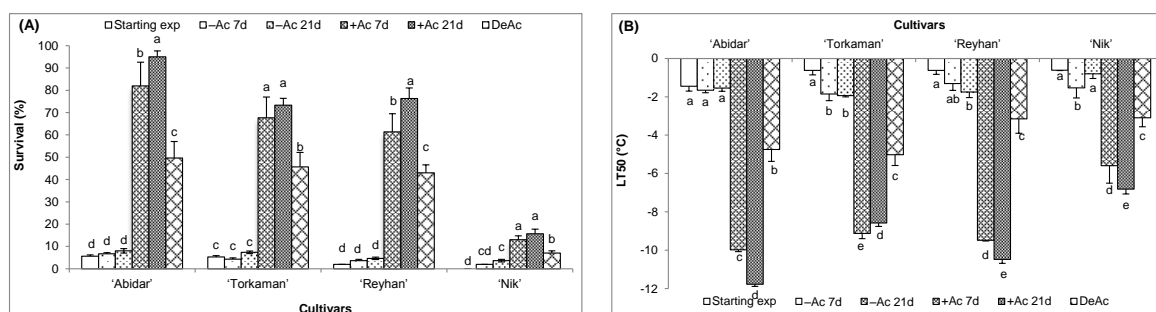


treatment significantly ( $P < 0.05$ ) increased electrolyte leakage up to 1.9 to 2.9 fold. Unexpectedly, +Ac21d plants had slightly or significantly ( $P < 0.05$ ) lower (16 to 35%) electrolyte leakage than +Ac7d ones (Figure 4-A). Chilling stress is frequently related to an increase in membrane permeability, affecting membrane integrity and cell compartmentation (Ruelland *et al.*, 2009). Increased rate of solute and electrolyte leakage has been used to evaluate membrane damage following chilling stress (Ruelland *et al.*, 2009). In the plants treated with freezing temperature, in contrast, electrolyte leakage was significantly lower in +Ac plants without a difference between +Ac7d and +Ac21d plants (Figure 4-B). This indicates the effectiveness of protection mechanisms induced by acclimation treatment. Expectedly, de-acclimation treatment decreased electrolyte leakage in the absence of freezing slightly or significantly (Figure 4-A) while increased it in plants treated with freezing temperature (Figure 4-B).

The survival rate in the -Ac (-Ac 21d) plants was as low as 4-8%, being higher ( $P < 0.001$ ) in 'Abidar' and 'Torkaman' than that in 'Reyhan' and 'Nik'. Acclimation treatment considerably increased survival of plants, and higher values were obtained in +Ac21d plants. Similar to the results of Exp. I, the highest survival rate was observed in 'Abidar', the lowest in 'Nik' and intermediate values in 'Torkaman' and 'Reyhan'. De-acclimation treatment decreased plants survival but this parameter

was still higher than that in the -Ac plants (Figure 5-A). Lethal Temperature ( $LT_{50}$ ) was also significantly ( $P < 0.001$ ) affected by acclimation treatments. The lowest  $LT_{50}$  value was observed in plants acclimated for 21 days while a shorter acclimation period (7 days) and de-acclimation, both, resulted in lower  $LT_{50}$  (Figure 5-B). At low non-freezing temperatures, plants can acquire an increased frost resistance that involves a variety of changes in gene expression, membrane lipid composition, compatible osmolytes, levels of antioxidants, and developmental traits (Ruelland *et al.*, 2009). Data of electrolyte leakage, survival and  $LT_{50}$  indicated that damage of freezing temperature was considerably declined by acclimation treatment. The effectiveness of acclimation treatment was not different between the studied cultivars. Considering electrolyte leakage as an indicator of frost tolerance, a prolonged acclimation period (21 days) was not more effective than a shorter acclimation period (7 days), however, freezing tolerance indicated by survival rate and  $LT_{50}$  was improved pronouncedly by an extended (21 days) acclimation period.

The ability to maintain a significant level of freezing tolerance despite exposure to temperatures more conducive to growth is an integral part of the overall cold tolerance of a particular species or genotype within a species (Arora and Rowland, 2011). Plants that are able to maintain a portion of their original freezing tolerance, i.e. de-acclimation resistance, do not suffer significant damage (Arora and Rowland,



**Figure 5.** Survival (%) (A) and  $LT_{50}$  (B) in four barley (*Hordeum vulgare* L.) cultivars subjected to two acclimation treatments. Treatments symbols are defined under Figure 1 and in the text. Data of each cultivar indicated by the same letter are not significantly different (LSD test,  $P \leq 0.05$ ).

2011). In the present work, de-acclimation treatment significantly decreased survival (38-55%,  $P < 0.001$ ) and increased  $LT_{50}$  (41-70%,  $P < 0.001$ ) and electrolyte leakage (11-21%,  $P < 0.05$ ) upon exposure to frost temperature. However, in de-acclimated plants survival rate was still higher and electrolyte leakage and  $LT_{50}$  were still lower than those in the -Ac plants. This suggests a high rate of de-acclimation resistance in the studied barley cultivars. Higher de-acclimation resistance has been observed in meadow fescue compared to barley (Rapacz *et al.*, 2000).

### CONCLUSIONS

The survival rate and  $LT_{50}$  data suggested that although the most freezing-tolerant cultivar ('Abidar') was a winter/facultative genotype and the most susceptible one ('Nik') was a spring cultivar, frost tolerance was not related to the growth habit when all 12 tested cultivars were compared. To evaluate tolerance to low temperature stress under field conditions among the three phenotypic parameters used in this work,  $Fv/Fm$  and survival rate seemed to be more reliable indicators than biomass production. Lack of any relationship between vegetative growth and survival rate revealed that the ability of plants for biomass production under low and non-freezing temperature was not associated with their survival after a frost event. In contrast, a close relationship between  $Fv/Fm$  and survival rate suggested that  $Fv/Fm$  was a fast and reliable method to estimate frost tolerance in barley cultivars. Our data demonstrated that the studied barley cultivars were different in the metabolic adjustments upon acclimation treatment. The obtained data indicated that the apoplastic carbohydrates was directly associated with frost tolerance of barley cultivars. A low proline content could also be responsible for a high susceptibility of some spring cultivars to freezing temperatures. Metabolic profiling approach aided by genomics and proteomics studies is

interesting topic for elucidation of frost tolerance mechanisms and its relationship with growth type in barley cultivars.

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## ظرفیت انطباق با سرما و تحمل یخ زدگی در برخی ارقام ایرانی جو

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### چکیده

دمای پایین یکی از شایع ترین تنش های محیطی است که عملکرد گندمیان را محدود می کند. در این پژوهش تحمل دمای پایین در ۱۲ رقم از گیاه جو با رفتار رشد متفاوت (زمستانه، بهاره و اختیاری) بررسی شد. گیاهان در معرض سرمای بالاتر از یخ زدگی (۵ درجه سانتی گراد) برای بوم پذیری قرار گرفتند سپس با دمای یخ زدگی (۵- درجه سانتی گراد) تیمار شدند. سنجش های فنوتیپی مختلفی از جمله وزن خشک و درصد زنده مانی انجام شد تا تنوع ژنوتیپی از نظر تحمل دمای پایین در ارقام انتخاب شده مشخص شود. علیرغم اینکه متحمل ترین رقم به یخ زدگی یک رقم زمستانه اختیاری (آبیدر) و حساس ترین آن، یک رقم بهاره (نیک) بود، ولی چنین ارتباطی زمانی که همه ۱۲ رقم با هم در نظر گرفته شدند، دیده نشد. براساس داده های بدست آمده برای درصد زنده مانی، چهار رقم با پاسخ متمایز انتخاب شده و تغییرات متابولیکی در این ارقام تحت تاثیر دو دوره زمانی بوم پذیری (۷ و ۲۱ روز) و رفع بوم پذیری (قرار گیری مجدد در ۲۵ درجه سانتی گراد) مطالعه شد. کربوهیدرات های محلول و پرولین در برگ ها در طی دوره بوم پذیری به ترتیب ۲۰-۷۰ درصد و تا ۳۷ برابر ( $P < 0.001$ ) انباشته شد و با رفع بوم پذیری ۹-۴۲ درصد ( $P < 0.05$ ) کاهش یافت. برخلاف پروتئین کل، پروتئین و کربوهیدرات ها در آپوپلاست برگ در طی تیمار بوم پذیری ۲-۴ برابر ( $P < 0.05$ ) انباشته شد. غلظت کربوهیدرات های آپوپلاستی با تحمل یخ زدگی همبستگی ( $r = 0.91$ ,  $P < 0.05$ ) نشان داد ولی این همبستگی با پروتئین کل و آپوپلاستی و یا قند کل مشاهده نشد. داده های ما پیشنهاد می کند که تطابق متابولیکی برای تحمل یخ زدگی بسته به رقم متفاوت است و تعدادی از این تغییرات متابولیسمی با درجه تحمل یخ زدگی ارتباط نشان می دهد.