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Commun.Fac.Sci.Univ.Ank.Ser. C Biology
Volume 29, Number 2, Pages 338-350 (2020)
ISSN 1303-6025 E-ISSN 2651-3749
https://dergipark.org.tr/en/pub/communc/issue//55192/679603

Received by the Editors: April 01, 2020; Accepted: September 30, 2020

ANTIOXIDANT AND PHYSIOLOGICAL ANALYSIS OF *TRITICALE* UNDER COLD ACCLIMATION CONDITIONS *IN VITRO* AND *EX VITRO*

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ABSTRACT. *Triticale* is an artificial species that originated about 130 years ago from between wheat and rye, and the first commercially viable cultivars were released in the 1960s. The crop exhibits high yield, promising long term potential, elevated grain quality, and better resistance to pathogens, desirable amino acid content, and high adaptation ability to adverse climate conditions. Sudden decreases in climate can pose significant losses in many crops including *Triticale*. Understanding plant response to cold acclimation could help developing crops resilient to cold. In this study, we aim to compare the antioxidants and physiological content of *Triticale* under cold acclimation *in vitro* and *ex vitro*. In our study, five triticale cultivars, *Ümran Hanım*, *Alper Bey*, *Mikham 2002*, *Tatlıcak*, and *Melez 2001* were used as the plant material. *Triticale* seeds were planted in 15 cm sand pods. They were maintained in 20/18^{0}C (day / night) greenhouse with a 12 h day length for 10 days to initiation germination. After 2 weeks the plants were transferred at 4±1^{0}C for cold acclimation for 30 days. Callus was transferred to a hormone-free MS medium for 1 month. All cultures were kept under fluorescent light with 15000 lux and 16 h/8 h light/dark cycle at 25±1^{0}C. The culture media was subsequently refreshed and kept under fluorescent light with 1500 lux and 16 h/8 h light/dark cycle at 4±1^{0}C under cold acclimation. Our results revealed that the cold acclimation changed the activities of APX (Ascorbate Peroxidase), SOD (Superoxide Dismutase), and CAT (Catalase) under both *ex vitro* and *in vitro* conditions. The highest correlation between enzyme activities and cold resistance was observed in the sugar content of in vitro stress callus. Our results indicated as closely related to proline, sugar content and antioxidant enzyme activities at cold acclimation in the evaluation of cold tolerance of *Triticale* cultivars.

Keyword and phrases. Antioxidant activity, callus, cold acclimation, proline, *Triticale*.

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1. INTRODUCTION

Low temperature is a notable abiotic stress that bring about important agricultural casualties around the world [1, 2]. The rapidly changing temperature is noted to adversely affecting plant growth and productivity. Plants are complex organisms, exhibiting a wide variation in their ability to maintain growth during chilling and lower temperatures [3]. Many plant species developed cold resistance to low non-freezing temperatures, a physiological process known as cold acclimation [4, 5]. Cold acclimation is a dynamic process in which plants acquire tolerance to sub-zero temperatures when exposed to low temperatures [6]. Plants respond to acclimation through several of biochemical, molecular genetics, ecological, and physiological alterations involving changes in carbohydrate, proline, protein content, and enzymatic activities [7].

Soluble carbohydrates and free proline may inhibit water loss during the acclimation [8]. Cold acclimation increases the level of proline via changes in enzyme activities in the proline metabolism pathway that in turn enhances cold-resistance [9]. Proline is also strongly associated with the plant cold stress since free proline increases during the acquisition of the cold resistance in plant species. The antioxidant enzymes in the plants are known to play a major role in the regulation against stress. Plant species produce various types of antioxidants, such as APX (Ascorbate Peroxidase), CAT (Catalase), and SOD (Superoxide Dismutase) to reduce the stress triggered by the elevated oxidative level [10, 11]. *Triticale* is used both for food and feed and is superior to other cereals in terms of nutritional quality [12]. Therefore, *Triticale* has gradually transformed into an important crop worldwide. *Triticale* can also contribute to environmental quality via better conservation of the soil. Resistance to diseases, the ability to grow in low pH, and persistence to drought are among other superiorities [13, 14]. The main breeding goals of *Triticale* breeding programs are increasing grain yield, nutritional quality, and plant height. Although traditional breeding methods have been routinely employed, incorporating desirable genes into the released cultivars to induce stress resistance via genetic engineering can be an alternative approach to enhance the *Triticale* breeding [15]. The selection and development of stress-resistant genotypes require an efficient screening method. Cell and tissue culture can be efficient methods for increasing the plant productivity and quality of plant materials. These methods can be also be used for understanding molecular and cellular basis of abiotic and biotic stress factors in plants and ultimately eliminating the crop yield losses due to abiotic and biotic stress factors. *In vitro*, culture techniques are among the available methods for improving cultivars resistant to a number of biotic and abiotic stress factors in the context of sustainable agriculture [16]. In recent decades, the *in vitro* plant tissue culture selection pressure method has been one of the most commonly used techniques for the selection of

genotypes resistant to environmental stress. *In vitro* culture has been used to obtain cold-resistant plants as there is a relation between cellular machinery and *ex vitro* plant with desirable traits. On the other hand, it is often uneasy to analyze the response of plants to various abiotic stresses in the field or in greenhouse conditions, due to the complex and unstable nature of these stresses. *In vitro* tissue culture is an extremely powerful tool to have a deeper understanding of physiology and biochemistry in molecular plant breeding under adverse environmental conditions [17].

The purpose of this study was to evaluate the cold acclimation stress in a number of *Triticale* plants using the proline content and antioxidant capacity of plants derived from *in vitro* culture and greenhouse (*ex vitro*).

2. Materials and Methods

Plant Material

In our study, the five most widely planted *Triticale* cultivars, *Ümran Hanım*, *Alper Bey*, *Mikham 2002*, *Tatlıcak*, and *Melez 2001* were used as the plant material. *Triticale* seeds were planted in 15 cm pods in the sand. They were maintained in $20/18^0$C (day/night) greenhouse with a 12 h day length for 10 days for the initiation of the germination. Two weeks later the plants were transferred at 4 ± 1^0C for cold acclimation and maintained there for 30 days.

Callus Induction

The mature seeds were sterilized with 1% NaOCl for 5 minutes, washed several times with sterile distilled water, and rinsed with several changes of sterile distilled water overnight at 4^0C. The mature embryos were cultivated in Petri dishes containing full MS medium. The plant material was kept in MS for 30 days at 25±1 and in 16 hours light/8 hour dark photoperiod at 1500 lux illumination intensity. Mature embryos were removed aseptically using forceps and placed on MS medium [18] with 2 mg L^{-1} glycine, 4 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 100 mg L^{-1} myo-inositol, 0,5 mg L^{-1} nicotinic acid, 0,5 mg L^{-1} pyridoxine HCl, 0,1 mg L^{-1} of thiamine HCl vitamins, 1,95 g L^{-1} of MES, 50 mg L^{-1} of ascorbic acid, 20 g L^{-1} of sucrose, solidified with 7 g L^{-1} of agar and the pH adjusted to 5.8 prior to autoclaving. In order to sterilize the vitamins and hormones, 0.22 μm of porous cellulose nitrate filters were used. The mature embryos were incubated in total darkness at 25 ± 1^0C temperature for one month.

Cold Acclimation

Callus was transferred to a hormone-free MS medium for one month. All cultures were kept under fluorescent light with 15000 lux and 16 h/8 h light/dark cycle at 25 ± 1^0C. The culture media were subsequently refreshed and kept under fluorescent light with 15000 lux and 16 h/8 h light/dark cycle at 4 ± 1^0C under cold acclimation. The total culture duration was one month.

Proline Estimation

Proline content was detected with the method of Bates et al. [19]. Briefly, 100 mg of plant material was homogenized in 5 mL of 3% aqueous sulfosalicylic acid and centrifuged at 4^0C for 15 min at 4800 rpm. 2 mL of extract was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in test tubes. Samples were kept for 1 h at 100^0C. The reaction was completed in an ice bath. 4 mL of toluene was used for reaction mixture extraction. The absorbance of color reaction product was measured at 520 nm using toluene for a blank. The proline concentration was determined from a calibration curve.

Soluble Sugars Determination

For soluble sugars determination, a total of 50 mg of tissue per embryogenic callus was grounded in a mortar, homogenized in 1 ml of ethanol (80%) and centrifuged at 5000 rpm for 10 min at 4^0C. Supernatants were transferred into other tubes and the pellets were homogenized again in 0,5 mL ethanol (80%),and centrifuged as above. The second supernatant was added to the first one. Total soluble sugars were measured by a modified method of Watanabe et al. [20]. Briefly, a total of 1 mL of extract was reacted with 3 mL freshly prepared anthrone reagent (50 mg anthrone, 50 mL of H_2SO_4 95%) at 100^0C for 10 min. After cooling on ice, the total sugar content was determined at 620 nm by a spectrophotometer using glucose as standard.

Enzyme Extraction and Assay

Samples for the assay of SOD (Superoxide Dismutase), APX (Ascorbate Peroxidase), and CAT (Catalase) contents were collected from the newly proliferated leaves at the end of 3 months. Fresh leaves tissue (500 mg) was homogenized in 5 mL 10 mM potassium phosphate buffer (pH:7.0) containing 4% (w/v) PVP (polyvinylpyrrolidon). The homogenate was centrifuged at 12000 rpm for 30 minutes at 4^0C, and the resulting supernatant was used as an enzyme extract. SOD (Superoxide Dismutase) activity was assayed by monitoring the inhibition of

photochemical reduction of NBT (nitroblue tetrazolium chloride) at 560 nm as described by Agarwal and Pandey [21] in a reaction mixture containing 13 mM methionine, 75 mM NBT (nitroblue tetrazolium chloride), 0.1 mM EDTA (ethylenediamine tetraacetic acid), 50mM phosphate buffer (pH:7.8), 2 μM riboflavin, and 0,02 cm^3 of enzyme extract. CAT (Catalase) activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH:7.5) containing 20 mM H_2O_2. One unit of CAT (Catalase) activity was defined as the amount of enzyme that was used 1 μmol H2O2 [22]. APX (Ascorbate Peroxidase) activity was measured according to Nakano and Asada [23]. The reaction mixture contained 50 mM potassium phosphate buffer (pH:7.0), 0.5 mM ascorbic acid, 0.1 Mm H_2O_2,and 0.1 mL of enzyme extract in a total volume of 1 mL. The concentration of oxidized ascorbate was calculated by 150 decreasing in absorbance at 290 nm.

Statistical Analysis

Each experiment was repeated three times. Analysis of variance was conducted using a one-way ANOVA test using SPSS 13.0 and means were compared following Duncan's multiple testing procedures test. The alpha cut-off value of 0.05 was considered throughout the statistical tests.

3. Results

Proline Assay

Proline level is increased in the leaves and callus of all the *Triticale* cultivars in both *in vitro* and *ex vitro* conditions after acclimatization. In fact, a dramatic increase in proline was observed in the acclimation period in all cultivars. Proline values indicated a narrow range of variation among cultivars for 7 days under *in vitro* conditions, ranging from 0,923 to 1,167 nmol g^{-1} FW (Fresh Weight), except the *Tatlıcak* cultivar (Figure 1). The highest proline value for 7 days, was found in *Tatlıcak* (1,370 nmol g^{-1} FW), which was followed by *Melez* 2001, *Mikham 2002*, *Alper Bey* and *Ümran Hanım*. All cultivars indicated a slight increase in proline concentration on the 14^{th} day compared to *ex vitro* (Figure 1). The lowest amount of proline was found in *Alper Bey*, but the highest amount of proline was noted in *Alper Bey* for 14-day assay. The highest amount of proline for 21-day assay was found in *Alper Bey* and *Tatlıcak*, and the lowest in *Mikham 2002* and *Melez 2001 in vitro* and *ex vitro* (Figure 1).

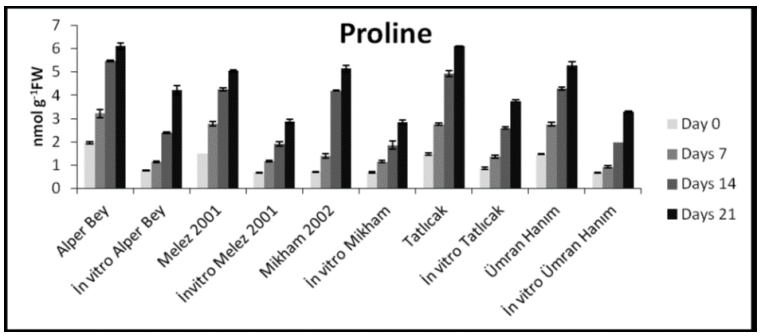

FIGURE 1. Changes of proline activity in five *Triticale* cultivars treated cold acclimation.

Sugar Content

The sugar content in 5 the cultivars of *Triticale* (*Ümran Hanım*, *Alper Bey*, *Mikham 2002*, *Melez 2001*, and *Tatlıcak*) were studied under low-temperature conditions. Figure 2 shows a gradual increase in sugar content in the leaves and callus of 5 the cultivars with increasing cold treatment. The callus and leaves of *Tatlıcak* showed 0.75 (leaves) to 1.11 (callus) nmol g^{-1} FW increase on the 7th day at *in vitro* conditions. Similarly, Tatlıcak showed 0.91 (leaves) to 1.29 (calli) nmol g-1 FW increase at the end of the 14th day under low-temperature conditions. Moreover, *Tatlıcak* slightly increased at the end of the 21st day by exhibiting 1.31 (leaves) to 1.39 (callus) nmol g^{-1} FW under the low-temperature conditions.

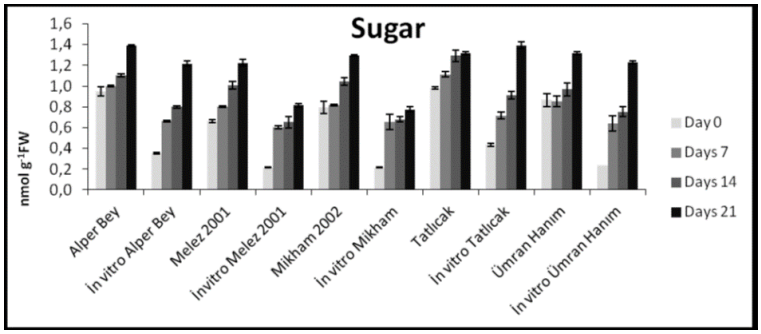

FIGURE 2. Changes of sugar content in five *Triticale* cultivars treated cold acclimation.

Antioxidant Enzyme Activity

Triticale leaves and callus were exposed to the cold stress, significantly affecting antioxidant enzyme activities after the 7th days of cold acclimation

(Fig. 3, 4-5). SOD (Superoxide Dismutase) activity was significantly higher in all tested cultivar leaves compared to cold-stressed callus. Among all the tested cultivars, *Tatlıcak* and *Alper Bey* indicated a higher SOD (Superoxide Dismutase) activity, whereas *Melez 2001*, *Mikham 2002*,and *Ümran Hanım* had a similar trend under *in-vitro* and *ex-vitro* conditions (Fig. 3).

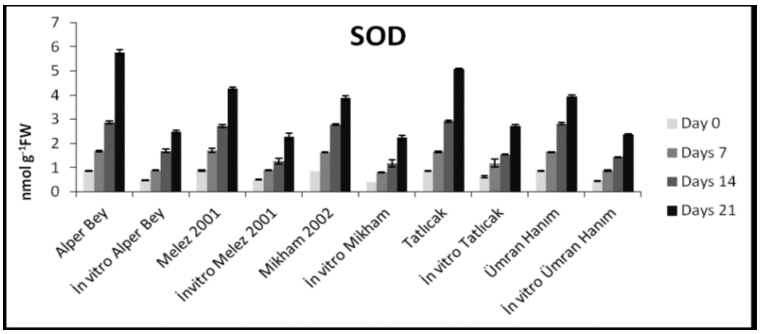

FIGURE 3. Changes of SOD (Superoxide Dismutase) in five *Triticale* cultivars treated cold acclimation.

Moreover, we observed that *Melez 2001* had higher activity at the end of the 7th day. As shown in Fig. 4, APX (Ascorbate Peroxidase) activity of five *Triticale* cultivars was obvious on the 6 activity days.

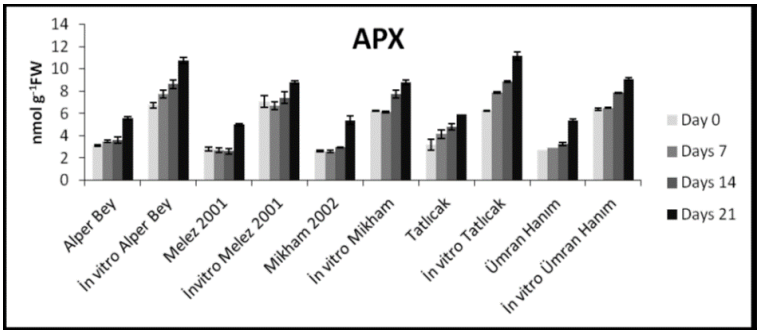

FIGURE 4. Changes of APX (Ascorbate Peroxidase) in five *Triticale* cultivars treated cold acclimation.

There was a detectable APX (Ascorbate Peroxidase) level difference between *ex vitro* plant and stressed callus. The APX (Ascorbate Peroxidase) activity in all tested cultivars was significantly increased by cold stress. Increase *ex vitro* tested plants

were higher than in the other cultivars. CAT (Catalase) activity also increased considerably under low-temperature conditions. The activity of CAT (Catalase) reduced progressively and significantly at the end of the 14th and the 21st day acclimation. Nevertheless, the activity increased significantly in callus stressed at the end of the 7th day (Fig. 5). The highest CAT (Catalase) activity was observed in the cold-acclimated leaves of the *Tatlıcak*, whereas the lowest level of activity at CAT (Catalase) was in *Mikham 2002 in vitro* callus (Fig. 5).

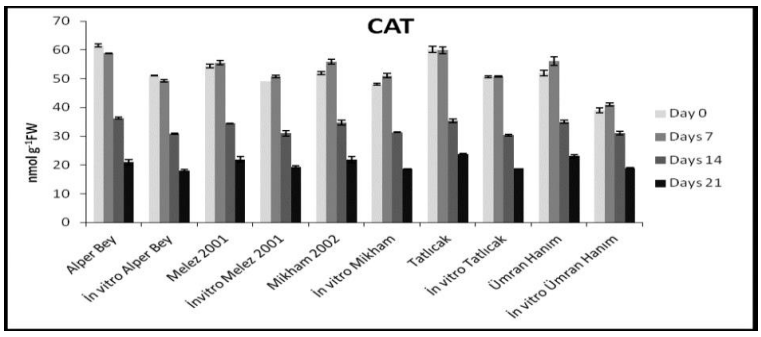

FIGURE 5. Changes of CAT (Catalase) in five *Triticale* cultivars treated cold acclimation

4. DISCUSSION

Earlier studies focusing on cold stress have indicated that the extent of cold stress adaptation in the whole plant is also reflected in callus tissue [11]. Extensive research has been conducted on understanding the physiological mechanisms underlining the cold stress in plants [24]. However, there have been a few studies on cellular level studies from cereal plants regarding cold acclimation at the tissue culture [25, 26]. The exposure of plants to cold stress induces many changes at the physiological and molecular levels [27, 28]. Pinpointing the cold resistance mechanisms under field conditions may not be straight forward due to several of other environmental factors. Cell and tissue culture have been useful for studying cold resistance mechanisms as they allow detection of relatively rapid responses, short generation time, and the use of controlled environmental conditions. Therefore, studies focusing on cold stress are preferably applied to in vitro cultures with the environmental control room set to low temperatures [29] Tissue culture is an important strategy that allows a controlled and uniform environment for studying physiological and molecular mechanisms in plants at the cellular differentiation under cold stress conditions [30]. In this study, physiological responses of *Triticale* under cold stress were studied. The results of the present study indicated that *Triticale* callus tissues respond quite differently to cold stress in comparison to the whole plant during acclimation. In this study,

through an analysis of the changes in biochemical properties in cultivated *Triticale* callus and whole plants, we found regular changes in proline, sugar content, and antioxidant enzyme activities at cold acclimation in the evaluation of cold tolerance of *Triticale* cultivars. These results suggested that the proline, sugar and antioxidant enzyme activities were important influential factors. The proline content in triticale increased in cold applications (7, 14 and 21days) (Figure 1). It has been reported that the proline levels in pea increases under cold acclimation [31]. In previous studies, it was determined that the proline content in wheat leaves increased significantly under cold stress. Proline levels have proven to be the main factors of cold resistance. The proline content in the *Triticale* plant and callus increased in cold applications. However the all *Triticale* cultivars exhibited different proline content of resistance to cold (Figure 1). The findings in our study are in coherence with the previous reports. Vera-Hernandez et al. [32] notified that cold stress increased the proline content in the amaranth. Esim and Atici [33] reported that cold stress remarkably improved proline content in wheat. The degree of cold resistance appeared to be directly related to the proline content of the callus and seedling confirm that tolerance is induced by cold levels. The results suggested that the cold-induced *proline content* might be an adaptive property for the survival and stability of the growth rate of *Triticale* seedlings under cold stress conditions. According to the carbonhydrate analysis, our study also has demonstrated that *in vitro* and *ex-vitro* were strongly improved with cold resistance. *Ex vitro* analysis showed similar changing trends that were gradually increased in planted *Triticale* cultivars, but callus was significantly increased and then recovered after being exposed to cold (Fig. 2). The results indicated that after 21st day of cold treatments at 4^{0}C, sugar content in *in vitro* of the *Tatlıcak, Ümran Hanım* and *Alper Bey* cultivars callus underwent drastic changes to acclimatize to the cold stress condition. Therefore, those cultivars have showed highly resistance in response to cold stress. This result indicated that the increases in sugar content were potentially an important factor in the cold stress of plant species. Carbohydrates have shown osmoprotectant properties as they prevent dehydration of cytoplasm and help to interact with lipid bilayer [34]. The interaction with lipid bilayer is key to providing resistance to cold, and this formation is closely related to sugar content in the cell membrane. The results shown in Fig 2 indicate that the *in vitro* acclimatization 21 days had the highest sugar content when compared to the *ex-vitro* treatments. This reveals the richness in tissue culture and the content of sugar, and thus it potentially improves cold stress response. This is consistent with a previous study in which Upadhyaya et al. [11] reported remarkably high sugar content in callus derived from rice plants exposed to cold stress comparing to acclimatized seedlings. The simultaneous activity of multiple antioxidant enzymes plays an important role in the conservation of the plant cell against stress factors. All cultivars tested in the present paper showed changes in antioxidant enzyme activities

during cold acclimation. This noticeable increase in SOD (Superoxide Dismutase) and APX (Ascorbate Peroxidase) was related to cold tolerance rather than with resistance against low temperature. This was in turn connected mainly with the increased ability to spread vital function during cold acclimatization. Changes in SOD (superoxide dismutase), APX (Ascorbate Peroxidase) and CAT (Catalase) levels of cold-induced activities during acclimation were more variable in *ex vitro* tested plants than callus. SOD (Superoxide Dismutase) is generally known as the first step of defense against oxidative stress [35]. We observed that SOD (Superoxide Dismutase) responses of *Triticale* seedlings exposed from cold stress were three times higher than from *Triticale* callus exposed from cold stress. Our results have confirmed that the plants under stressful conditions undergo oxidative stress (Fig. 3). These findings have been consistent with studies, suggesting SOD (Superoxide Dismutase) activity increases in *Passiflora alata*, and wheat and pea plants under cold stress [31, 36, 37]. APX (Ascorbate Peroxidase) activity demonstrated the same trends as SOD (Superoxide Dismutase) activity. Samples from leaves of *ex vitro* plants showed roughly half of the APX (Ascorbate Peroxidase) observed in *in vitro* callus. Callus also indicated lower APX activity when compared to the leaves of triticale. APX (Ascorbate Peroxidase) level was gradually increased in *Triticale* cultivars grown *in vitro* and *ex-vitro* at days 7, 14 and 21, due to the activity of APX (Ascorbate Peroxidase) required to protect against the cold stress. The observed differences in APX (Ascorbate Peroxidase) activity among cultivars included in this research might be the results of their genetic properties in terms of cold-resistance. Similar results were noted in *Brassica napus* under cold stress [38]. Joudmand [39] reported that under low-temperature conditions, the application of silicon can effectively mitigate the negative effects of cold stress on barley plants. The results obtained in their study demonstrated that the activity of antioxidative enzymes and concentrations of soluble carbohydrates and proteins in the leaf apoplasm were increased upon cold acclimation and particularly on Si treatment. However, further and more detailed studies are needed, particularly on the involved mechanisms. CAT (Catalase) enzyme requires no supply of reducing equivalents [40] that provide an important advantage especially in the stress conditions where photosynthesis rate reduces and the plant energy reservation. Thus, there is a strong correlation between CAT (Catalase) activity and stress tolerance in plants. Our results confirmed that cultivar *Tatlıcak* as a cold resistant cultivar displayed a higher level of this corporation under cold treatment. Our results are inconsistent with the results reported in wheat where the spring type displayed lower CAT activity than the winter type under cold acclimation [41] and soybean plants under cold treatment [42]. High activity of CAT (Catalase) displayed cold resistance in some of the wheat cultivars [43]. Based on our observations, antioxidant enzyme mechanisms after cold-acclimation seems to be linked to the re-establishment of the cold resistance.

In conclusion, the findings reported in the present study clearly show that cold exposure can improve cold resistance in triticale, which might be attributed partly to the elevation of the sugar and proline levels as well as to the activities of antioxidant enzymes. The obtained results clarify that the antioxidant enzyme level was higher in the *ex vitro* plants, a fact that might result from the environmental factors that are not present for the *in vitro* growing conditions.

Acknowledgement. This study was approved by Erzurum Technical University Review Board (Project no: 263 BAP 2015/10), and supported by Erzurum Technical University Research Fund.

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