EFFECT OF COLD STRESS ON ANTIOXIDANT MECHANISM OF WINTER AND SPRING TYPE BARLEY (*Hordeum vulgare* L.) CULTIVARS

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"EFFECT OF COLD STRESS ON ANTIOXIDANT MECHANISM OF WINTER AND SPRING TYPE BARLEY *(Hordeum vulgare L.)* CULTIVARS"

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ABSTRACT

EFFECT OF COLD STRESS ON ANTIOXIDANT MECHANISM OF WINTER AND SPRING TYPE BARLEY (*Hordeum vulgare* L.) CULTIVARS

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In this study, effect of cold stress on physiology and biochemistry of two Turkish barley cultivars, winter type Tarm-92 and spring type Zafer-160, was studied. For chilling stress treatment, cultivars were exposed to +4 ºC for 1, 3 and 7 days, and for freezing stress application acclimated cultivars (+4 ºC for 3 days) were treated with -3 ºC and -7 ºC. After freezing stress treatment, a recovery period was applied for 4 days at 4 ºC.

Following analyses were performed on leaf and root tissues: growth parameters (length, wet-dry weights), malondialdehyde (MDA) content, proline content, hydrogen peroxide content (H_2O_2) electrolyte leakage, PS II fluorescence (Fv/Fm), antioxidant enzyme activities such as catalase (CAT: EC 1.11.1.6), ascorbate peroxidase (APX: EC 1.11.1.11) and glutathione reductase (GR: EC 1.6.4.2).

It was observed that effect of cold was more at freezing temperatures than chilling temperature. Cold dependent damage was more obvious as the duration of chilling temperature increased. Growth retardation, membrane damage, leaf catalase

deactivation were more apparent and leaf glutathione reductase activity increase was less in spring type cultivar Zafer than in winter type Tarm. These results indicated that winter type barley cultivar is more cold tolerant than spring type barley.

Key words: Barley, cold stress, membrane damage, antioxidant enzyme activities

ÖZ

SOĞUK STRESİNİN KIŞLIK VE YAZLIK ARPA (*Hordeum vulgare* L.) ÇEŞİTLERİNİN ANTİOKSİDAN MEKANİZMASINA ETKİSİ

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Bu çalışmada soğuk stresinin kışlık (Tarm-92) ve yazlık (Zafer-160) Türk arpa çeşitlerinin fizyoloji ve biyokimyasına etkisi çalışılmıştır. Üşüme stresi için, bitkiler 1,3, ve 7 gün boyunca +4 ºC' ye maruz bırakılmış; donma stresi için ise soğuk uyumlandırılmış (3 gün +4 ºC) bitkiler -3 ºC ve -7 ºC ile muamele edilmiştir. Donma stresinden sonra +4 ºC' de 4 günlük iyileşme periyodu uygulanmıştır.

Yaprak ve kök dokularında; büyüme parametreleri (uzunluk, yaş-kuru ağırlık), malondialdehit (MDA) miktarı, prolin miktarı, hidrojen peroksit miktarı (H_2O_2) elektrolit geçirgenliği, PS II florasans (Fv/Fm), katalaz (CAT: EC 1.11.1.6), askorbat peroksidaz (APX: EC 1.11.1.11) and glutatyon redüktaz (GR: EC 1.6.4.2) antioksidan enzim aktiviteleri ölçülmüştür.

Soğuk stresi uygulamalarının arpayı üşüme sıcaklıklarına göre donma sıcaklıklarında daha fazla etkilediği gözlenmiştir. Aynı zamanda strese maruz kalma süresi arttıkça üşüme hasarı artmıştır. Zafer çeşidinde büyümenin engellenmesi, hücre zarı hasarı, yaprak katalaz deaktivasyonu Tarm' a göre daha

fazlayken, yaprak glutatyon redüktaz aktivitesindeki artış Tarm' a göre daha azdır. Bu sonuçlar kışlık Tarm bitkisinin yazlık Zafer bitkisine göre soğuğa karşı daha dirençli olmasından kaynaklanıyor olabilir.

Anahtar kelimeler: Arpa, soğuk stresi, hücre zarı hasarı, antioksidan enzim aktiviteleri

To the people I love

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
CAT	Catalase
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
O_2^1	Singlet oxygen
$O_2^{\cdot -}$	Superoxide radical
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
$OH^{\cdot}$	Hydroxyl radical
ROOH	Lipid hydroperoxide
ROS	Reactive oxygen species
SEM	Standard error of mean
SOD	Superoxide dismutase

CHAPTER 1

INTRODUCTION

1.1 Barley

Barley is one of the first domesticated cereals. Archaeological research show that it has been most likely originated in the Fertile Crescent area of the Middle East consisting of Turkey, Iran, Iraq, and Lebanon. There is considerable evidence that the initial cultivation has been found dating back to 8000 B.C. (Badr *et al.,* 2000).

Barley (*Hordeum vulgare* L.) is the world's forth most important cereal after wheat, maize and rice. Barley is more adaptable than other cereals, tolerating many diverse environments. In the arid and semi-arid areas, it is the major winter crop, since it is more tolerant to dryness, poor soils and salinity than wheat, and usually gives a higher grain yield (ICARDA, 2002). Barley, despite this proven tolerance, is however may be susceptible to extreme environmental sresses such as drought and cold (Stanca *et al.*, 1996).

Russia is the leader of top barley producers in the world. France, Canada, and Germany are the followers in the list. Turkey (9 million metric tons) and Ukraine also produce significant amounts (FAOSTAT, 2005) (Figure 1.1).

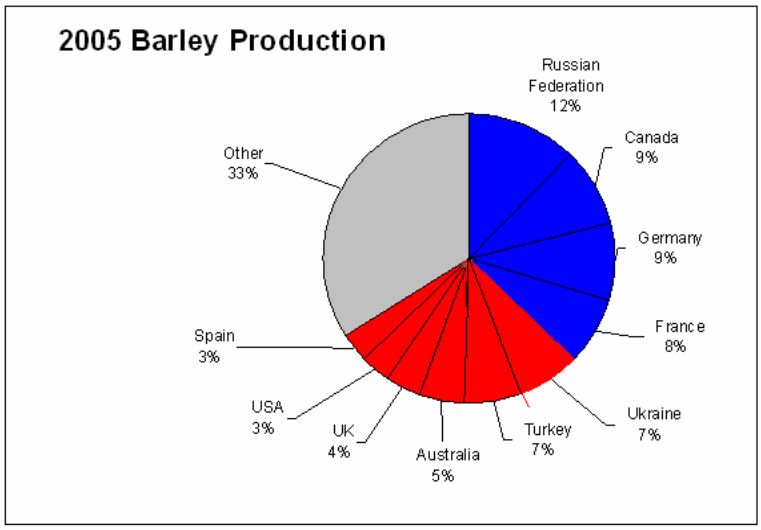


Figure 1.1. Barley Production

The grain is widely used for animal feed. The second important use of barley is for malting. Malt is mostly used to produce beer. Barley is also present in breakfast cereals, infant foods, and pharmaceuticals. It has several health advantages like regulating blood sugar levels in diabetics, lovering cholesterol and reducing the heart disease risk (Small, 1999).

Hordeum vulgare L. belongs to the phylum *Magnoliophyta* (angiosperms), class *Liliopsida* (monocotyledons), subclass *Commelinidae*, order *Poales*, and familiy *Poaceae* (*Graminae*-grasses).

Cultivated barley, *Hordeum vulgare* is descended from wild barley, *Hordeum spontaneum*. Both are diploid species with 2n=14 chromosomes (Komatsuda *et al.,* 1999). They are morphologically similar, wild barley has a brittle rachis (main axis) and occurs only in the two-row form. Cultivated barley has a nonbrittle rachis and six-rowed (Komatsuda *et al.*, 2007).

1.2 Environmental Stresses

Environmental stresses such as high light, drought, salinity, heavy metals, UV radiation, atmospheric pollutants, mechanical and physical stress, high temperature, and low temperature can adversely affect the plant growth since these stress factors lead to the increase in the amount of ROS in plant cells.

1.2.1 Generation of ROS

Free radicals are generated when a covalent bond between entities is broken and one electron remains with each newly formed atom. The presence of unpaired electrons makes these molecules very reactive. Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). When a ROS steal an electron from a surrounding compound or molecule a new free radical is generated in its place and in turn it steals electrons from cellular structures or molecules. Any circumstance in which cellular redox homeostasis is disrupted can lead to generation of ROS within the organism (Edreva, 2005).

1.2.1 1 Types of ROS

Reduction of atmospheric oxygen (O_2) generates ROS. They typically result from the excitation of O_2 to form singlet oxygen (O_2^1) or from the transfer of one, two or three electrons to O_2 to form, respectively, a superoxide radical (O^-_2), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH^-) (Fig.1.1). Unlike atmospheric oxygen, ROS are capable of unrestricted oxidation of various cellular components and can lead to the oxidative destruction of the cell through chain reaction (Figure 1.2).

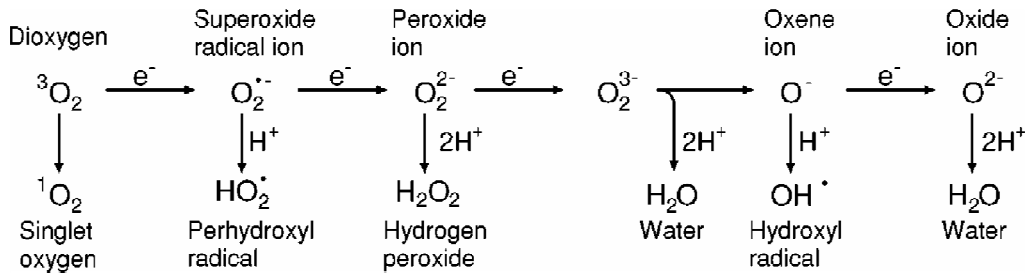


Figure 1.2. Oxidation level of reactive oxygen species.

Different kinds of ROS are produced in both stressed and unstressed cells, and in various locations (Scandalios, 2005).

Singlet oxygen

In electronic ground state all electrons are paired and there is no electronic spin. Most molecules have a singlet state as electronic ground state. However, molecular oxygen (O_2^3) which has triplet state as electronic ground state is an exception. It becomes an electronic singlet (O_2^1) in the electronically excited state. Reactions of singlet oxygen with organic molecules are not spin forbidden, and have much less activation energy and more reactive (Matysik *et al.,* 2002).

The protein-bound chlorophyll pigments associated with the electron transport system are the main source of singlet oxygen. For instance P680, the primary electron donor in reaction centers of photosystem II, acts as photosensitizer and is the primary source of singlet oxygen in plants (Figure 1.3). Under high light stress, the possibility of singlet oxygen production increases, since photons are absorbed faster than electrons pumped. Singlet oxygen being highly reactive destructs most biological molecules at near diffusion-controlled rates (Ledford and Niyogi, 2005).

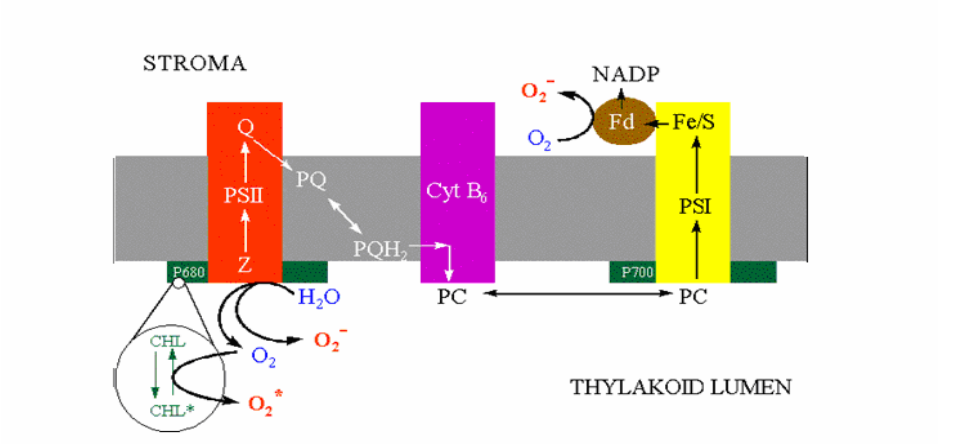


Figure 1.3 Production of superoxide radical and singlet oxygen in chloroplast at the site of PSI and PSII.

Superoxide

In chloroplasts, Ferredoxin on the reducing side of PSI is the primary superoxide radical (O^{-}_{2}) producer. It reduces molecular oxygen to the superoxide oxygen under conditions where NADP is limiting (Fig.1.3).
The three possible pathways for oxygen reduction and superoxide formation in plant mitochondria are as follows (Arora *et al.,*2002):

1. Oxygen consumption via cytochrome oxidase to produce water, a process which accounts for more than 95% oxygen consumption.
2. Direct reduction of oxygen to superoxide anions in the flavoprotein region of NADH dehydrogenase segment of the respiratory chain.
3. Oxygen reduction to superoxide anions in the ubiquinone cytochrome region of the respiratory chain.

Plant microsomes are another superoxide generation site. Cytochrome P_{450} dependent oxygenases in microsomes are involved in metabolism of both endogenous and exogenous compounds. Their action is dependent on oxygen and reducing equivalents provided by NAD(P)H or NADH. This electron transport chain may result in the formation of superoxide radical. A superoxide-generating NAD(P)H oxidase activity has also been identified on plasma membrane. Wounding, heat shock and xenobiotics transiently activate this superoxide generating activity on plasma membrane, and consequently, it has been proposed that these superoxide generating reactions may serve as a signal in plant cells to elicit responses to biological, physical or chemical stress (Simontacchi and Puntarulo, 1992).

Hydrogen peroxide

Superoxide is rapidly converted to hydrogen peroxide and oxygen. This reaction occurs spontaneously or is catalyzed by the enzyme superoxide dismutase (SOD). Large amounts of hydrogen peroxide (H_2O_2) are produced during photorespiration by glycolate oxidase in a two electron transfer from glycolate to oxygen and also produced in β-oxidation of fatty acids by oxidases in peroxisomes. In leaf peroxisomes, most of the H_2O_2 produced can be removed by catalase. Catalase

has not been found in chloroplasts. The hydrogen peroxide in chloroplasts and cytosol is scavenged by ascorbate peroxidase using the the electron donor ascorbate (Arora *et al*., 2002). Superoxide is a charged molecule and for that reason it can not readily travel across membranes. On the other hand, hydrogen peroxide is a relatively stable molecule that can travel freely across membranes and possibly signal stress (Ledford and Niyogi, 2005).

Hydroxyl radical

In fact, hydrogen peroxide and superoxide radical themselves are relatively less damaging, but they can result in the production of most reactive oxygen species hydroxyl radicals (Ledford and Niyogi, 2005). They can initiate lipid peroxidation and also attack DNA, proteins and many small molecules. Fenton, in the late nineteenth century described the oxidizing potential of hydrogen peroxide with ferrous salts. Forty years later, Haber and Weiss showed that in the presence of a trace amount of iron, the reaction of superoxide and hydrogen peroxide will form hydroxyl radical (Aroara *et al.,* 2002).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$ (Fenton reaction).

$H_2O_2 + O_{2.}^{-} \rightarrow OH^{-} + O_2 + OH^{\cdot}$ (Haber - Weiss reaction).

1.2.1.2 ROS Damage to Plant

The presence of various reactive oxygen species (ROS) such as singlet oxygen (O_2^1), superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), etc., generated can cause severe oxidative damage to biomolecules like lipids, proteins and DNA. The presence of high concentrations of these ROS can thus disrupt the normal physiological and cellular functions (Jungklang *et al.*, 2004).

ROS damage to membrane

The unsaturated double bonds of the biological membrane are the sites of ROS attack. The more double bonds in a fatty acid side chain, the easier lipid are peroxidized. That is why polyunsaturated fatty acids are readiliy peroxidized. Lipid

peroxidation in biological membranes distrupts membrane functioning, changes fluidity, inactivates membrane-bound receptors and enzymes, and increases nonspecific permeability to ions. ROS start peroxidation reaction by abstracting a single hydrogen atom from unsaturated fatty acids (Halliwell and Chirico, 1993). The hydrogen abstraction mechanism of ROS is best shown by lipid peroxidation of linolenic acid in cell membranes. ROS abstracts an H atom from carbon-11 of the fatty acid between the two double bonds of linolenic acid. The electron deficiency is shared among carbons 9 to 13 in a resonance structure. Triplet oxygen that has two unpaired electrons may attach to this structure at either carbon -9 or -13 forming a peroxy radical. This peroxy radical will abstract another hydrogen atom from a second linoleic acid molecule in a propagation reaction forming a lipid hydroperoxide (Frankel, 1984) (Figure.1.4).

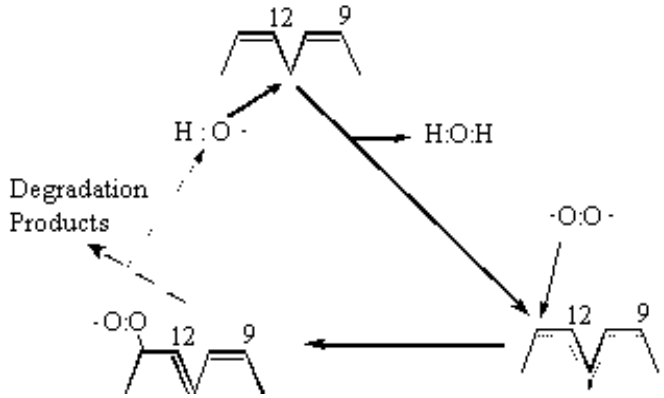


Figure 1 4 The peroxidation of linoleic acid.

A lipid hydroperoxide (ROOH) is unstable in the presence of metal catalysts such as Fe and Cu since they will participate in a Fenton reaction which will result in the formation of reactive alkoxy radicals. Among the degradation products of ROOH are aldehydes, such as malondialdehyde (MDA), and hydrocarbons, such as ethane and pentane which are commonly measured end products of lipid peroxidation. Measuring the end products of lipid peroxidation such as malondialdehyde (MDA) is one of the most widely accepted assays for oxidative damage and has been extensively used. The TBA test for MDA measurement is

cheap and simple. Sample whose MDA content is being evaluated is heated with TBA at low pH, and a pink chromogen is measured by its absorbance at 532nm (Halliwell and Chirico, 1993).

ROS damage to proteins

Protein oxidation can occur by ROS either through direct covalent modification (Møller and Kristensen, 2004) or indirectly, involving end products of lipid peroxidation (Halliwell and Chirico, 1993). ROS attack to the polypeptide backbone, abstract a hydrogen atom and form a carbon centered radical. Although all amino acids can be oxidized, some are more susceptible. Cysteine can be oxidized to cystine, and both proline and arginine are converted to glutamyl semialdehyde. Such modifications can affect the function of proteins. The damaged protein may be degraded by some proteases (Møller and Kristensen 2004).

The oxidative degradation of protein is enhanced in the presence of metal cofactors such as Fe. In these cases, the metal binds to a divalent cation binding site on the protein. The metal then reacts with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical which is extremely reactive and rapidly oxidizes an amino acid residue at or near the cation binding site of the protein (Berlett and Stadtman, 1997).

ROS damage to DNA

DNA is an important target for reactive oxygen species. Both the sugar and the base moieties on DNA are susceptible to oxidation which results in base degradation, single strand breakage, and cross-linking to protein. Oxidation may cause deletions, mutations and other lethal genetic effects on DNA (Henle and Linn, 1997).

Free superoxide is relatively unreactive with DNA. However, superoxide can dismutate to ROS such as hydroxyl radical and hydrogen peroxide via spontaneous or enzymatic reactions. In the presence of a trace amount of iron, the reaction of superoxide and hydrogen peroxide can also form hydroxyl radical (Keyer K. And Imlay J. A., 1996). The principle cause of single strand breaks is

oxidation of the sugar moiety by the hydroxyl radical. *In vitro* neither hydrogen peroxide alone nor superoxide cause strand breaks under physiological conditions, and therefore, their damage *in vivo* is most likely due to Fenton reactions with a metal catalyst. Active metal is bound to DNA, probably chelated to phosphodiester linkage. If the bound metal is reduced by a small diffusible molecule, such as NAD(P)H or superoxide, it will react with hydrogen peroxide and yield the hydroxyl radical. The short-lived, reactive hydroxyl radical then oxidizes an adjacent sugar or base causing breakage of the DNA chain. Cross-linking of DNA to protein is another consequence of free radical attack on either DNA or its associated proteins. In order to remove deleterious effects of DNA oxidation, the cell has a number of DNA repair enzymes (Henle and Linn, 1997).

1.2.2 High-light exposure

Although light is a requirement for photosynthesis, high light conditions, often combined with other stresses can cause a decrease in photosynthetic activity. The decrease in photosynthetic activity which is observed upon excess light is called photoinhibition (Yordanov and Velikova, 2000). Under excess light, enzymatic processes for CO_2 fixation become rate limiting and, photosynthesis yields more NADPH and ATP than needed. As a result, redox and energy equivalents accumulate and reduce the plastoquinone pool and/or inhibit the watersplitting complex, inevitably leading to PSII inactivation. An increase in the rate of O_2 photoreduction with high irradiance has been reported for *Vinca major*, *Schefflera arboricola*, and *Mahonia repens*. Exposure of *Arabidopsis* seedlings to excess light (>2000 µmol photons $m^{-2}s^{-1}$) for 1 h resulted in photoinhibition, and accompanied by H_2O_2. When exposed to high light (>300 µmol photons $m^{-2}s^{-1}$), leaves of catalase-deficient (Cat1AS) tobacco plants become bleached as a result of H_2O_2 accumulation in the peroxisomes (Dat *et al.*,2000).

1.2.3 Drought

During drought, water becomes limited. In order to prevent further water loss, the plant experiences stomatal closure and limit the carbon dioxide available for fixation by photosynthesis. As a result, quantum efficiency decreases and an excess of excitation energy at the reaction centers of the photosynthetic machinery

occurs. In this situation NADP becomes limited and ferredoxin selectively reduces oxygen instead, so $O_2^{\cdot-}$ radical is produced owing to the electron transport by photosystem I to O_2 (Reddy *et al.,*2004). Alteration in the oxidative balance leads to lipid peroxidation, which is an indicator of oxidative stress. Pea chloroplastic Cu/ZnSOD and cytosolic APX activities rise during drought (Dat *et al.,*2000). Gr activity increased in drought stress applied wheat and cotton plants. Also according to various studies, drought tolerant genotypes of *Hordeum* species showed an increase in Gr and APX (Arora *et al.*, 2002).

1.2.4 Salt stress

Salt stress decreases water availability and causes osmotic stress. As a result, oxidative stress takes place in salt damage (Parida and Das, 2004). The generation of ROS during salt stress is probably similar to that during drought and is mainly attributed to increased leakage of electrons to O_2, ensuing from a decline in CO_2 fixation. Upon exposure to salt stress, ROS are produced and cause lipid peroxidation in citrus plant. Concentrations of H_2O_2 in shoot tissue of rice significantly increase upon salt stress. Tobacco plants deficient in catalase (Cat1AS) were also more susceptible to salt stress. Mitochondrial and chloroplastic SOD and APX isozymes increase during salt stress in pea (Dat *et al.*,2000).

1.2.5 Heavy metals

When heavy metals such as, copper, aluminum, cadmium, zinc, and iron concentration excessively rises, metal toxicity occurs, cellular homeostasis is distrupted and ROS production is enhanced. Generally, heavy metal related oxidative stress results from direct transfer of electrons (Demirevska-Kepova *et al.,* 2004). The differential activation of SODs in tobacco and soybean after Cu treatment shows ROS accumulation during Cu stress. Zinc application of *Phaseolus vulgaris* rises the levels of H_2O_2 in roots and lipid peroxidation in primary leaves. Iron toxicity occurs due to its potential for reacting with H_2O_2 and $O_2^{\cdot-}$ to generate the more toxic radical $OH^{\cdot}$. Increased activity of catalase and APX in tobacco seedlings after iron excess supports this model (Dat *et al.*, 2000).

1.2.6 UV radiation

Excitation of electrons by electronic transition in appropriate photosensitizers, UV radiation causes production of $O_2^{\cdot-}$. SOD can then dismutate the produced $O_2^{\cdot-}$ to H_2O_2. Accordingly, H_2O_2 accumulation significantly rises 60–90 min after UV-C irradiation of *Rosa damascena* cell suspensions. Exposure of *N. plumbaginifolia* to UV-B strongly rises Cat2. However, APX and SOD transcript levels remain relatively same. Similarly, SOD transcript levels of *Pisum sativum* decrease after UV-B application, whereas those of GR are several–fold higher (Dat *et al*., 2000).

1.2.7 Atmospheric pollutants

Atmospheric pollutants such as ozone (O_3) and sulphur dioxide (SO_2) are involved in free-radical formation (Arora *et. Al.*, 2002). The phyto-toxicity of O_3 is due to its oxidizing potential and the consequent formation of reactive oxygen species (Wu and Tiedemann*.,* 2002). Upon ozone treatment, Bluegrass and ryegrass accumulated, $O_2^{\cdot-}$ and H_2O_2 respectively. O_3 was shown to cause an oxidative burst by activating an NADPH-dependent oxidase in *Arabidopsis*. After low-level exposure to ozone, expression of the cytosolic *APX*1 gene of *Arabidopsis* increases. Similarly, cytosolic APX, Cu:ZnSOD, and GST mRNA increase with the onset of visible injury in tobacco, *Arabidopsis*, and bean (Dat *et al.,*2000). Exposure to SO_2 may also trigger the ROS formation in plant tissues (Arora *et al.*, 2002). In tobacco plants SO_2 exposure, increased the Cat2 and GPX transcript levels, whereas other antioxidants are either repressed or unaltered by the treatment. In *Arabidopsis*, APX and POD activities rised, whereas in *Cassia siamea*, SOD and POD activities increase (Dat *et al.*, 2000).

1.2.8 Mechanical and physical stress

Physical and mechanical wounding responses involves ROS accumulation. The source of ROS during such conditions is believed to be NAD(P)H dependent, because wound-induced ROS accumulation in various species is inhibited by diphenyleneiodonium, an NADPH-oxidase inhibitor. Wounding stimulates H_2O_2 generation systemically in tomato leaves (Orozco and Ryan, 1999). ROS accumulate after slicing tissues of various species (Schopfer, 1994), mechanical

wounding of mesocarp tissue of winter squash (Watanabe and Sakai, 1998) or potato tissues (Doke *et al.,*1994; Doke,1997], and mechanical stress in red macroalgae (Colle´n and Pederse´n, 1994).

1.2.9 High temperature

High temperature distrupts normal physiological processes and result in generation of ROS. (Almeselmani *et al.*, 2006). The major site of ROS production at high temperature is probably the chloroplast via leakage of electrons following a decline in CO_2 fixation. Alternatively, an NAD(P)H dependent oxidase might be activated upon heat treatment. Potato leaf tissues generate an oxidative burst within 15 min after heat shock. Exposure of whole tobacco seedlings to 40 °C for 1 h in the light results in a significant increase in H_2O_2. Similarly after a heat treatment, H_2O_2 accumulation is observed in mustard seedlings (Dat *et al.,* 2000). Under heat stress, catalase levels decreased in many plant species (Almeselmani *et al.*, 2006). Heat protection of wheat is enhanced by keeping APX and GR activities high, and GR activity is significantly increased during heat-induced thermoprotection in mustard seedlings. Moreover, exposure of alfalfa to supra-optimal temperature rises APX and catalase activities (Dat *et al.,* 2000).

1.2.10. Cold stress

Cold stress is a major environmental constraint which limits growth, development, crop productivity of plants (Xin and Browse, 2000). Each year about $100 million is expended to minimize cold injury to crops and annual losses of $10-100 million or higher from cold injury (Pearse, 1999). Cold stress like other environmental stresses results in enhancement in ROS formation and change in morphological, biochemical and physiological characters (Dat *et al.*, 2000) (discussed in section 1.4).

1.3. Antioxidant Defense Systems

Because of the deleterious effects of ROS, plants have developed various protective mechanisms involving antioxidative enzymes and antioxidant molecules to protect the cell. Increase in activities and concentrations of these enzymes and molecules under oxidative stress confer resistance to plants (Yan *et al.,* 2003).

1.3.1. Non-enzymatic System

The non-enzymatic defense system scavengers include glutathione, ascorbate, carotenoids and vitamin E (α-tocopherol). These molecules are the major cellular redox buffers and they non-enzymatically help cells to keep ROS concentrations under control (Apel and Hirt, 2004).

1.3.1.1 Glutathione

In most plants, the tripeptide glutathione (γ-Glu- Cys-Gly, GSH) is the major low molecular weight thiol compound. However in some legumes, the homologous peptide homoglutathione (γ-Glu-Cys-Ala, hGSH) may partly or wholly replace glutathione. GSH is found in most cellular compartments: chloroplast, cytosol, endoplasmic reticulum, vacuole and mitochondria. It functions as antioxidant under oxidative stress. The high reductive potential of GSH originates from sulfhydryl group of cysteine, which forms a disulfide bond with a second molecule of GSH to form oxidized glutathione (GSSG) upon oxidation. The enzyme glutathione reductase (GR) turns the oxidized glutathione (GSSG) back to its reduced form (GSH). GSH can function as an antioxidant in several ways. It can directly react with free radicals or react with ascorbic acid, which is another antioxidant. GSH recycles ascorbic acid from its oxidized (Dehydroascorbate) to its reduced form (Ascorbate) by the enzyme dehydroascorbate reductase (Arora *et al.,*2002).

1.3.1.2 Ascorbate

L-Ascorbic acid (vitamin C) is an important vitamin in the human diet and is abundant in plant tissues such as chloroplasts, cytosol, vacuole and apoplastic space. Ascorbate is synthesized from D-glucose. It has significant roles in several physiological processes in plants like cell division and elongation (Horemans *et al.,* 2000). It is also one of the most important antioxidant in plants. It can directly scavenge free radicals or reduce hydrogen peroxide (H_2O_2) to water via ascorbate peroxidase reaction. Due to the fact that chloroplasts lack catalase, which scavenges hydrogen peroxide in the peroxisomes, ascorbate has a central importance in eliminating the H_2O_2 from chloroplasts (Arora *et al.,* 2002). In addition to its role as a primary antioxidant, ascorbate has a significant secondary antioxidant function. The ascorbate pool represents a reservoir of antioxidant potential that is used to regenerate other membrane-bound antioxidants such as α-tocopherol and zeaxanthin in xanthophylls cycle. These scavenge lipid peroxide and singlet oxygen, respectively (Horemans *et al.,* 2000).

1.3.1.3 Carotenoids

In nature, the majority of the 600 carotenoids contain 40 C-atoms. They are located in the plastids of plant tissues. Carotenoids can be classified into carotenes (pure carbohydrates without additional groups) and the xanthophylls (carotenoids containing oxygen). Carotenoids possess antioxidant properties; they can protect the photosystems (Krinsky, 1998);

1. By reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen.
2. By scavenging singlet oxygen and dissipating the energy as heat.
3. By reacting with lipid peroxidation products and terminate chain reactions.

1.3.1.4 α-Tocopherol

In photosynthetic membranes α-Tocopherol with other antioxidants reduces ROS levels (mainly $^1O_2$ and OH^-) and limit the extent of lipid peroxidation by reducing the lipid peroxyl radicals to the corresponding hydroperoxides. α-Tocopherol can physically quench and deactivate $^1O_2$ in chloroplasts. In addition to this physical quenching property, α-tocopherol can chemically scavenge $^1O_2$ and lipid peroxyl radicals. The chemical scavenging of $^1O_2$ by α-tocopherol irreversibly results in the formation of their quinones and epoxides. On the other hand, the scavenging of lipid peroxyl radicals results in the formation of tocopheroxyl radicals, which can be recycled back to α-tocopherol by ascorbate. α-Tocopherol action is important for preservation of an adequate redox state in chloroplasts, and it maintains the thylakoid membrane structure and function during plant development, and in plant responses to stress (Munné-Bosch, 2005).

1.3.2. Enzymatic Systems

Antioxidant enzymes are the most efficient scavenging mechanism against oxidative stress. The main antioxidative enzymes are catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.1.1.11), glutathione reductase (GR; EC 1.6.4.2) superoxide dismutase (SOD; EC 1.15.1.1), and other ascorbate-glutathione cycle enzymes (monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1).

1.3.2.1 Catalase

Catalase (CAT) is a tetrameric heme-containing enzyme. It is found in all aerobic eukaryotes. Catalyse converts hydrogen peroxide into water and oxygen, thus, protecting the cell from the damaging effects of H_2O_2 accumulation.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

CAT is mostly localized in peroxisomes and is important in the removal of hydrogen peroxide which is mainly generated by oxidases involved in ß-oxidation of fatty acids and the glyoxylate cycle (photorespiration) in peroxisomes. CAT has a very

high Michaelis constant (K_m) and therefore, it is not easiliy saturated with its substrate H_2O_2 (Scandolios, 2005). Catalase is sensitive to light. It is photoinactivated in moderate light under conditions to which plants are adapted. Although its turnover is continuous, stress conditions like low temperature rapidly decrease its steady state level (Dat *et al.,* 2000).

1.3.2.2 Enzymes of Ascorabate-Glutathione Cycle

Removal of H_2O_2 is catalyzed by catalase, which is mostly localized in peroxisomes. However, catalase has a very low affinity for H_2O_2 and it is successful in the removal of bulk of H_2O_2. CAT activity is either extremely low or not detectable in the cytosol and chloroplast. In chloroplast and cytosol, plant cells possess a more efficient detoxification mechanism against H_2O_2, called the ascorbate-glutathione or Halliwell-Asada cycle (Figure 1.5) (Dat *et al.,* 2000). The cycle involves enzymes ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase (Edreva, 2005).

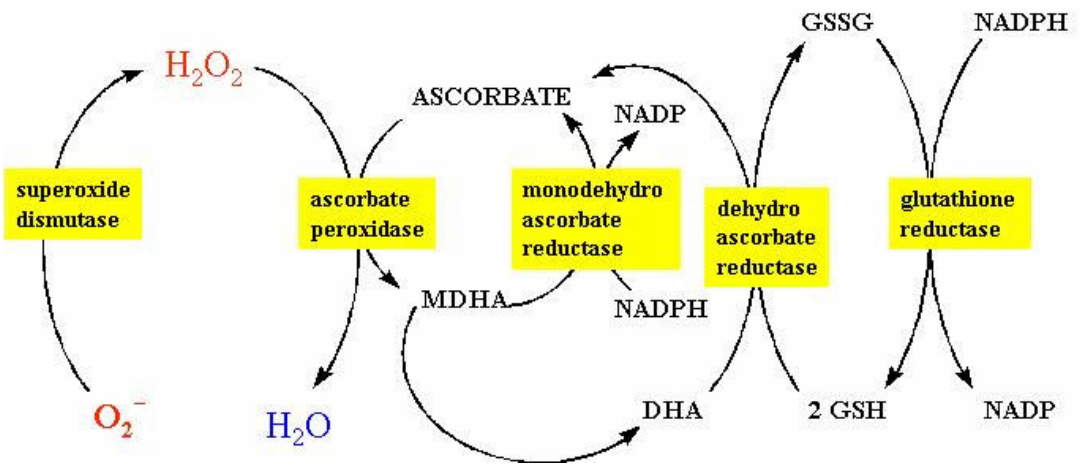


Figure 1.5. Asada–Halliwell pathway of hydrogen peroxide scavenging and ascorbic acid regeneration involving various antioxidant enzymes.

Ascorbate peroxidase (APX) activity has mainly been reported in chloroplast, cytosol and mitochondria (Sharma and Dubey, 2004). APX has high specificity and affinity for ascorbate as reductant. Both ascorbate peroxidase and catalase are heme containing enzymes. The initial step in the catalytic mechanism of both CAT and APX is heterolysis of the oxygen-oxygen bond of hydrogen peroxide. This causes the release of one water molecule and coordination of second oxygen atom to the iron center, forming an intermediate, compound I. In catalase reaction, a second peroxide molecule is used as, a reducing agent for compound I (Blokhina, 2001). In APX reaction reduction of the compound I occurs via the formation of another intermediate, compound II. Ascorbate is the reductant. (Jakopitsch, 2003).

In ascorbate-glutathione cycle, APX oxidizes ascorbic acid and generates monodehydroascorbate. Monodehydroascorbate is reduced to ascorbate by the action of NAD(P)H-dependent monodehydroascorbate reductase (MDHAR) or spontaneously disproportionates to dehydroascorbate. In order to prevent loss of ascorbate pool following oxidation, the chloroplast possess efficient mechanisms of recycling both monodehydroascorbate and dehydroascorbate, and these ensure that the ascorbate pool is maintained largely in reduced form. Glutathione (GSH) will enzymatically reduce dehydroascorbate back to ascorbate by enzyme dehydroascorbate reductase (DHAR). DHAR uses reduced glutathione (GSH) as an electron donor for the reduction of dehydroascorbate to ascorbate (Junklang *et al.*, 2004).

GSH is regenerated by glutathione reductase (GR) in a NADPH-dependent reaction. GR turns the oxidized glutathione (GSSG) back to its reduced form (GSH) (Israr *et al.,* 2005). It maintains a high ratio of GSH/GSSG, which is required for the regeneration of ascorbate. It is located in chloroplast, mitochondria and cytosol (Scandalios, 2006).

1.3.2.3 SOD

Superoxide dismutase (SOD) catalyzes the dismutation of two superoxide radicals into hydrogen peroxide and oxygen

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

SOD and hydrogen peroxide scavenging enzymes (APX, CAT) work together while removing superoxide radicals. Hydrogen peroxide produced as a result of SOD activity is removed by hydrogen peroxide removing enzymes. SOD is present in all aerobic organisms and most subcellular compartments that generate activated oxygen (Holmberg and Bülow, 1998).

There are three known types of SOD are present. These are classified according to their metal cofactor: The copper/zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD) forms. The mechanism of catalysis involves attraction of negatively charged superoxide radicals to the active site of the enzyme where positively charged amino acid residues are present. The transition metal at the active site then carries an electron to the superoxide radical and produces hydrogen peroxide (Alscher R. G. *et al.*, 2002).

1.4 Cold stress injury

There are two types of stresses a plant can suffer upon exposure to cold. The first type is called chilling stress that occurs temperatures from 15 to 0 ºC. The second is called freezing stress which occurs when the external temperature drops below the freezing point of water (Kocsy *et al.*, 2001). Cold stress injury is defined as damage occur due to physiological and biochemical alterations induced by low temperatures. A temperature which is optimum for one plant may be stressful for another plant. The duration of cold exposure which initiates injury varies from plant to plant and also depends upon the sensitivity of a plant to cold stress. Most tropical and subtropical plants are injured when exposed to chilling stresses below 10 ºC. On contrary, temperate plants have evolved some mechanisms by which

they can increase their ability to survive at freezing temperatures when exposed to a period of chilling. This process is called cold acclimation. This process can be studied in laboratory conditions by exposing plants to low temperatures (2-6 ºC) (Xin and Browse, 2000).

1.4.1. Symptoms of cold injury

Several phenotypic symptoms in response to cold include reduced growth, leaf expansion, wilting, chlorosis (yellowing of leaves) and it may lead to necrosis (death of tissue). Cold stress may also negatively effect the reproductive development of the plant and sometimes leads to sterility (Mahajan and Tuteja, 2005).

1.4.1.1. Ultrastructural symptoms

Ultrastructural studies show that cold-temperature related changes involve several cell components, and plastids are one of the most extensively investigated organelles. Plastids and thylakoid membranes swell and become disorganized, thylakoids and peripheral reticulum vesiculate, plastoglobuli or lipid droplets accumulate, and finally the whole plastid becomes disorganized leading to the disintegration of the envelope. Significant changes have also been investigated in other cell components. Mitochondria swell, the population of ribosomes decreases, endoplasmic reticulum (ER) dilates, cytoplasmic membranes vesiculate, the nuclear chromatin condenses forming clumps, plasmalemma invaginates and there is an increase in vacuolation and the number of membraneous vesicles (Lee *et al.*,2002).

1.4.1.2. Membrane symptoms

The plasma membrane which is composed of phospholipids and glycolipids is a primary target in cold stress. Low temperature affects the acyl tails of the phospholipid molecules in cell membranes and causes the lateral phase separation of these lipids from highly fluid, liquid phase to a more rigid gel phase. Due to the presence of a gel phase domain in the membrane bilayer, permeability and leakiness increase. (Parkin *et al*., 1988). Protoplasm viscosity also decreases as

temperature drops. The more sensitive a plant to cold, the higher is the temperature at which “gelation” occurs, and the faster is the rise in the cytoplasm viscosity. A significant increase in viscosity slows down biochemical reactions in the cytoplasm and disturbs metabolism, which leads to physiological disorders in nutrition, respiration, and growth (Lukatkin, 2005). In addition to phase separation ice formation occurs when temperature drops belo zero. Ice formation is the most important cause of freeze-induced injury. When ice starts to form in the apoplastic space, the unfrozen cytoplasmic water migrates from cells to the apoplast and cause severe dehydration. Growing ice crystals can also rupture cells. When subjected to cold, protein-protein and protein-lipid interactions are distrupted and proper functioning of integral membrane proteins is prevented. Loss of turgor, leakage of cytoplasmic solutes, lack of energy metabolism, disruption of the photosystems, cell autolysis and death occur (Parkin *et al*., 1988).

1.4.1.3 Photoinhibition

Photoinhibition which is characterized as a reduction of photosynthesis in the presence of light occur when plants are exposed to cold. Low temperature reduces metabolic reactions due to kinetic effects, especially those involved in CO_2 fixation, and those involved in regulating stomatal aperture. As a result, exposure of leaves to low temperature reduces the demand for chemical energy, and the ability of the stomata to regulate water loss and CO_2 exchange. In this situation, the photosynthetic apparatus captures photons in excess of the requirements for chemical energy and the electron transport through photosystem II is inhibited (Parkin *et al.*, 1988). After inactivation of PSII electron transport, the D1 protein, one of the heterodimeric polypeptides of the PSII reaction center complex is irreversibly damaged. Restoration of PSII electron transport activity after such photoinhibitory damage can only occur via degradation and *de novo* synthesis of the D1 protein and reactivation of the PSII complex. Additionally, the rapid reversible photoinhibition or “down-regulation” of PSII offers a photoprotection at high photon flux densities and dissipates of excess excitation energy. This reversible inhibition state relaxes without D1 protein synthesis (Aro *et al.*, 1994). The Fv/Fm ratio represents the maximum quantum yield of the primary photochemical reaction of PSII and is an important parameter of the physiological

state of the photosynthetic apparatus. Measuring the PSII fluorescence by Fv/Fm method is a nice tool to evaluate photoinhibition and degree of chilling injury (Kocheva *et al.*,2004).

1.4.1.4 Oxidative stress

When plants are exposed to low temperatures, membranes are damaged and metabolism is altered. ROS are produced due to the blocking or uncoupling of electron transporting systems in chloroplast and mitochondria (Lukatkin, 2005). ROS also may be generated via inhibition of CO_2 fixation (Dat *et al.*, 2000). Literature data show that exposure of callus tissue of *A*. *thaliana* to 4°C leads to conditions of oxidative stress. A correlation between H_2O_2 accumulation and chilling has been investigated in *A. thaliana* callus tissue, cucumber, winter wheat, rice, and maize (Dat *et al.*,2000). The development of cold injury symptoms is frequently coincident with lipid peroxidation. Lipid peroxidation would change the physical properties of membrane lipids and increase the permeability to ions and water. Those changes might finally result in the distruption of normal physiological processes in the cell (Lukatkin, 2005). Malondialdehyde (MDA) is one of the degradation products of lipids, and has been considered a marker for lipid peroxidation and cold injury (Morsy *et al.*, 2007). Another method for evaluation of degree of cold injury is electrical conductivity test which detertmines the increased rates of solute and electrolyte leakage in chilled tissues (Prášil and Zámečík, 1998).

1.4.2 Cold Tolerance

In order to avoid negative physiological and biochemical alterations which occur as a result of cold injury at low temperatures, plants have adapted several mechanisms like unsaturation of lipids, antioxidant mechanism, and accumulation of solutes.

1.4.2.1 Unsaturation of Lipids

In physiological membrane, the fatty acyl chains of phospholipids and glycolipids are important for chilling tolerance. The saturated fatty acids with double bond introduce bends in the fatty acyl chains and maintain membrane fluidity by preventing tight packing of adjacent lipid molecules (Cyril *et al.*, 2002). Cold -resistant plants contain more unsaturated fatty acids in membrane lipids than those cold-sensitive plants and prevent membrane transition from liquid crystalline to gel phase. For instance, phospholipids in alfalfa and wheat are more unsaturated when they are grown at low temperatures. Cold-resistant alfalfa varieties have also greater degree of unsaturation in phospholipids than cold-sensitive types (Dogras *et al*.,1977).

1.4.2.2 Antioxidant Mechanism

ROS accumulated as a result of chilling could be removed by the increased amount or activity of antioxidants and antioxidant enzymes. In most chilling sensitive plants, chilling treatment cause deactivation of antioxidant enzymes (Lukatkin, 2005). However, chilling tolerant plants show higher antioxidant activities than chilling sensitive plants. For instance, chilling tolerant cultivars of rice, cucumber and maize have higher activities of antioxidant enzymes than their chilling sensitive ones (Guo *et al.,*2006). In cucumber leaves, the accumulation of H_2O_2 can be induced by the increase of total SOD activity or alterations in the relative distributions of SOD isoforms. The newly accumulated H_2O_2, in turn, may trigger a protective mechanism against chilling that increases the activity of several enzymes such as peroxidase, APX and GR under catalase deactivation (Lee and Lee, 2000). Exposure to low temperatures causes an increase in CAT and GR activities in maize seedlings and induces chilling tolerance (Prasad, 1997). Similarly, decreased CAT, APX and MDHAR activities reduces chilling tolerance at the early stages of development in some maize types (Hodges *et al*., 1997).

1.4.2.3 Accumulation of Solutes

When plants are exposed to extreme environmental stresses such as salt, drought, and low-temperature, they accumulate highly soluble organic compounds of low molecular weight, called osmoprotectants or compatible solutes. The accumulation of these substances might play a role in increasing internal osmotic pressure and preventing loss of water from the cell when environmental stresses cause water deficit. These solutes can be amino acids such as proline, amino acid derivatives such as glycinebetaine, mannitol, and other sugar alcohols (Iba, 2002). Amino acid proline is present widely in plants and accumulates in response to environmental stresses. In addition to its role as an osmolyte for maintaining high water levels, it may also scavenge ROS and buffer cellular redox potential. In most of the plants, accumulation of proline under stress has been correlated with stress tolerance and its concentration are generally higher in stress tolerant plants than in stress sensitive plants. Despite the strong correlation between stress tolerance and its accumulation, this relationship is not unique and sometimes it accumulates as a result of stress injury rather than an indicator of tolerance. For instance, when some genotypes of rice and sorghum plants are exposed to salt, proline accumulation occur as a symptom for salt injury (Ashraf and Foolad, 2007).

Some proteins like chaperons, LEA, COR, and dehydrins are involved in cold tolerance. These proteins accumulate during acclimation period. They stabilize structures and by this way maintain membrane integrity and cellular function (Mahajan and Tuteja, 2005) under low temperature or water deficit. There are also some other antifreeze proteins (AFP). AFPs have the ability to adsorb onto the surface of crystals and modify their growth. They inhibit ice crstal growth and depress the freezing temperature of the solution (Atıcı and Nalbantoğlu, 2003)

1.5 Aim of the study

The aim of this study is to determine physiological and biochemical responses of two Turkish barley cultivars, winter type Tarm-92 and spring type Zafer-160 to chilling (+4 ºC) and freezing stresses (-3 ºC and -7 ºC).

For this purpose, the following physiological and biochemical analyses were performed to the shoot and root tissues of both cold treated and control barley seedlings:

i) Determination of antioxidant enzyme activities (CAT, APX, and GR).

ii) Determination of oxidative stress parameters (membrane leakage test, MDA, proline, hydrogen peroxide and chlorophyll florescence (Fv/Fm).

iii) Determination of some physiological parameters (Length, wet-dry weight)

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals were obtained from Sigma Chemical Company, Merck Chemical Company or Applichem Chemical Company.

2.1.2 Plant Material

In this study, two Turkish cultivars of barley (*Hordeum* vulgare), Tarm-92 and Zafer-160 were used. Tarm-92 was designated as winter type and Zafer-160 was designated as spring type by Turkish Ministry of Agriculture and Rural Affairs. The seeds were obtained from the Central Research Institute for Field Crops, Ankara.

2.2 Methods

2.2.1 Growth of Plants

Seeds were surface-sterilized with 30% sodium hypochloride solution for 12 minutes. Then they were washed and imbibed in distilled water for 24 hours at room temperature. Then, 15 seeds were planted onto perlit containing ½ strength Hoagland's solution (pH 5.8) (Hoagland and Arnon, 1950). Plants were grown for 8 days in a growth chamber at 23±2 ^{o}C with 16 hours light and 8 hours dark photo-cycle at light intensity 40 µmol $m^{-2}s^{-2}$.

2.2.2 Application of Cold Stress

The leaf and root tissues of both control and cold treated plants were analyzed for parameters like: length, wet weight, dry weight, electrolyte leakage and chlorophyll fluorescence (Fv/Fm); MDA, proline, and H_2O_2 contents; APX, CAT and GR activities.

2.2.2.1 +4 ºChilling Stress Treatment

On the 8th day of normal growth at 23 ºC, barley cultivars, Tarm-92 and Zafer-160 were treated with +4 ºC chilling stress in cold room for 1, 3 and 7 days (16 hours light and 8 hours dark photo- cycle). Control plants continued to grow at 23 ºC.

2.2.2.2 -3 ºC Freezing Stress Application

After growing at 23 ºC for 8 days, barley cultivars, Tarm-92 and Zafer-160 were acclimated at +4 ºC in cold room for 3 days, and then treated with -3 ºC freezing stress for 3 hours (Nuve ES 110 cooled incubator). After -3 ºC freezing stress treatment, recovery period applied plants were put back to +4 ºC and grown for 4 days. Control plants were grown at +23 ºC for 11 days. After growing at +23 ºC for 8 days, acclimated control plants were grown at +4 ºC for 4 days without -3 ºC freezing stress application. For the comparison of physiological parameters of recovery period applied plants, another control group were grown at +23 ºC for 15 days.

2.2.2.3 -7 ºC Freezing Stress Application

After growing at 23 Cº for 8 days, barley cultivars, Tarm-92 and Zafer-160 were acclimated at +4 ºC for 3 days, and treated with -3 ºC freezing stress for ½ hours and then with -7 ºC freezing stress for 1,5 hours (Nuve ES 110 cooled incubator). After -7 ºC freezing stress treatment, recovery period applied plants were put back to +4 ºC and grown for 4 days. Control plants were grown at +23 ºC for 11 days. After growing at +23 ºC for 8 days, acclimated control plants were grown at +4 ºC for 4 days without freezing stress application.

2.2.3 Length, Wet and Dry Weight

The shoot and root wet weight, dry weight, and length of control and cold treated plants were measured. Shoot length was measured from base to the tip of primary leaf. For root length the longest root was evaluated. After measuring the wet weights of shoots and roots, they were let to dry in an oven at 50 ºC for 24 hours, and then dry weights of the samples were measured.

2.2.4 Determination of Proline Content

The proline content was determined according to a modified method of Bates *et al.*, (1973). 0.2 g leaf and root tissues were homogenized with liquid nitrogen and suspended in 2 ml of 3% sulphosalicylic acid. The homogenates were transferred into eppendorf tubes and centrifuged at 14000 rpm (Shimadzu UV mini 1240) for 5 minutes at room temperature. 0.1 ml supernatant from each homogenate was added to the eppendorf tubes containing 0.2 ml acid ninhydrin (0.31 g ninhydrin, 7.5 ml acetic acid and 5 ml 6 M phosphoric acid), 0.2 ml 96% phosphoric acid and 0.1 ml 3% sulphosalicylic acid. Tubes were incubated at 96ºC for 1 hour. After incubation 1 ml toluene was added to each tube and vortexed. Then, tubes were centrifuged at 14000 rpm for 5 minutes. The pink-red upper phase was transferred to the cuvette and absorbance at 520 nm was measured against toluene. A standard curve for proline containing the range 0.01 µM - 1.5 mM was constructed to determine the proline concentration in each sample (Appendix A).

2.2.5 Determination of MDA Content

The MDA content was determined according to the method of Ohkawa *et al*., (1979). 0.2 g leaf and root tissues from control and treated plants were homogenized with liquid nitrogen and suspended in 5% trichloro acetic acid (TCA) solution. The homogenates were transferred into eppendorf tubes and centrifuged at 12000 rpm for 15 minutes at room temperature. Equal volumes of supernatant and 0.5% thiobarbituric acid in 20% TCA (freshly prepared) were added into a new eppendorf tube and incubated at 96^{o}C for 25 minutes. The tubes were transferred

into ice bath and then centrifuged at 10000 rpm for 5 minutes. The absorbance of the supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. 0.5% thiobarbituric acid in 20% TCA was used as blank. MDA content was determined using the extinction coefficient 155 $mM^{-1}cm^{-1}$.

2.2.6 Electrolyte Leakage Test

Electrolyte leakage of shoot tissues was measured according to the method of Sairam *et al*. (1997) with minor modifications. One cm leaf segments were cut. After washing the segments with ultra pure water, they were placed in tubes including 5 ml ultra pure water. The tubes were shaken at 100 rpm for 24 h at room temperature and after incubation electrical conductance were measured (C_1) using Metter-Toledo Mpc 227 conductivity meter. Then, the samples were incubated in liquid nitrogen for 20 minutes to kill the tissue completely and the second conductance (C_2) was taken to measure the total ion concentration after complete membrane disintegration. The cold injury to the membranes were calculated by using the formula $(C_1/C_2)*100$.

2.2.7 Chlorophyll Fluorescence Analysis

In dark adapted leaf tissue, Chlorophyll fluorescence (Fv/Fm) which is an indicator of the efficiency of excitation energy capture by PSII reaction centers (Rizza *et al.,* 2001) was measured by using OS5-FL Modulated Fluorometer .

2.2.8 Determination of Hydrogen Peroxide Content

The hydrogen peroxide (H_2O_2) content was measured according to the modified method of Bernt and Bergmeyer (1974). 0.5 g leaf and root tissue were homogenized with liquid nitrogen and suspended in 1.5 ml of 100 mM potassium phosphate buffer, pH 6.8. The homogenate was then centrifuged at 18000 g for 20 min at 4 ^{o}C. 0.250 ml of supernatant was mixed with 1.25 ml of peroxidase

reagent, consisting of 83 mM potassium phosphate, pH 7.0, 0.005% (w/v) o-dianizidine, 40 µg peroxidase/ml and incubated for 10 minutes at 30 ^{o}C in a water bath. The reaction was stopped by adding 0.250 ml of 1 N perchloric acid and centrifuged at 5000 g for 5 minutes. The supernatant was read against peroxidase reagent at 436 nm and H_2O_2 content was measured by construction of a H_2O_2 standard curve (Apeendix B).

2.2.9 Enzyme Assays

0.5 g leaf and root samples were homogenized with liquid nitrogen and suspended in 50mM potassium phosphate buffer (pH 7.5) containing 2% (w/v) PVP, 1mM EDTA and 1mM ascorbate. The homogenate was centrifuged at 15000 g for 20 min at +4° C. The supernatant was used for protein content and enzyme (ascorbate peroxidase, catalase ve glutathione reductase) activity determination.

2.2.9.1 Protein Determination

The soluble protein concentration in leaf and root crude extracts was determined according to Bradford method (Bradford, 1976). Concentrated Bradford reagent (5X) was prepared by dissolving 500 mg of Coomassie Brilliant Blue G-250 in 250 ml 95% ethanol. After addition of 500 ml 85% phosphoric acid, the solution was diluted to 1 liter with distilled water and filtered. It was stored at 4ºC. Before use, 5X Bradford reagent was diluted 5 times. 10 µl of leaf and 20 µl root sample was diluted to 500 µl with distilled water in a test tube. Then 5 ml 1X Bradford reagent was added and vortexed. After 10 minutes incubation period, the absorbance was measured at 595 nm with Schimadzu UV-1240 spectrophotometer against blank containing 500 µl distilled water and 5 ml Bradford reagent. Bovine Serum Albumin (BSA) with concentrations 10, 20, 30, 40, 50, 60 µg/ml was used for construction of the standard curve.

2.2 9.2 Determination of CAT Activity

Catalase activity was determined according to the method of Chance *et al.* (1995) with minor modifications. Assay medium contained 50 mM potassium phosphate buffer (pH 7.0), 100 mM H_2O_2, and the enzyme extract containing 100 µg protein for leaf and 50 µg protein for root determined by Bradford method (1976). The reaction was initiated by the addition of 100 mM H_2O_2 at root temperature. The decrease in absorbance was recorded at 240 nm for 2 minutes by using Schimadzu double-beam spectrophotometer at room temperature. Assay medium without enzyme extract was used as blank. The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of H_2O_2 = 40 mM^{-1} cm^{-1}). One enzyme unit was defined as µmol ml^{-1} H_2O_2 destroyed per min.

2.2.9.3 Determination of APX Activity

APX activity was determined according to the method Wang *et al.*, (1991). Assay medium contained 50 mM potassium phosphate buffer (pH 6.6), 0.25 mM ascorbate, 10 mM H_2O_2 and enzyme extract containing 100 µg protein for leaf and 50 µg protein for root determined by Bradford method (1976). The reaction was initiated by the addition of 10 mM H_2O_2 peroxide at room temperature. The decrease in ascorbate concentration was recorded at 290 nm with Schimadzu double-beam spectrophotometer for 2 minutes. Assay medium without enzyme extract was used as blank. The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of ascorbate = 2.8 $mM^{-1}cm^{-1}$ at 290 nm). One enzyme unit was defined as µmol ml^{-1} ascorbate oxidized per min.

2.2.9.4 Determination of GR Activity

Glutathione reductase activity was determined according to the method of Sgherri *et al.,* (1994) with minor modifications. The GSSG-dependent oxidation of NADPH was monitored by the decrease in absorbance at 340nm. The assay mixture was consist of 200 mM potassium phosphate buffer (pH 7.5) containing 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.50 mM GSSG, 50 µM NADPH and enzyme extract containing 50 µg protein determined by Bradford method. The reaction was initiated by NADPH addition at room temperature. Assay medium without enzyme

extract was used as blank. The enzyme activity was calculated from the initial rate of the enzyme. (Extinction coefficient of NADPH = 6.2 $mM^{-1}cm^{-1}$ at 340 nm). One enzyme unit was defined as µmol ml^{-1} GSSG oxidized per min.

2.2.10 Statistical Analysis

The significance of difference between mean values was determined by one-way analysis of variance at 95 % confidence interval. The standard deviations among means were calculated by descriptive statistics test by using Minitab software program. Experiments were repeated for 5 times with independently grown seedlings.

CHAPTER 3

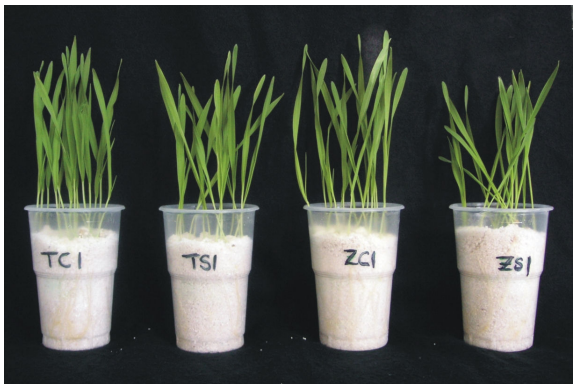
RESULTS

The leaf and root tissues of both control and cold treated two barley cultivars winter type Tarm-92 and spring type Zafer-160 were analyzed for parameters like: length, wet weight, dry weight, electrolyte leakage and chlorophyll fluorescence (Fv/Fm); MDA, proline, and H_2O_2 contents; APX, CAT and GR activities. All of the experiments were repeated for 5 times with independently grown seedlings.

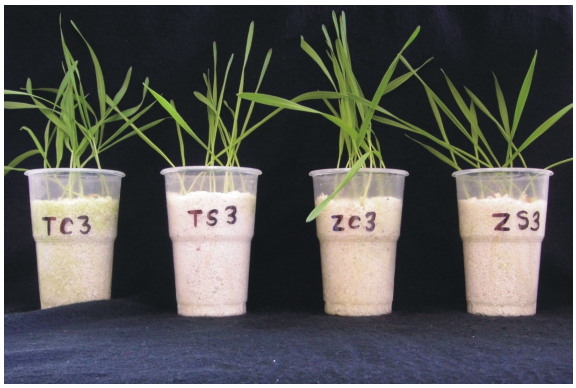
3.1 Physiological Changes under Cold Stress

The physiological changes under cold stress were analyzed by measuring the shoot and root length, wet weight and, dry weight of control and treated plants. The photographs of 1, 3 and 7 days of +4 ºC chilling stress applied barley seedlings and their controls are shown in Figure 3.1. As shown in Figure 3.2, treatment of -3 ºC and -7 ºC freezing stresses without an acclimation period directly killed both winter type Tarm and spring type Zafer. For that reason, only acclimated plants were used in the experiments. The physiological appearance of acclimated barley cultivars after -3 ºC and -7 ºC freezing stress and recovery period applied plants are shown in Figure 3.3. Treatment of +4 ºC stress and -3 ºC and -7 ºC stresses resulted in growth retardation in both cultivars. In addition to growth inhibition, application of -3 ºC and -7 ºC stresses caused wilting. Both growth retardation and wilting symptoms were more severe in Zafer than in Tarm.

a



b



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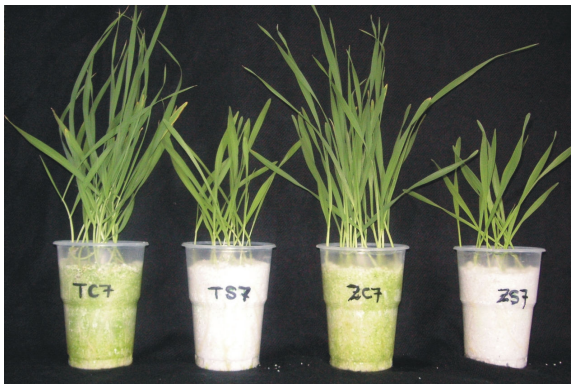

Figure 3.1. +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). a: TC1:Tarm control, TS1: 1 day stress treatment, ZC1, Zafer control, ZS1: 1 day stress treatment. b: TC3:Tarm control, TS3: 3 days stress treatment, ZC3, Zafer control, ZS3: 3 days stress treatment. c: TC7:Tarm control, TS7: 7 days stress treatment, ZC7, Zafer control, ZS7: 7 days stress treatment.

Figure 3.2. Unacclimated -3 ºC and -7 ºC freezing stress treated barley cultivars. (Winter type Tarm-92 and spring type Zafer-160 cultivars).

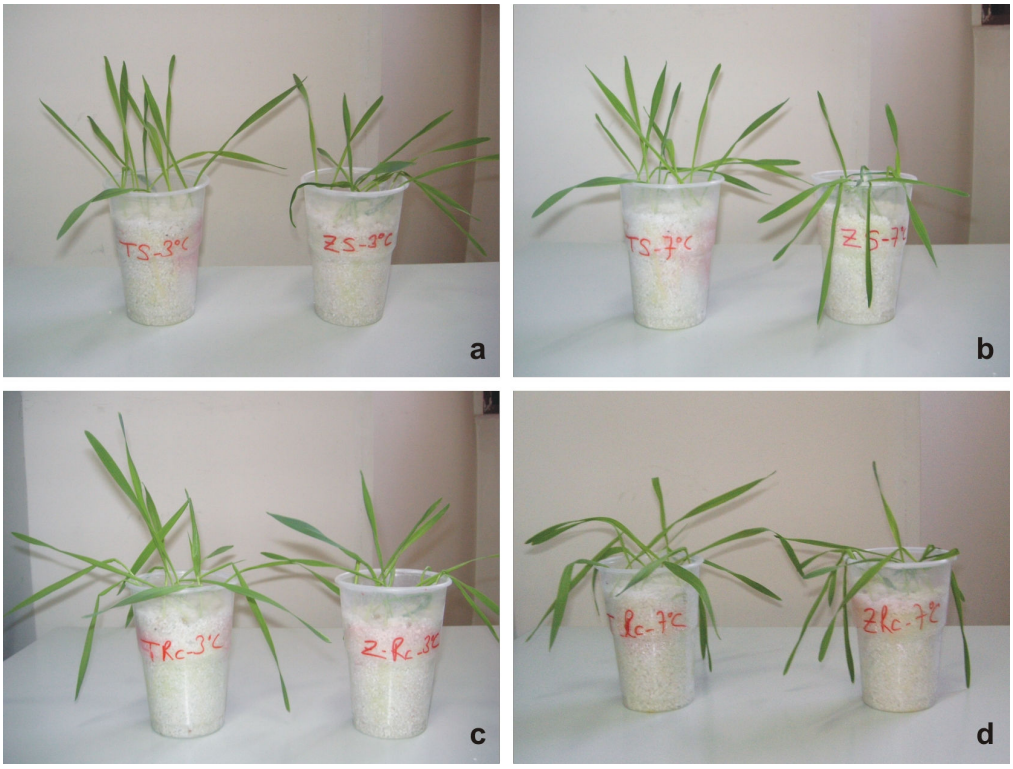

Figure 3.3. Acclimated -3 ºC and -7 ºC freezing stress and recovery period applied barley cultivars. (Winter type Tarm-92 and spring type Zafer-160 cultivars).**a:** -3 ºC freezing stress treated Tarm and Zafer. **b:** -7 ºC freezing stress treated Tarm and Zafer. **c:** recovery period applied Tarm and Zafer after -3 ºC freezing stress treatment. **d:** recovery period applied Tarm and Zafer after -7 ºC freezing stress treatment.

3.1.1 Length

Effect of cold application on shoot and root lengths of winter type, Tarm-92 and spring type cultivar, Zafer-160 were assessed after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.1.1.1 Effect of +4 ºC cold stress treatment

The effect of +4 ºC cold stress application was illustrated in Figure 3.4. Shoot growth of both Tarm and Zafer seedlings were retarded after +4 ºC cold stress treatment. Shoot length decreased significantly after 1, 3, and 7 days of +4 ºC stress application in both cultivars. The degree of shoot growth inhibition increased in both Tarm and Zafer as the period of +4 ºC cold exposure increased.

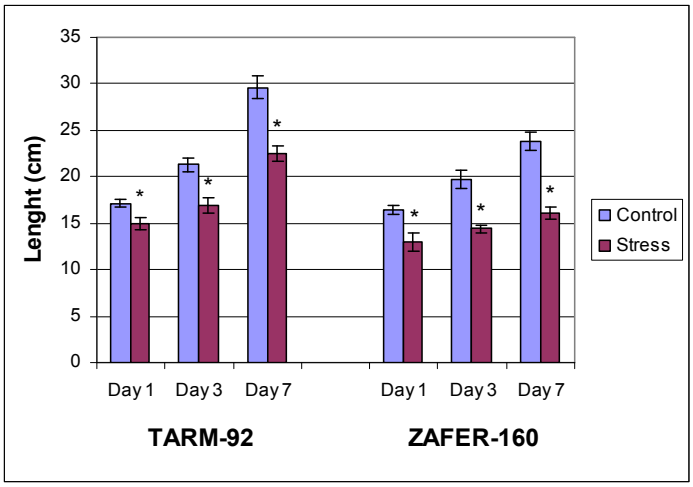

Figure 3.4. Shoot length of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

Application of +4 ºC cold stress caused retardation in root length of both cultivars (Figure 3.5). The decrease in root length after 1, 3 and 7 days of +4 ºC cold stress was not statistically significant in Tarm cultivar. However, Zafer root growth was significantly decreased after 3 days and 7 days of +4 ºC application.

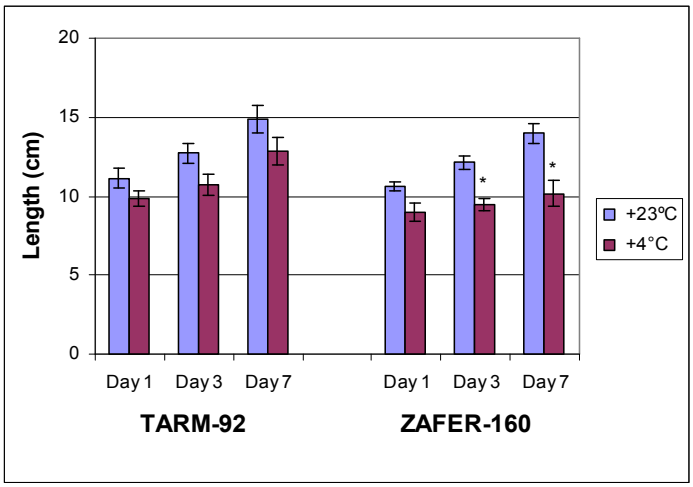

Figure 3.5. Root length of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.1.1.2 Effect of -3 ºC and -7 ºC cold stress treatment

Compared to +23 ºC control plants, application of both -3 ºC and -7 ºC cold stresses showed similar effects and resulted in significant decrease in shoot length of Tarm and Zafer cultivars. On the other hand, no meaningful change was observed in shoot length of both cultivars when stress treated plants were compared with acclimated control plants. During recovery period, -3 ºC and -7 ºC stress applied Tarm cultivar continued to grow and showed a significant increase in shoot length. However, shoot length of Zafer increased only slightly after recovery period application. Effect of -3 ºC cold stress on shoot length was shown in Figure 3.6 and effect of -7 ºC on shoot length was shown in Figure 3.7.

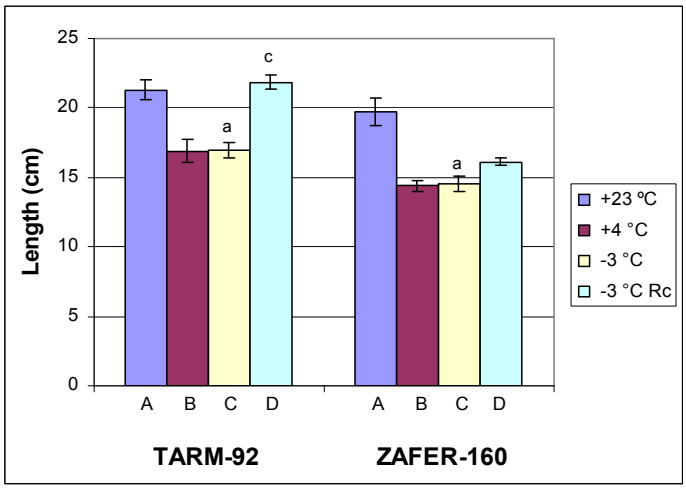

Figure 3.6. Shoot length of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

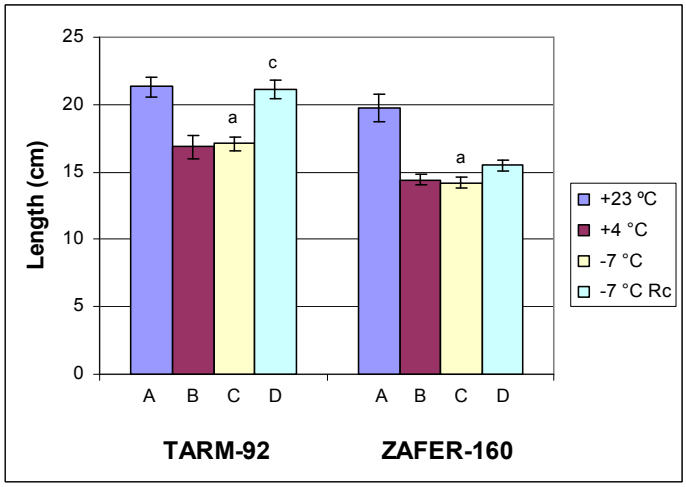

Figure 3.7. Shoot length of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control . [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

When compared with +23 ºC control, Tarm root length decreased after -3 ºC and -7 ºC cold stress applications, but the level of decrease is statistically insignificant. On the other hand, -3 ºC and -7 ºC cold stresses led to a significant decrease in Zafer root length. No meaningful change was observed in root length of both cold stress treated cultivars compared to acclimated controls. After recovery period application, -3 ºC and -7 ºC treated Tarm and Zafer cultivars showed a non significant increase in root length. However, Tarm root length increased more than Zafer root length. Root length change in Tarm and Zafer after -3 ºC stress application was illustrated in Figure 3.8 and root length change after -7 ºC cold stress was illustrated in Figure 3.9.

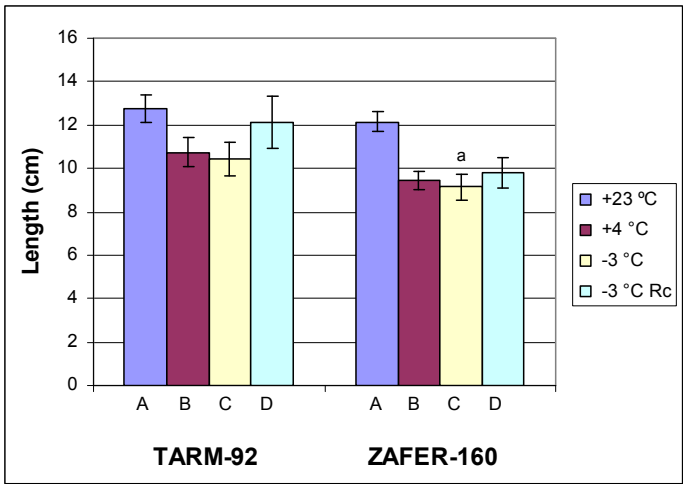

Figure 3.8. Root length of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$).

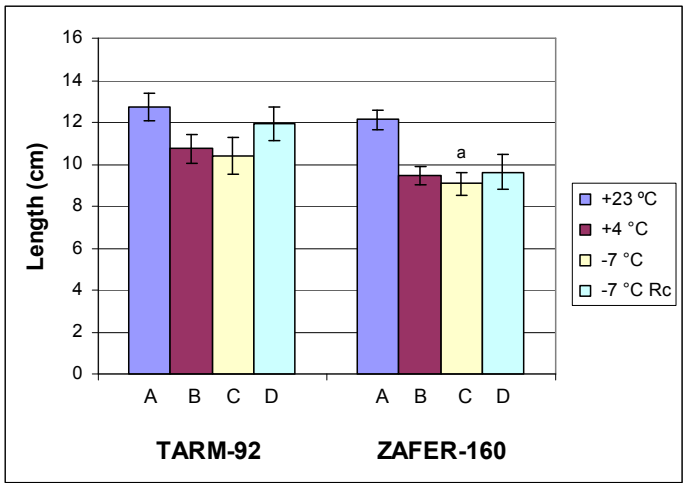

Figure 3.9. Root length of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control.

3.1.2 Wet weight

Effect of cold treatment on shoot and root wet weights of winter type, Tarm-92 and spring type cultivar, Zafer-160 were assessed after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.1.2.1 Effect of +4 ºC cold stress treatment

Shoot wet weights of both Tarm and Zafer seedlings decreased after +4 ºC cold stress treatment (Figure 3.10). Although shoot wet weight difference between stress treated and control plants were not significant after 1 day, it was significant after 3 and 7 days of +4 ºC exposures in both cultivars.

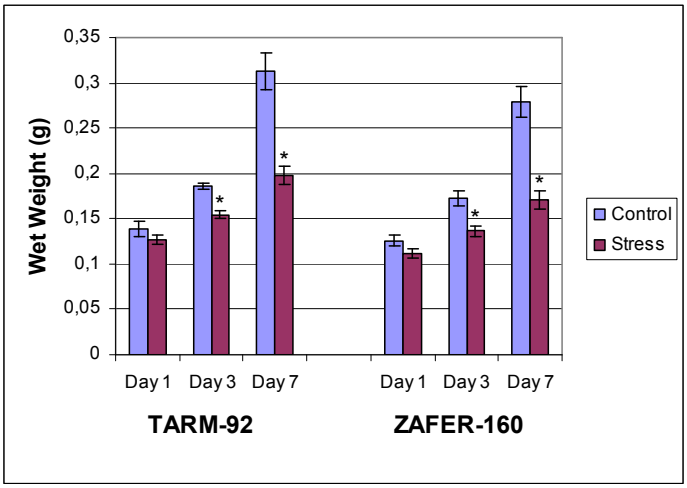

Figure 3.10. Shoot wet weight of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

Effect of +4 ºC cold stress application on root wet weight was illustrated in Figure 3.11. Treatment of +4 ºC reduced wet weight of roots in both Tarm and Zafer seedlings. The root wet weight reduction was significant after 3 and 7 days of +4 ºC cold stress treatment in both cultivars. The observed decrease in root wet weight was higher in Zafer than in Tarm seedlings.

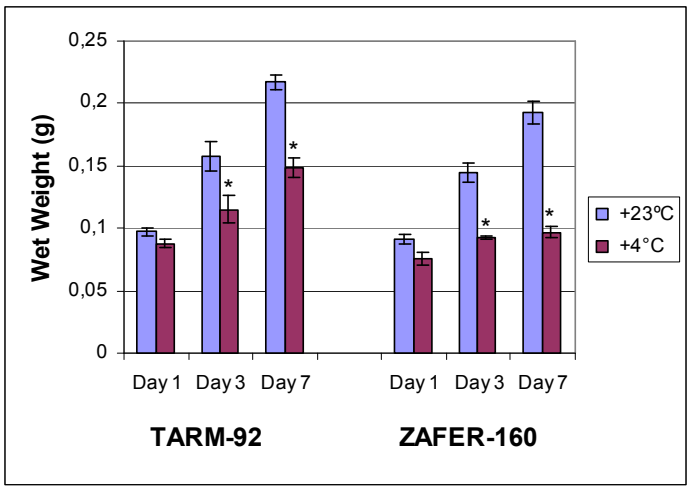

Figure 3.11. Root wet weight of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.1.2.2 Effect of -3 ºC and -7 ºC cold stress treatment

Shoot wet weight of Tarm cultivar decreased after -3 ºC and -7 ºC cold stress treatments compared to 23 ºC control. It was observed that the decrease was significant only after -7 ºC stress application. When compared with 23 ºC control, shoot wet weight of Zafer showed a statistically significant decrease after both -3 ºC and -7 ºC stresses. -3 ºC and -7 ºC cold stress applications did not have any effect on shoot wet weight of Tarm and Zafer compared to acclimated control plants. Recovery period after -3 ºC and -7 ºC treatments resulted in a significant increase in shoot wet weights of both Tarm and Zafer cultivars. Wet weight change in Tarm and Zafer shoots after -3 ºC cold stress treatment was illustrated in Figure 3.12. Shoot wet weight change of Tarm and Zafer cultivars after -7 ºC cold stress application was shown in Figure 3.13.

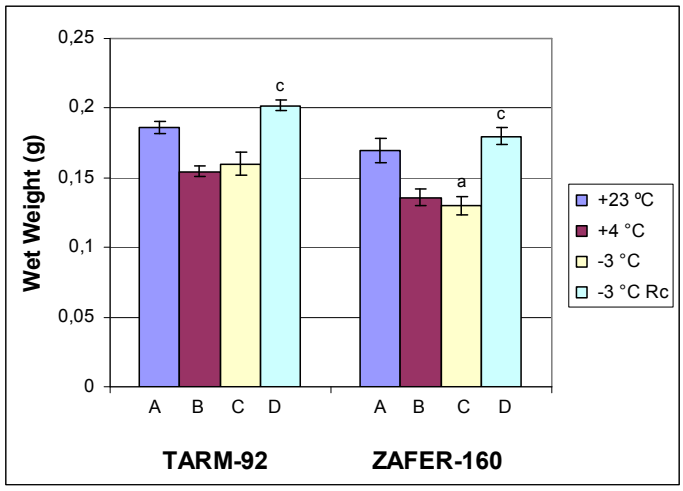

Figure 3.12. Shoot wet weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

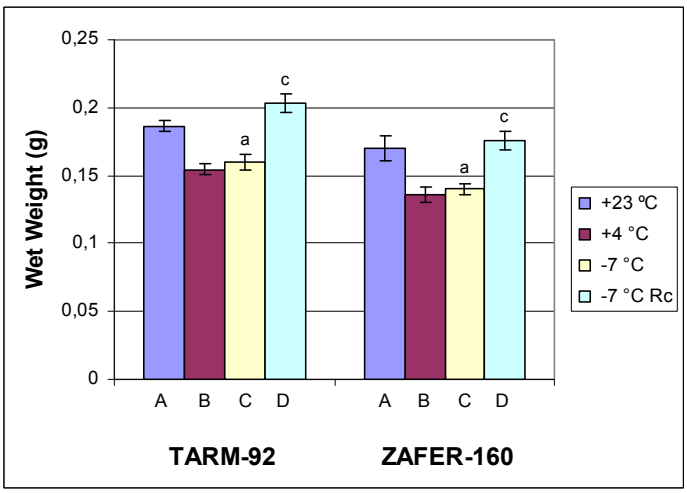

Figure 3.13. Shoot wet weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

When compared with 23 ºC controls, -3 ºC and -7 ºC treated Tarm and Zafer seedlings showed a significant decrease in root wet weight. It was observed that the decrease in root wet weight was not meaningful when -3 ºC and -7 ºC applied cultivars were compared with acclimated controls. Recovery period application after -3 ºC and -7 ºC cold stress treatments caused an important increase in root wet weight of Tarm cultivar and the increase was measured to be statistically significant in recovery period after -7 ºC stress application. After -3 ºC and -7 ºC cold stress treatments, recovery period also elevated root wet weight of Zafer. However, the increase was insignificant and less than in Tarm roots. Root wet weight change of Tarm and Zafer cultivars after -3 ºC cold stress treatment was shown in Figure 3.14. Effect of -7 ºC cold stress application on root wet weight of Tarm and Zafer was given in Figure 3.15.

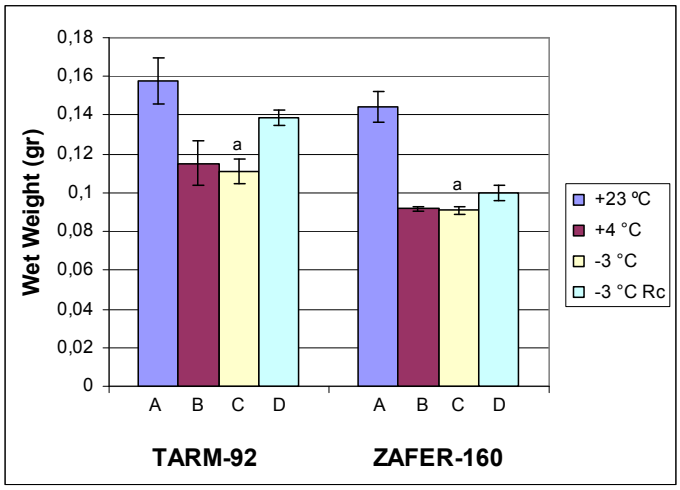

Figure 3.14. Root wet weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress applied plants for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control.

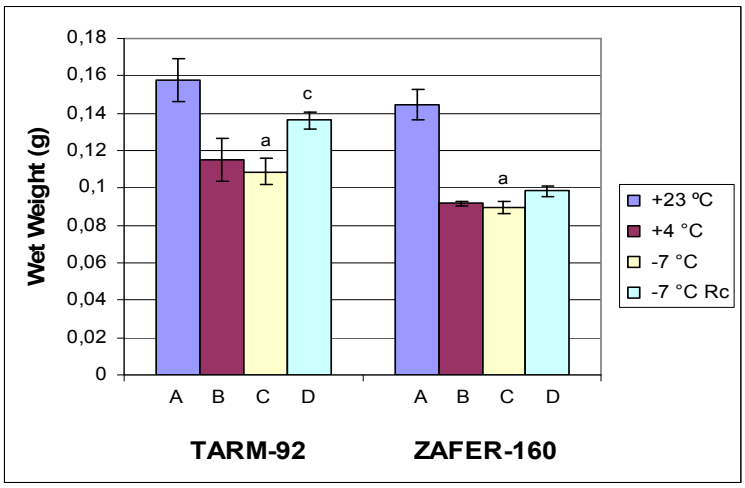

Figure 3.15. Root wet weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

3.1.3 Dry weight

Effect of cold application on shoot and root dry weights of winter type, Tarm-92 and spring type cultivar, Zafer-160 were observed after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress treatments.

3.1.3.1 Effect of +4 ºC cold stress treatment

Similar to shoot wet weights, shoot dry weight of Tarm and Zafer seedlings decreased after +4 ºC cold stress treatment. Although shoot wet weight change was not significant after 1 day of +4 ºC exposure, it was significant after 3 and 7 days of stress treatments in both cultivars. Effect of +4 ºC cold stress application was illustrated in Figure 3.16.

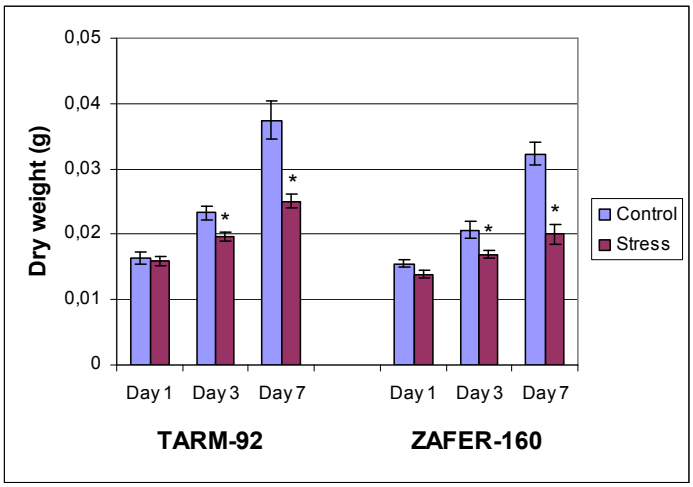

Figure 3.16 Shoot dry weight of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

Similar to root wet weight, application of +4 ºC cold stress decreased root dry weight in both Tarm and Zafer cultivars. The reduction in root dry weight was significant after 3 and 7 days of +4 ºC cold stress treatment in both cultivars. It was observed that decrease in root dry weight was higher in Zafer than in Tarm seedlings. Wet weight change after 1, 3 and 7 days of +4 ºC cold stress in roots of Tarm and Zafer was shown in Figure 3.17.

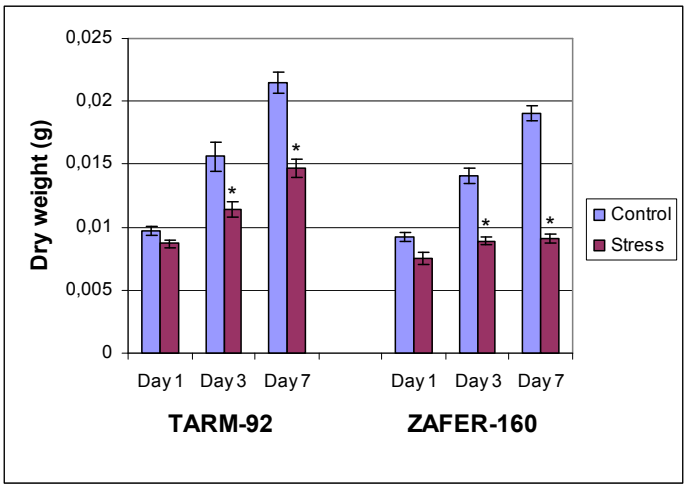

Figure 3.17 Root dry weight of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.1.3.2 Effect of -3 ºC and -7 ºC cold stress treatment

Shoot dry weights of Tarm and Zafer cultivars significantly reduced after -3 ºC and -7 ºC cold stress applications when compared with 23 ºC controls. Treatments of -3 ºC and -7 ºC cold stresses did not cause a meaningful decrease on shoot dry weight of Tarm and Zafer compared to acclimated control plants. Both -3 ºC and -7 ºC treated Tarm seedlings increased the amount of dry matter production in shoots after recovery period. After recovery period, -3 ºC stress applied Zafer cultivar managed to increase shoot dry weight content in tissues, whereas recovery period after -7 ºC stress application did not have any effect on shoot dry weight. Dry weight content in Tarm and Zafer shoots after -3 ºC cold stress treatment was illustrated in Figure 3.18. Shoot dry weight change of Tarm and Zafer seedlings after -7 ºC cold stress application was shown in Figure 3.19.

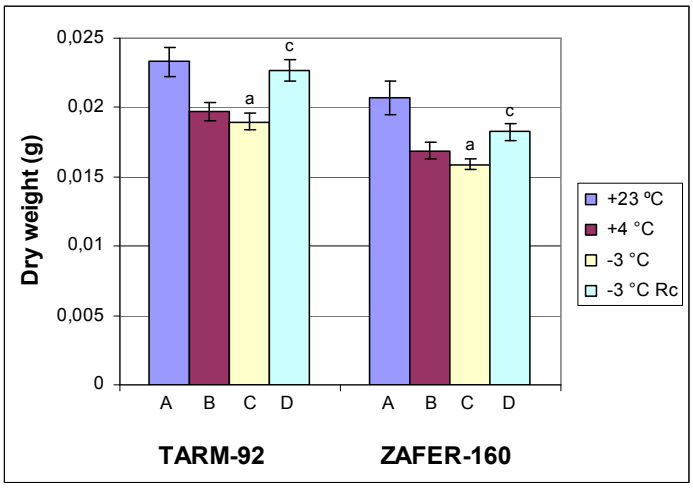

Figure 3.18. Shoot dry weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control.

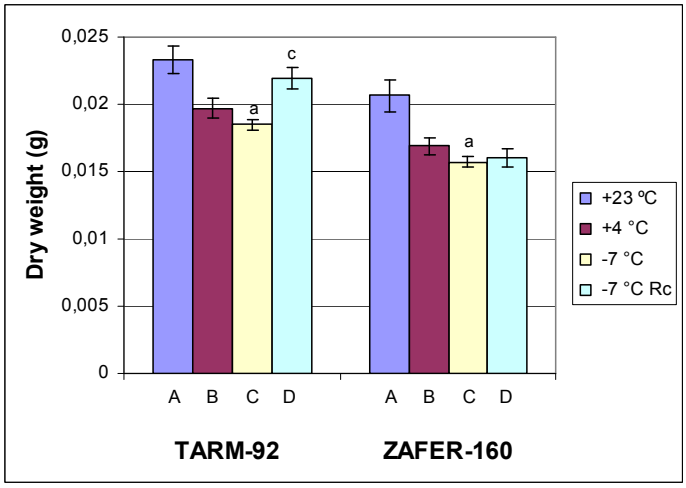

Figure 3.19. Shoot dry weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

When compared with 23 ºC controls, -3 ºC and -7 ºC cold stress treated Tarm and Zafer seedlings showed a significant reduction in root dry weight. On the other hand, the change in root dry weight content was not meaningful when -3 ºC and -7 ºC cold stress treated cultivars were compared with acclimated controls. Recovery period after -3 ºC and -7 ºC cold stress applications led to a significant increase in root dry weight of Tarm cultivar. After -3 ºC and -7 ºC cold stress treatments, no meaningful elevation in root dry weight of Zafer seedlings was observed in recovery period. Effect of -3 ºC cold stress application on root wet weight of Tarm and Zafer was shown in Figure 3.20. Root dry weight change of Tarm and Zafer cultivars after -7 ºC cold stress treatment was illustrated in Figure 3.21.

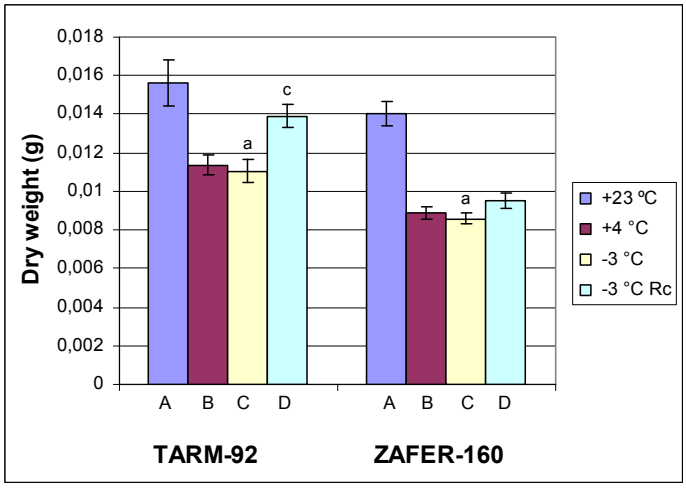

Figure 3.20. Root dry weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

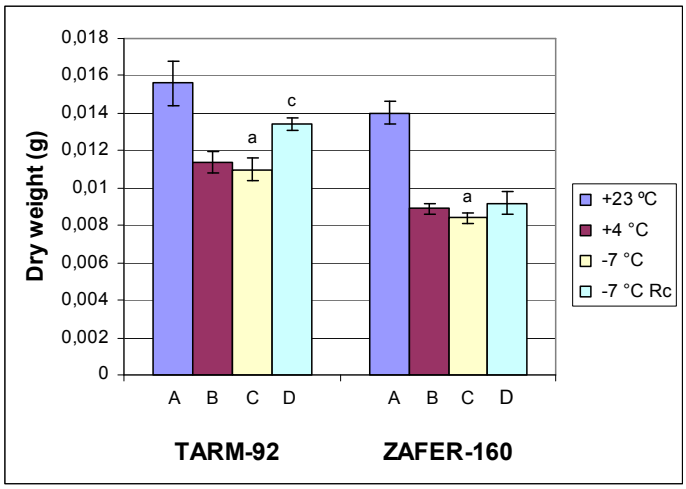

Figure 3.21. Root dry weight of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

3.1. Proline Content under Cold Stress

Effect of cold treatment on leaf and root proline content of winter type, Tarm-92 and spring type cultivar, Zafer-160 were analyzed after 1, 3 and 7 days of +4 ºC cold stresses, and -3 ºC and -7 ºC cold stress applications.

3.2.1 Effect of +4 ºC cold stress treatment

Although exposure to +4 ºC cold stress for 1 day did not cause an increase in leaf proline content, treatment of +4 ºC stress for 3 and 7 days resulted in important proline accumulation in leaves of both cultivars (Figure 3.22). The accumulation is significant in Tarm cultivar after 3 and 7 days of stress application. However, the leaf proline content is statistically significant only after 7 days of +4 ºC stress treatment in Zafer cultivar.

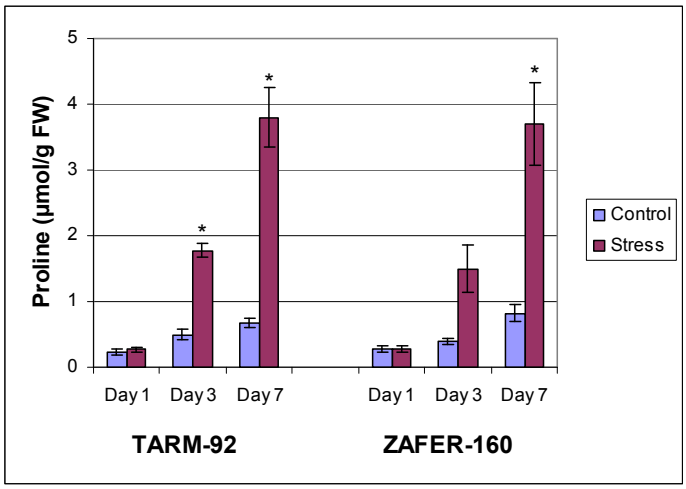

Figure 3.22. Leaf proline content of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

Application of +4 ºC stress for 1, 3 and 7 days elevated the root proline content of Tarm and Zafer (Figure 3.23). Proline accumulation in root tissues was significant in both cultivars after 3 and 7 days of +4 ºC cold stress exposure.

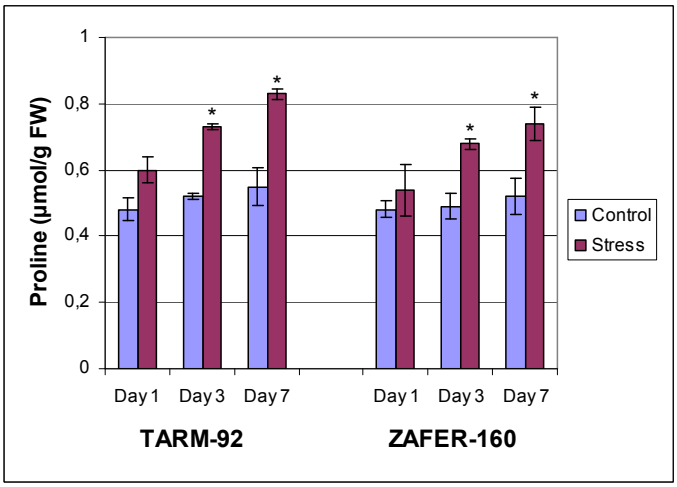

Figure 3.23. Root proline content of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.2.2 Effect of -3 ºC and -7 ºC cold stress treatment

Both -3 ºC and -7 ºC cold stress treatments caused significant proline accumulation in leaves of Tarm and Zafer seedlings compared to 23 ºC controls. -3 ºC stress treated Tarm and Zafer cultivars also increased leaf proline content when compared with acclimated control plants. The difference was significant in Tarm leaves. After -7 ºC cold stress application, there was no meaningful change in leaf proline amount of both cultivars compared to acclimated controls. Recovery period after -3 ºC and -7 ºC cold stress applications resulted in significant proline accumulation in leaves of Tarm and Zafer cultivars, but recovery period after -3 ºC stress led to more proline accumulation than that after -7 ºC cold stress treatment. Leaf proline accumulation in Tarm and Zafer seedlings after -3 ºC cold stress application was illustrated in Figure 3.24 Effect of -7 ºC cold stress treatment on leaf proline content was illustrated in Figure 3.25.

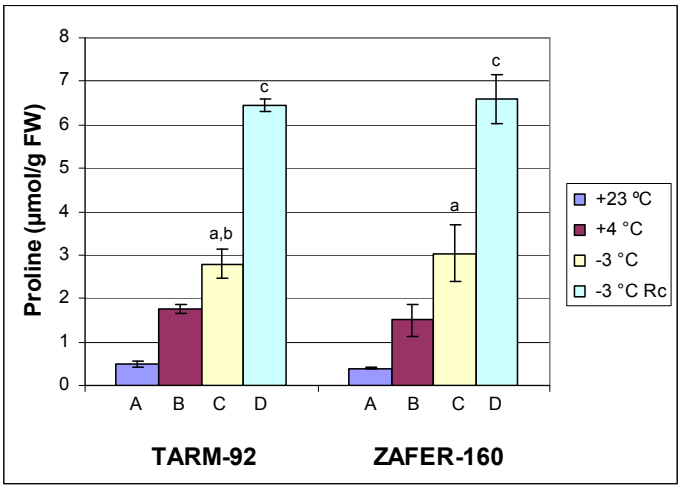

Figure 3.24. Leaf proline content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

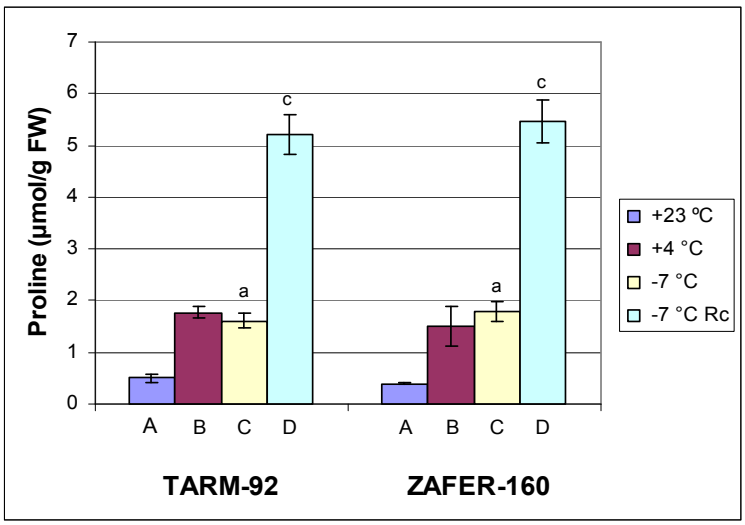

Figure 3.25. Leaf proline content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

Similar to leaves, -3 ºC and -7 ºC cold stress applications led to significant proline accumulation in roots of both Tarm and Zafer cultivars compared to 23 ºC controls. When compared with acclimated control, -3 ºC stress treated Tarm cultivar significantly elevated leaf proline content, whereas the increase was not significant in roots of Zafer. After -7 ºC cold stress application, neither Tarm nor Zafer roots meaningfully changed proline contents when compared with acclimated controls. Recovery period after -3 ºC and -7 ºC cold stress treatments insignificantly increased proline content in roots of Tarm and Zafer cultivars. It was also observed that proline accumulation in recovery period was more in roots of Tarm than that of Zafer after application of -7 ºC. Effect of -3 ºC cold stress treatment on root proline accumulation in Tarm and Zafer seedlings was shown in Figure 3.26 and effect of -7 ºC cold stress treatment on root proline content was illustrated in Figure 3.27.

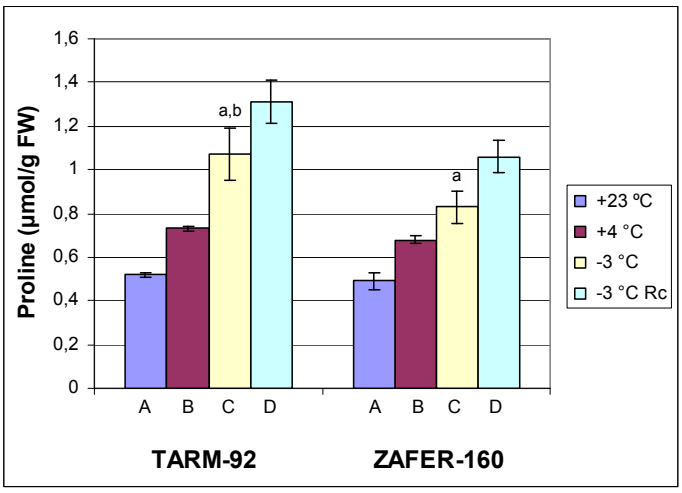

Figure 3.26. Root proline content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

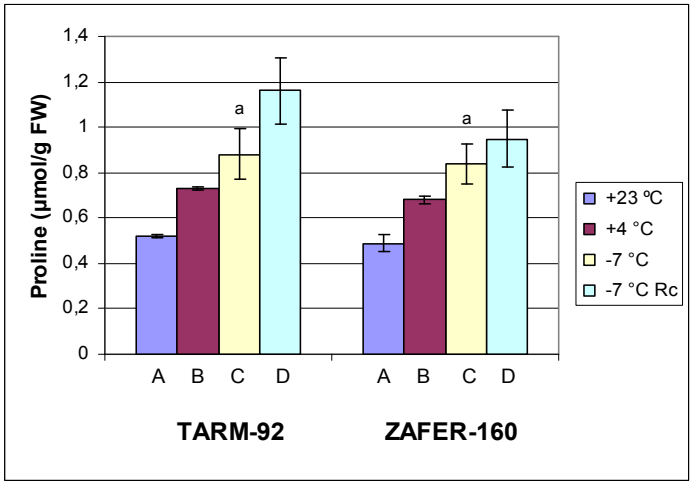

Figure 3.27. Root proline content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control.

3.3 Malondialdehyde (MDA) Content under Cold Stress

Effect of cold treatment on leaf and root lipid peroxidation was determined by measuring the MDA content of winter type, Tarm-92 and spring type cultivar, Zafer-160 were analyzed after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.3.1 Effect of +4 ºC cold stress treatment

Except for the significant increase in the MDA amount in leaves of Zafer cultivar after 1 day of +4 ºC cold stress treatment, no meaningful change was observed in leaf MDA content of both Tarm and Zafer cultivars after 1,3 and 7 days of +4 ºC cold stress exposure. Effect of +4 ºC cold stress treatment on MDA content in leaves of Tarm and Zafer was shown in Figure 28.

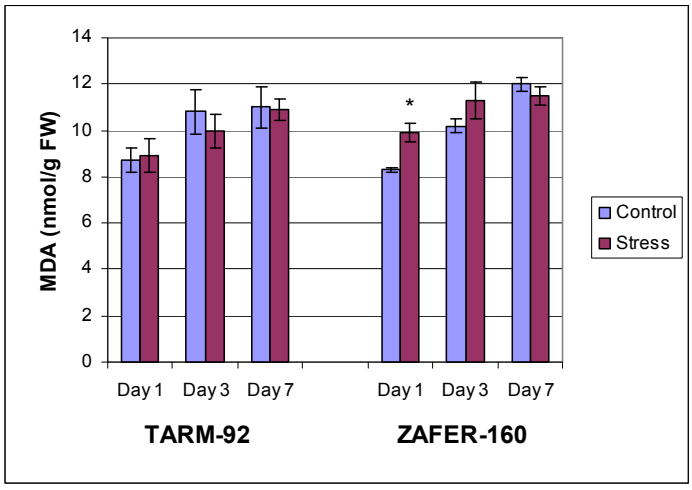

Figure 3.28. Leaf MDA content of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

It was observed that exposure of +4 ºC cold stress caused lipid peroxidation in roots and elevated MDA content in Tarm and Zafer. While the increase in root MDA content was not statistically important on day 1 of +4 ºC stress treatment, it

was significant on day 3 and 7 in both cultivars. The effect of +4 ºC stress exposure on root MDA content in Tarm and Zafer was shown in Figure 3.29. The difference in MDA content between stress applied and control plants was measured to be highest after 3 days of +4 ºC cold treatment.

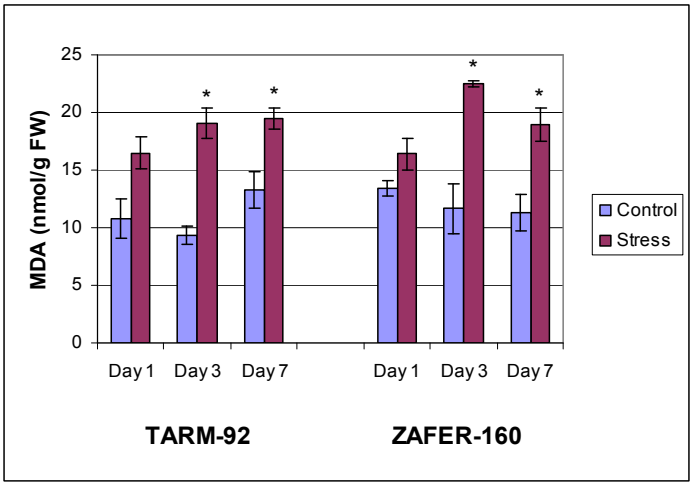

Figure 3.29. Root MDA content of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.3.2 Effect of -3 ºC and -7 ºC cold stress treatment

Application of -3 ºC and -7 ºC cold stresses showed a similar effect on MDA amount of leaves in Tarm and Zafer cultivars. After -3 ºC and -7 ºC treatments, lipid peroxidation increased and the level of MDA in leaves significantly elevated compared to 23 ºC and acclimated controls. Leaf MDA produced in Zafer cultivar after -7 ºC stress application was more than that produced after -3 ºC stress. In recovery period, neither Tarm nor Zafer leaves did not change MDA content after -3 ºC stress application. Despite the fact that MDA levels slightly increased in Tarm leaves in recovery period after -7 ºC stress application, the increase was not significant. Leaf MDA content after recovery period remained unchanged in -7 ºC stress treated Zafer cultivar. MDA content in Tarm and Zafer leaves after -3 ºC cold stress treatment was illustrated in Figure 3.30 and leaf MDA content after -7 ºC cold stress treatment was shown in Figure 3.31.

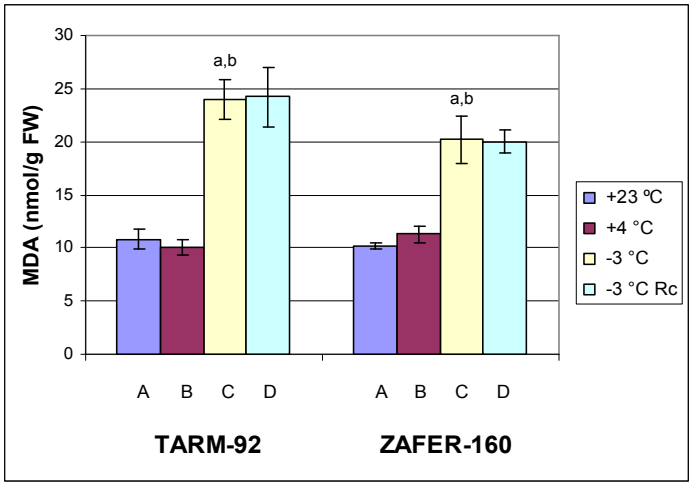

Figure 3.30. Leaf MDA content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

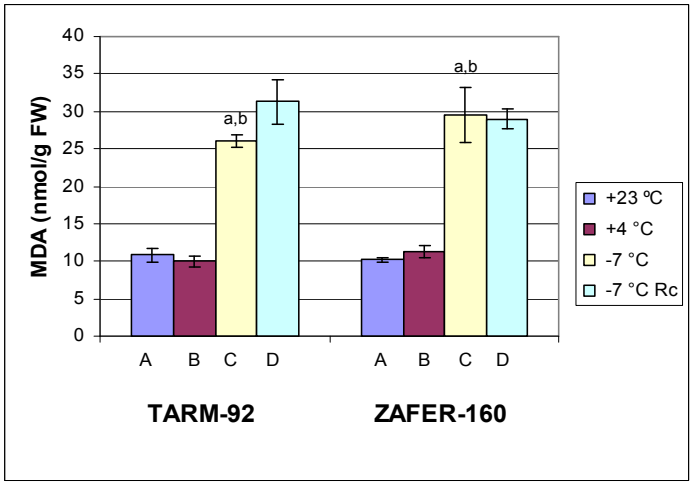

Figure 3.31. Leaf MDA content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

Treatments of -3 ºC and -7 ºC cold stresses resulted in lipid peroxidation and significantly elevated MDA content in roots of Tarm and Zafer cultivars compared to 23 ºC controls. It was also observed that the level of increase in root MDA content was higher after -7 ºC stress application in both of cultivars. -3 ºC cold stress did not cause a meaningful change in root MDA content of Tarm and Zafer compared to acclimated control plants. On the other hand, -7 ºC stress treatment increased the amount of MDA in roots of both cultivars compared to acclimated controls. However, the rise in MDA level was statistically insignificant. Although recovery period after -3 ºC stress exposure did not cause an important change in MDA content, recovery period after -7 ºC stress decreased MDA level in roots of Tarm and Zafer. Root MDA content in Tarm and Zafer after -3 ºC cold stress treatment was shown in Figure 3.32 and root MDA content after -7 ºC cold stress application was shown in Figure 3.33.

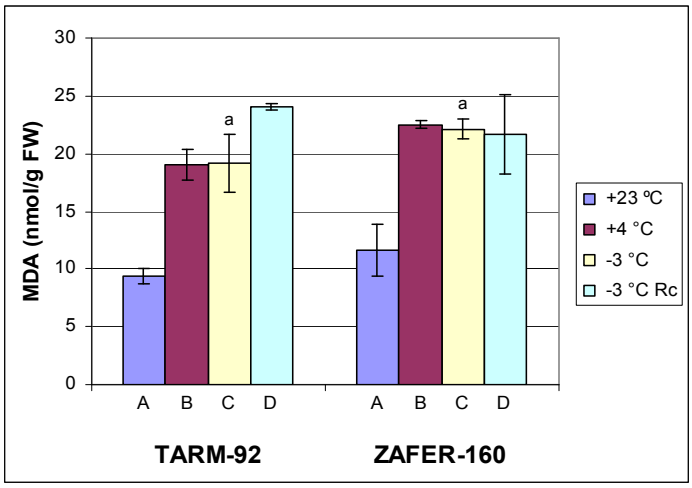

Figure 3.32. Root MDA content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

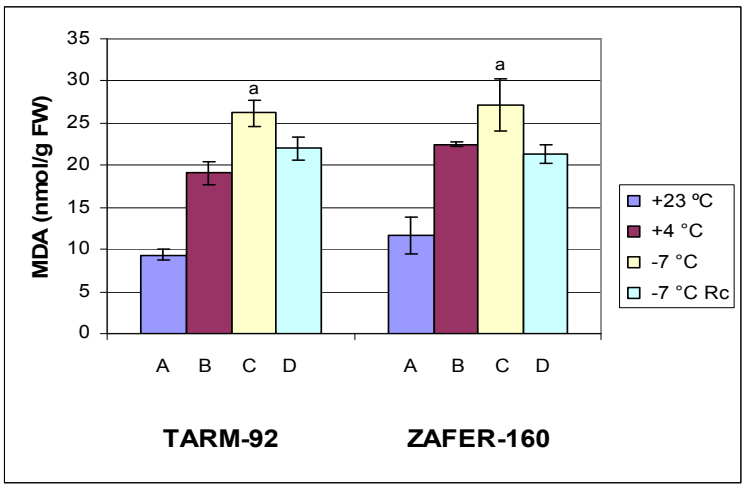

Figure 3.33. Root MDA content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control.

3.4 Electrolyte Leakage Test

Effect of cold stress exposure on electrolyte leakage from leaves of winter type, Tarm-92 and spring type cultivar, Zafer-160 were measured after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress applications.

3.4.1 Effect of +4 ºC cold stress treatment

+4 ºC cold stress treatment did not result in a meaningful change in electrolyte leakage of Tarm and Zafer leaves, but a slight, insignificant increase in electrolyte leakage was observed in leaves of both cultivars after 3 days of 4 ºC exposure. Effect of +4 ºC cold application on electrolyte leakage values was illustrated in Figure 3.34.

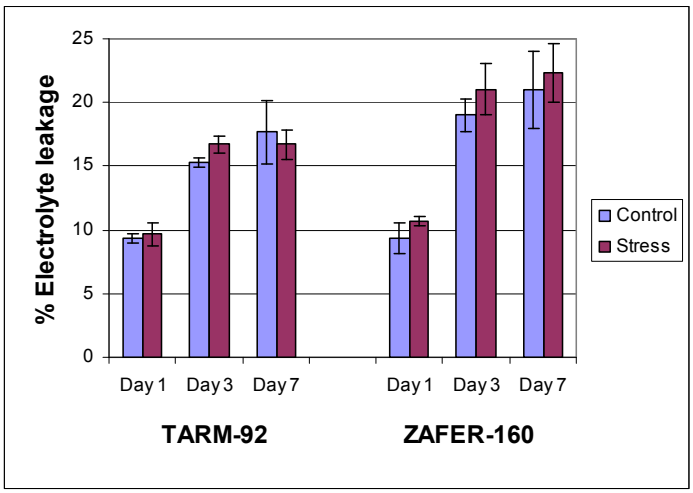

Figure 3.34. Leaf electrolyte leakage of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values.

3.4.2 Effect of -3 ºC and -7 ºC cold stress treatment

In Tarm cultivar, -3 ºC cold stress application did not have any effect on electrolyte leakage from leaves when compared with both +23 ºC and acclimated controls. However in Zafer cultivar, -3 ºC cold stress treatment led to an insignificant increase in electrolyte leakage compared to both +23 ºC and acclimated controls. In recovery period after treatment of -3 ºC stress, electrolyte leakage amount from leaves of Tarm remained same, but electrolyte leakage from leaves of Zafer slightly increased. Effect of -7 ºC cold stress application on electrolyte leakage was more obvious. Electrolyte leakage in both Tarm and Zafer cultivars remarkably increased after -7 ºC stress treatment compared to +23 ºC and acclimated controls. In Tarm, the increase in electrolyte leakage was statistically significant compared to +23 ºC control and in Zafer, the increase was statistically significant compared to +23 ºC and acclimated controls. After -7 ºC cold stress application, the electrolyte leakage from leaves continued to increase insignificantly in recovery period in both cultivars and the electrolyte leakage increase was higher in Zafer than in Tarm. Effect of -3 ºC cold stress treatment on leaf electrolyte leakage in Tarm and Zafer seedlings was shown in Figure 3.35 and effect of -7 ºC cold stress treatment on leaf electrolyte leakage was illustrated in Figure 3.36.

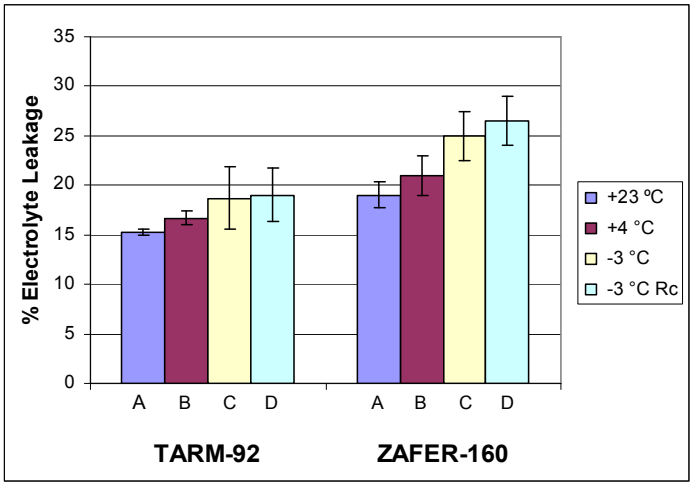

Figure 3.35. Leaf electrolyte leakage of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values.

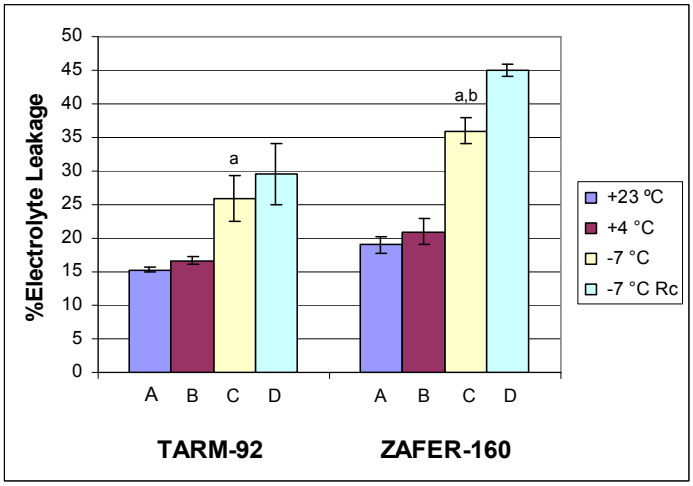

Figure 3.36. Leaf electrolyte leakage of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

3.5 Chlorophyll Fluorescence Analysis

In dark adapted leaf tissue, Chlorophyll fluorescence (Fv/Fm) of winter type, Tarm-92 and spring type cultivar, Zafer-160 were analyzed after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.5.1 Effect of +4 ºC cold stress treatment

+4 ºC cold stress treatment reduced the level of chlorophyll fluorescence (Fv/Fm) in leaves of Tarm and Zafer cultivars. The decrease in Fv/Fm value was not significant on the first day of +4 ºC application, but it was significant after 3 and 7 days of +4 ºC treatment in both cultivars (Figure 3.37). The reduction in chlorophyll fluorescence was higher in Zafer than Tarm.

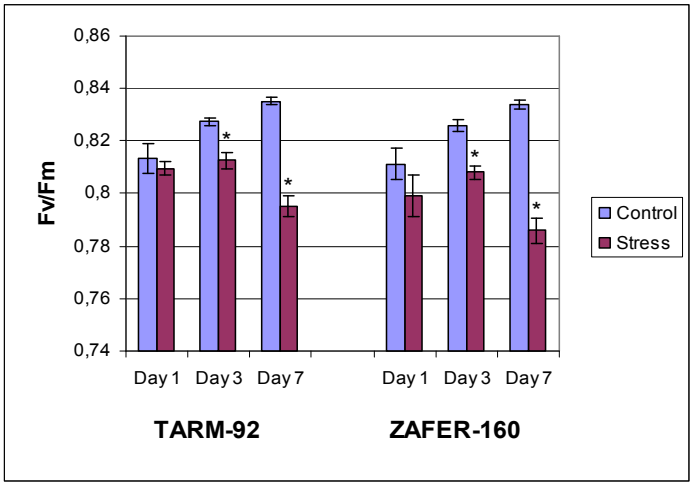

Figure 3.37. Leaf chlorophyll fluorescence (Fv/Fm) of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.6 Hydrogen Peroxide Content under Cold Stress

Effect of cold treatment on leaf and root hydrogen peroxide content of winter type, Tarm-92 and spring type cultivar, Zafer-160 were analyzed after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.6.1 Effect of +4 ºC cold stress treatment

Effect of +4 ºC cold stress treatment on hydrogen peroxide content in leaves of Tarm and Zafer was shown in Figure 3.38. Treatment of +4 ºC cold stress for 7 days caused a decrease in hydrogen peroxide content in leaves of both Tarm and Zafer cultivars, but 1 and 3 days of +4 ºC stress application did not result in an important decrease.

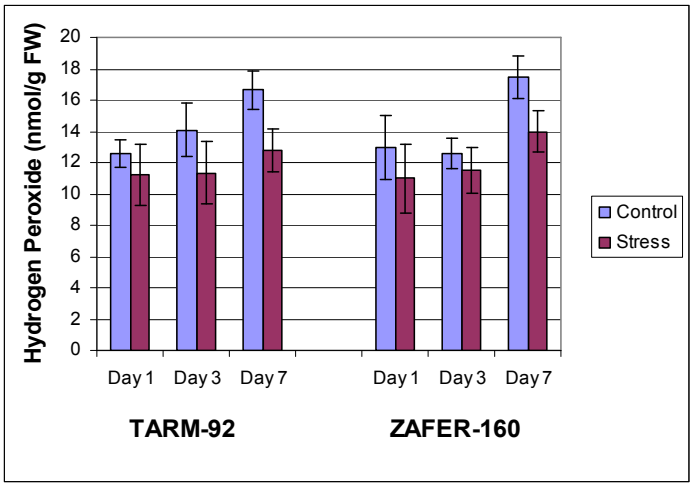

Figure 3.38. Leaf hydrogen peroxide content of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values.

Except for the slight increase on day 3, treatment of +4 ºC stress for 1 and 7 days did not cause a meaningful change in the roots of Tarm. Zafer roots increased the hydrogen peroxide content after 1, 3 and 7 days of +4 ºC stress treatment. The increase was significant on day 7. Hydrogen peroxide in roots of Tarm and Zafer cultivars after +4 ºC stress application was illustrated in figure 3.39.

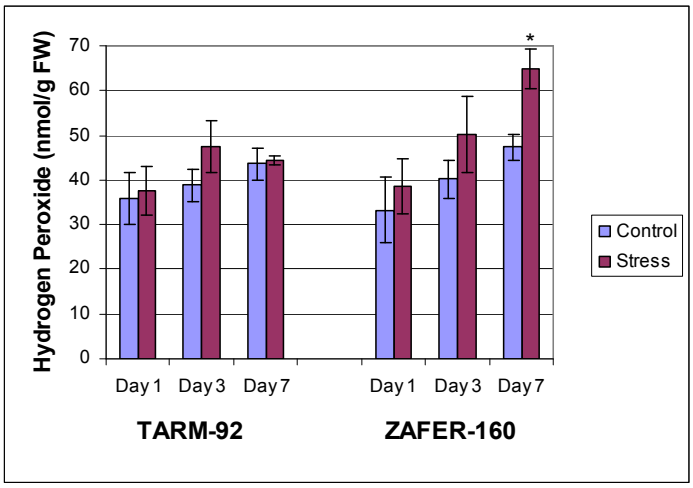

Figure 3.39. Root hydrogen peroxide content of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.6.2 Effect of -3 ºC and -7 ºC cold stress treatment

Hydrogen peroxide content in leaves increased after -3 ºC and -7 ºC cold stress treatment in both Tarm and Zafer cultivars. In Tarm leaves, -3 ºC cold stress application led to an increase in H_2O_2 compared to 23 ºC and acclimated controls. -7 ºC stress treatment also caused an increase in H_2O_2 Tarm leaves compared to 23 ºC and acclimated controls, but the increase after -7 ºC stress was significant. After both -3 ºC and -7 ºC cold stress treatments, amount of leaf H_2O_2 in Zafer significantly increased. Recovery period after -3 ºC cold stress did not have any effect on hydrogen peroxide level in leaves of Tarm. On the other hand, recovery period after -3 ºC stress treatment led to an elevation in leaf H_2O_2 content in Zafer cultivar. -7 ºC stress treated both Tarm and Zafer cultivars slightly increased leaf H_2O_2 content in recovery period. Zafer cultivar accumulated more leaf H_2O_2 than Tarm in recovery period after -7 ºC stress application. H_2O_2 change in Tarm and Zafer leaves after -3 ºC cold stress treatment was illustrated in Figure 3.40 and effect of application of -7 ºC cold stress was shown in Figure 3.41.

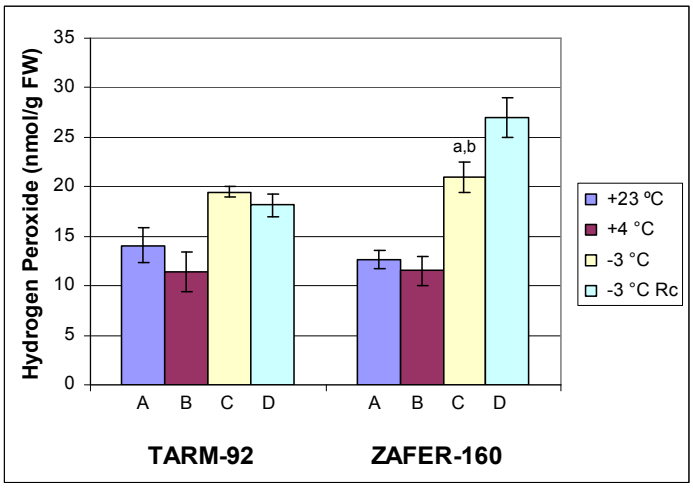

Figure 3.40. Leaf hydrogen peroxide content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

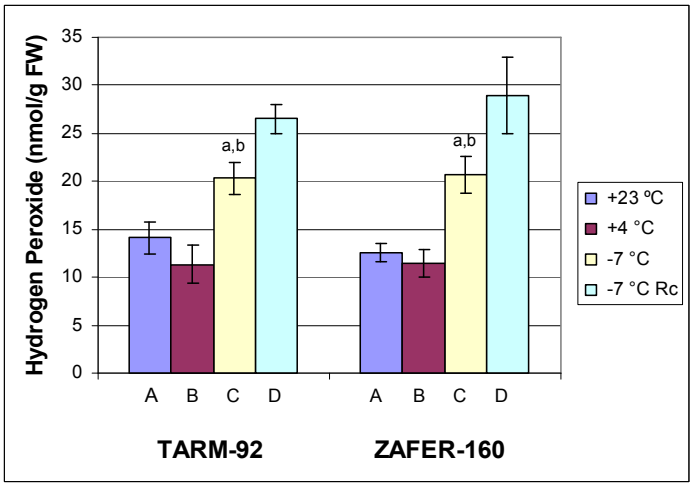

Figure 3.41. Leaf hydrogen peroxide content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

Similar to leaves, roots increased H_2O_2 content after application of -3 ºC and -7 ºC cold stress in Tarm and Zafer. The increase was significant with respect to 23 ºC controls in both cultivars, but it was statistically insignificant with respect to acclimated controls. In recovery period, -3 ºC stress treated Tarm and Zafer showed an increase in root H_2O_2 content and it was observed that the increase was statistically significant in Zafer roots. Recovery period after -7 ºC stress application caused a significant increase in roots of both Tarm and Zafer cultivars. H_2O_2 change in Tarm and Zafer roots after -3 ºC cold stress application was shown in Figure 3.42 and effect of treatment of -7 ºC cold stress was shown in Figure 3.43.

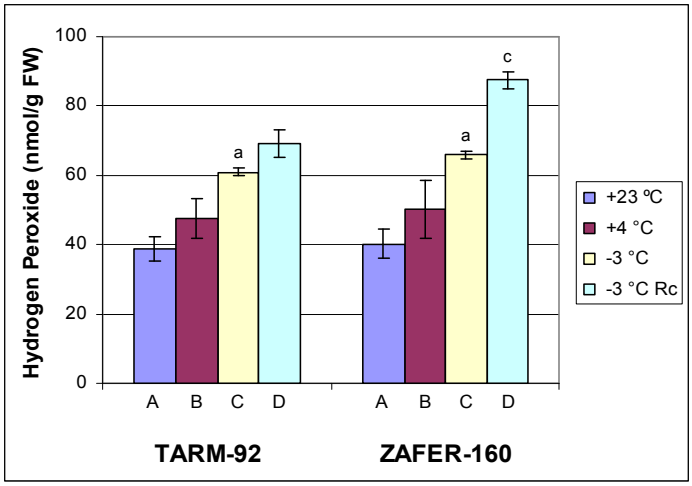

Figure 3.42. Root hydrogen peroxide content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

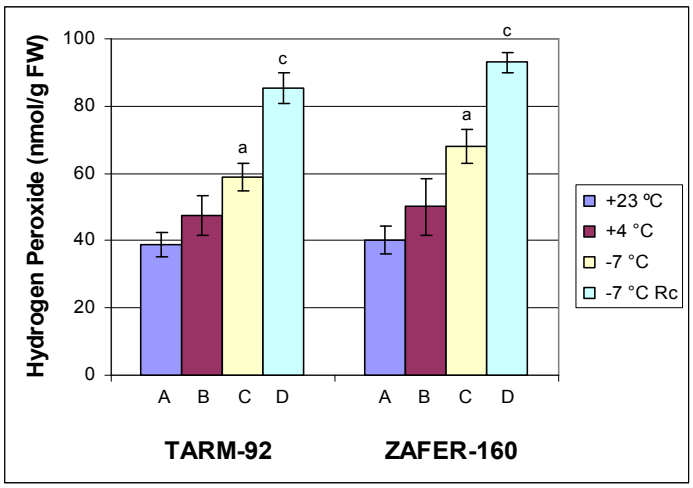

Figure 3.43. Root hydrogen peroxide content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

3.7 Total Soluble Protein Content under Cold Stress

Effect of cold treatment on leaf and root soluble protein content of winter type, Tarm-92 and spring type cultivar, Zafer-160 were determined after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.7.1 Effect of +4 ºC cold stress treatment

1,3 and 7 days of +4 ºC cold stress exposure did not cause a significant difference in leaf total soluble protein content of both Tarm and Zafer cultivars. Effect of +4 ºC cold stress treatment was shown in Figure 3.43.

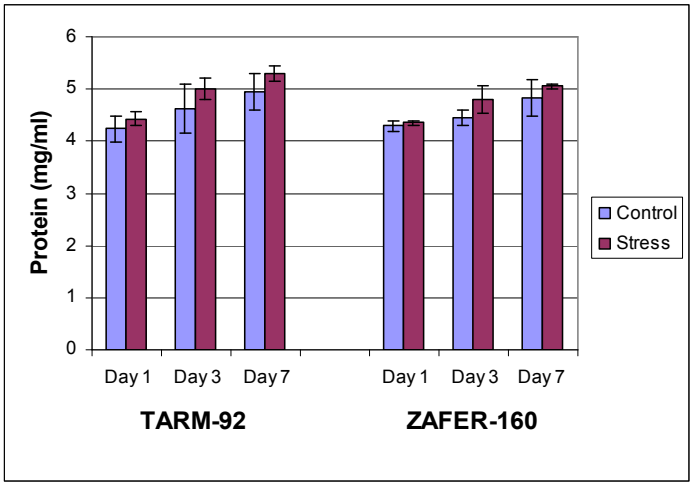

Figure 3.44. Leaf protein content of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values.

Similarly, treatment of +4 ºC for 1, 3 and 7 days cold stress exposure did not cause a significant difference in root total soluble protein content of both Tarm and Zafer cultivars. The effect of +4 ºC cold treatment on root total soluble protein was illustrated in Figure 3.45.

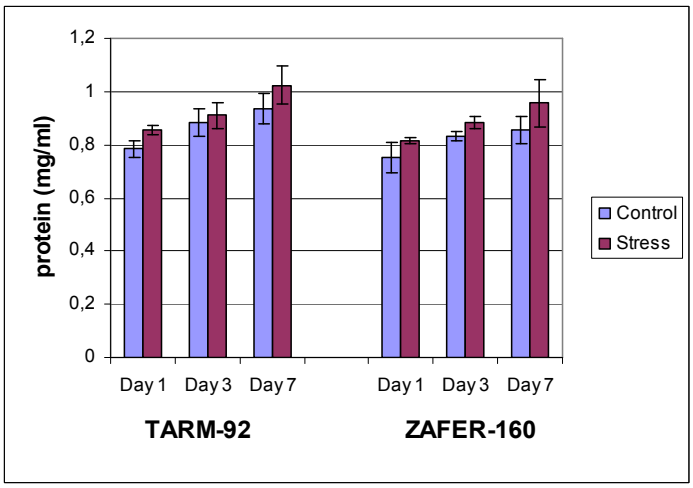

Figure 3.45. Root protein content of control and 1, 3 and 7 days of +4 ºC cold stress treated barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values.

3.7.2 Effect of -3 ºC and -7 ºC cold stress treatment

When compared with 23 ºC and acclimated controls, application of -3 ºC and -7 ºC cold stress did not cause a significant change in leaf total protein content in Tarm and Zafer. It was also observed that protein content in leaves of -3 ºC and -7 ºC treated Tarm and Zafer cultivars remained same after recovery period. Change in protein content of Tarm and Zafer leaves after -3 ºC cold stress application was shown in Figure 3.46 and effect of treatment of -7 ºC cold stress was shown in Figure 3.47.

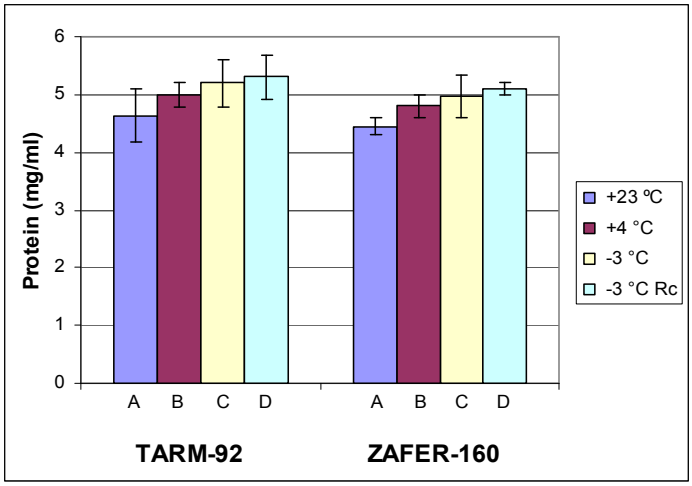

Figure 3.46. Leaf protein content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values.

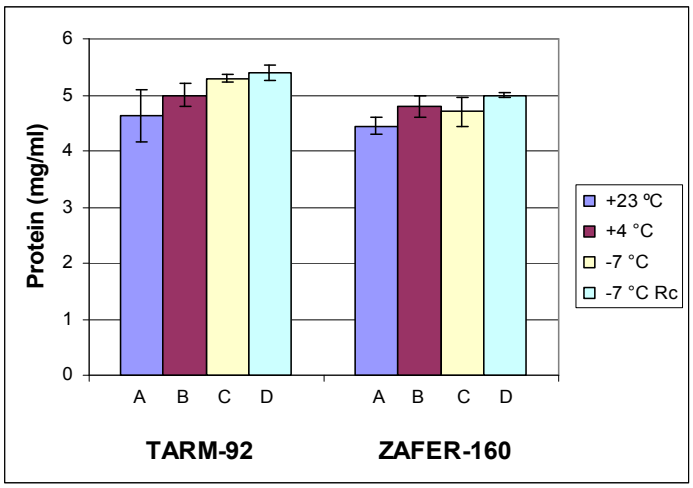

Figure 3.47. Root protein content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values.

-3 ºC and -7 ºC cold stress treatments did not result in a significant difference in root total protein content of Tarm and Zafer when compared with 23 ºC and acclimated controls. Moreover, recovery period after -3 ºC and -7 ºC cold stress applications did not have any effect on protein content of roots of both cultivars. Effect of -3 ºC cold stress treatment on root protein content in Tarm and Zafer seedlings was shown in Figure 3.48 and effect of -7 ºC cold stress application on root protein content was illustrated in Figure 3.49.

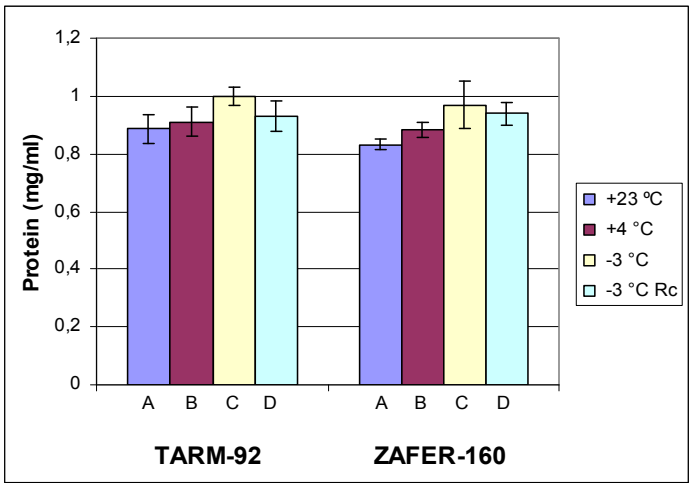

Figure 3.48. Root protein content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress applied plants treated with -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values.

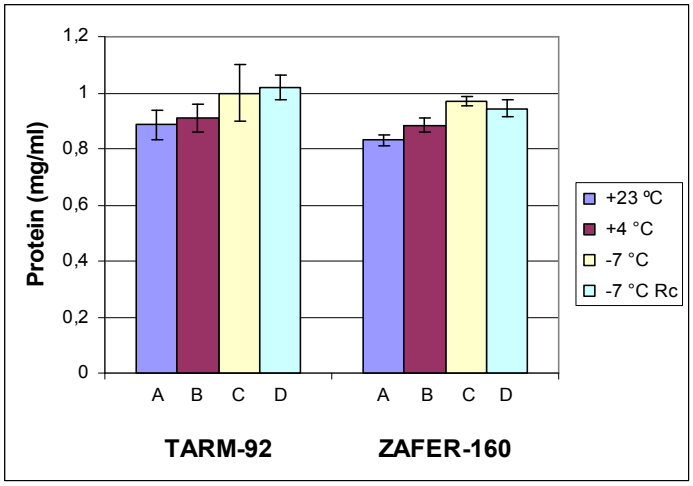

Figure 3.49. Root protein content of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values.

3.8 CAT Activity under Cold Stress

Effect of cold treatment on leaf and root CAT activity of winter type, Tarm-92 and spring type cultivar, Zafer-160 were determined after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.8.1 Effect of +4 ºC cold stress treatment

+4 ºC treatments for 1 day did not have any effect on catalase activity in both Tarm and Zafer. However, +4 ºC stress application after 3 and 7 days caused a decrease in leaf catalase activity in both cultivars and the decrease was significant on day 7 of +4 ºC stress treatment. Effect of +4 ºC cold stress treatment on leaf CAT activity of Tarm and Zafer was illustrated in Figure 3.50.

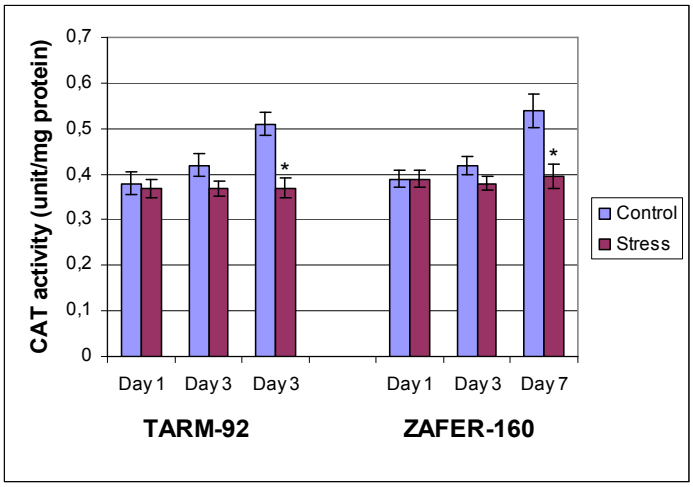

Figure 3.50. Leaf CAT activity of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

Effect of +4 ºC cold stress application on root CAT activity of Tarm and Zafer cultivars was shown in Figure 3.51. Treatment of +4 ºC chilling stress for 1, 3 and 7 days did not cause a meaningful change in root catalase activity in Tarm cultivar. Although 1 and 3 days of +4 ºC treatment did not affect root catalase activity of Zafer, 7 days treatment caused a statistically insignificant decrease in activity.

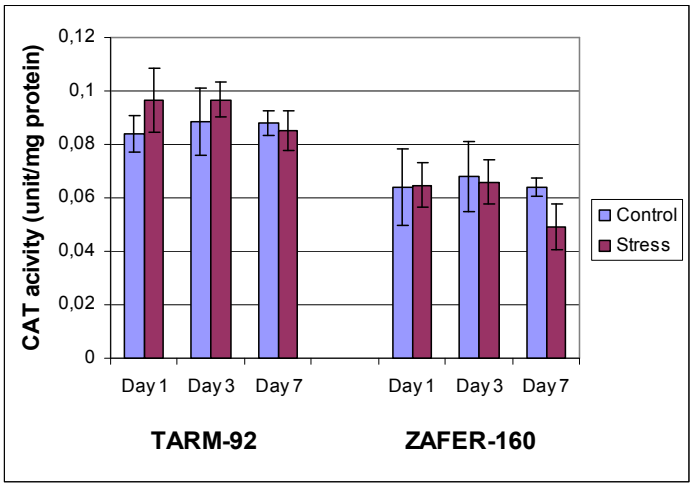

Figure 3.51. Root CAT activity of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values.

3.8.2 Effect of -3 ºC and -7 ºC cold stress treatment

When compared to leaf CAT activities of +23 ºC and acclimated controls, leaf CAT activities of -3 ºC and -7 ºC cold stress treated Tarm and Zafer cultivars were significantly reduced. After recovery period, -3 ºC stress applied Tarm seedlings were able to elevate CAT activity in leaf tissues. Moreover, the increase was significant. On the other hand, recovery period after -3 ºC stress treatment did not cause any change in activity of CAT in leaves of Zafer cultivar. It was observed that leaf CAT activity significantly decreased in recovery period after -3 ºC stress application in both Tarm and Zafer cultivar. Leaf CAT activity change in Tarm and Zafer cultivars after -7 ºC cold stress application was shown in Figure 3.52. Effect of -7 ºC cold stress treatment on leaf CAT activity of Tarm and Zafer was given in Figure 3.53.

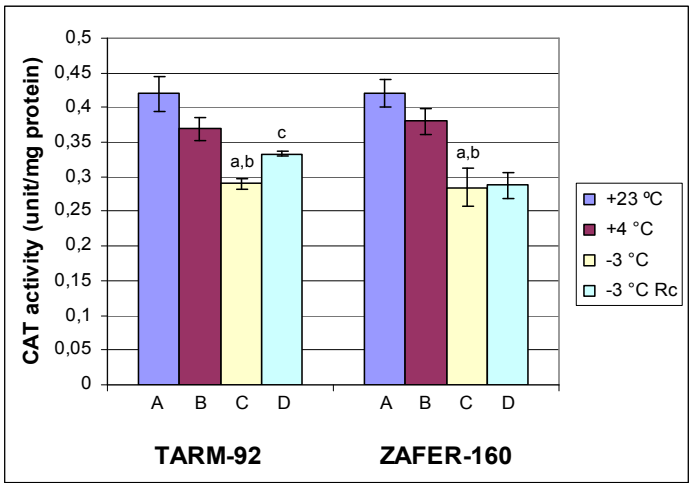

Figure 3.52. Leaf CAT activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

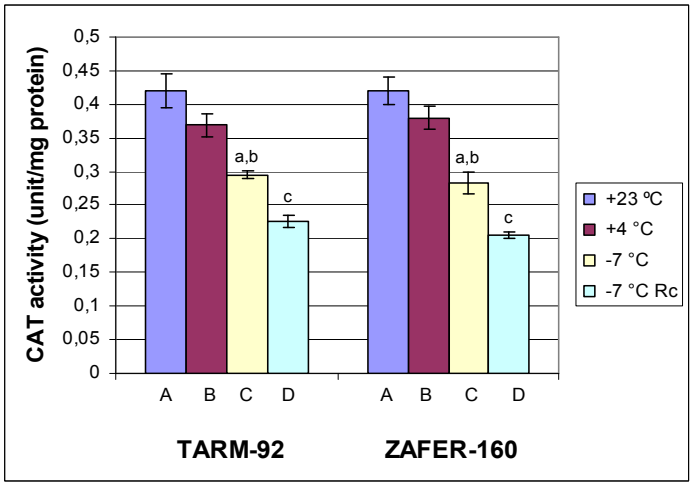

Figure 3.53. Leaf CAT activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

-3 ºC cold stress treated Tarm cultivar did not change root CAT activity compared to 23 ºC control, but root CAT activity decreased slightly when compared with acclimated control. Root CAT activity of Zafer cultivar did not show a difference compared to both 23 ºC and acclimated controls. After -7 ºC cold stress application, root CAT activity of Tarm and Zafer reduced when compared with 23 ºC and acclimated controls. The reduction in root Cat activity was higher and significant in Tarm than in Zafer. In recovery period after -3 ºC and -7 ºC cold stress applications, root CAT activity of Tarm and Zafer declined. The decrease in activity in recovery period was higher in Zafer cultivar after both -3 ºC and -7 ºC treatments. Effect of -3 ºC cold stress treatment on root CAT activity in Tarm and Zafer seedlings was shown in Figure 3.54 and effect of -7 ºC cold stress application on root CAT activity was illustrated in Figure 3.55.

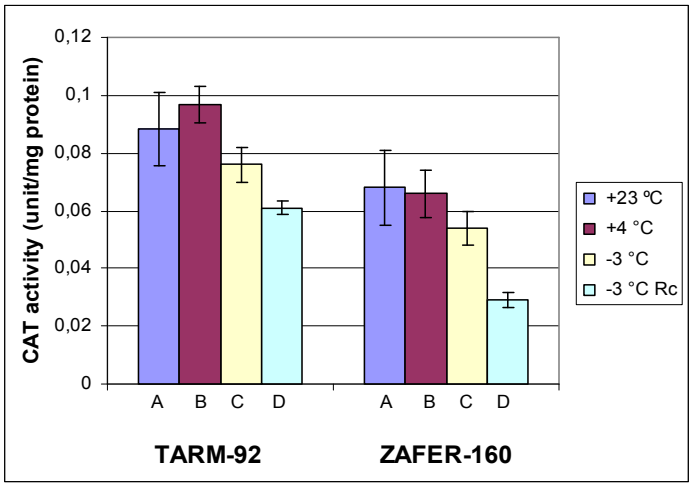

Figure 3.54. Root CAT activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values.

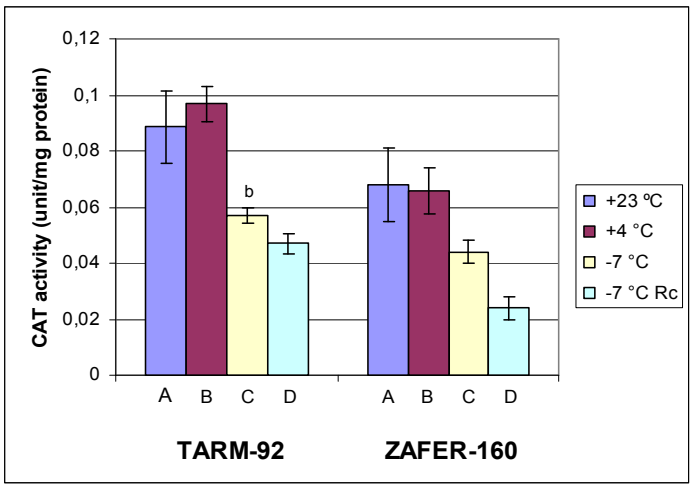

Figure 3.55. Root CAT activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

3.9 APX Activity under Cold Stress

Effect of cold treatment on leaf and root APX activity of winter type, Tarm-92 and spring type cultivar, Zafer-160 were determined after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.9.1 Effect of +4 ºC cold stress treatment

Although +4 ºC applications for 1 and 3 days did not cause a difference in leaf APX activity in Tarm and Zafer, +4 ºC stress application for 7 days resulted in a decrease in leaf APX activity in both cultivars. The effect of +4 ºC cold stress treatment for 1,3 and 7 days was illustrated in Figure 3.56.

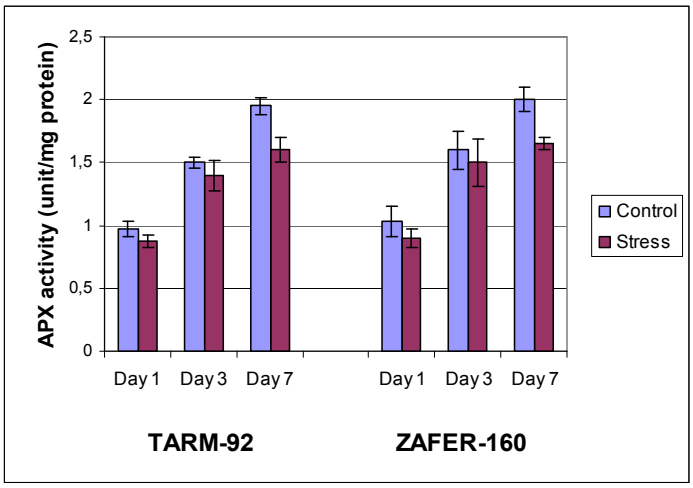

Figure 3.56 Leaf APX activity of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values.

Treatment of +4ºC cold stress for 1, 3 and 7 days did not cause a meaningful change in root APX activity of both Tarm and Zafer cultivars. The effect of +4 ºC cold stress application for 1, 3 and 7 days was shown in Figure 3.57.

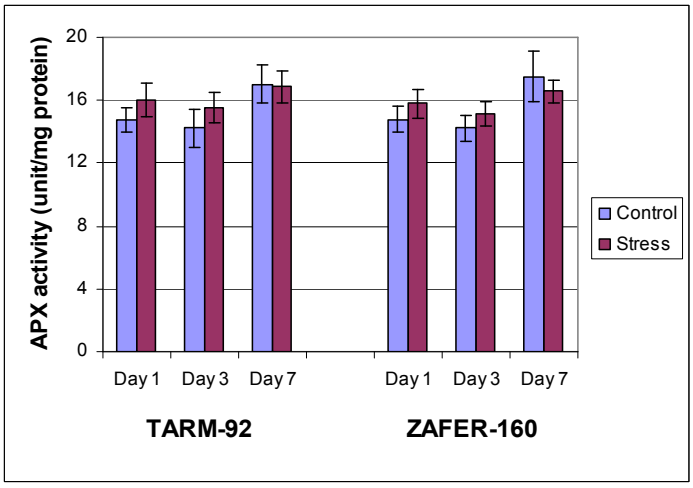

Figure 3.57.Root APX activity of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values.

3.9.2 Effect of -3 ºC and -7 ºC cold stress treatment

After treatment of -3 ºC and -7 ºC cold stresses, leaf APX activity showed an increase in Tarm cultivar when compared to 23 ºC and acclimated controls. -3 ºC stress application caused only a slight increase in activity of Zafer leaf compared to 23 ºC and acclimated controls. When compared to 23 ºC control, there was almost no effect of -7 ºC application on leaf APX activity of Zafer. Leaf APX activity of Zafer insignificantly increased when compared with acclimated control. No meaningful difference was observed in leaf APX activity of both Tarm and Zafer cultivars in recovery period after -3 ºC and -7 ºC cold stress treatment. APX activity change in leaves of Tarm and Zafer after -3 ºC was shown in Figure 3.58 and APX activity change in leaves of Tarm and Zafer after -7 ºC was illustrated in Figure 3.59.

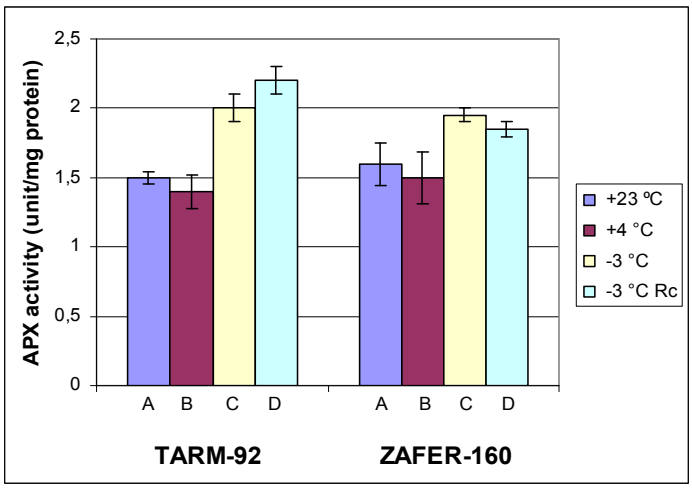

Figure 3.58. Leaf APX activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress applied plants treated with -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values.

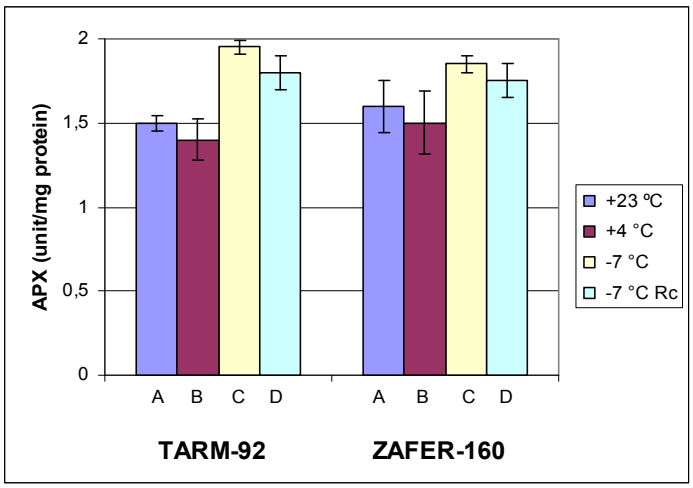

Figure 3.59. Leaf APX activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM

Treatment of -3 ºC and -7 ºC cold stress showed similar effects and significantly elevated root APX activity of both Tarm and Zafer cultivars compared to +23 ºC and acclimated controls. It was observed that no significant change in root APX activity was observed in recovery period after -3 ºC stress application in Tarm and Zafer. In contrast, recovery period after -7 ºC cold stress treatment caused significant decrease in root APX activity in both cultivars. Root APX activity measurements after -3 ºC stress application was shown in Figure 3.60 and root APX activity change after -7 ºC stress treatment was illustrated in Figure 3.61.

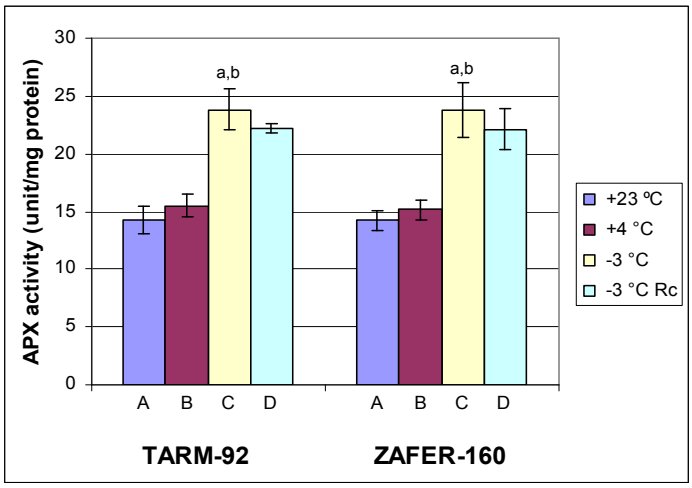

Figure 3.60. Root APX activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress applied plants treated with -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control.

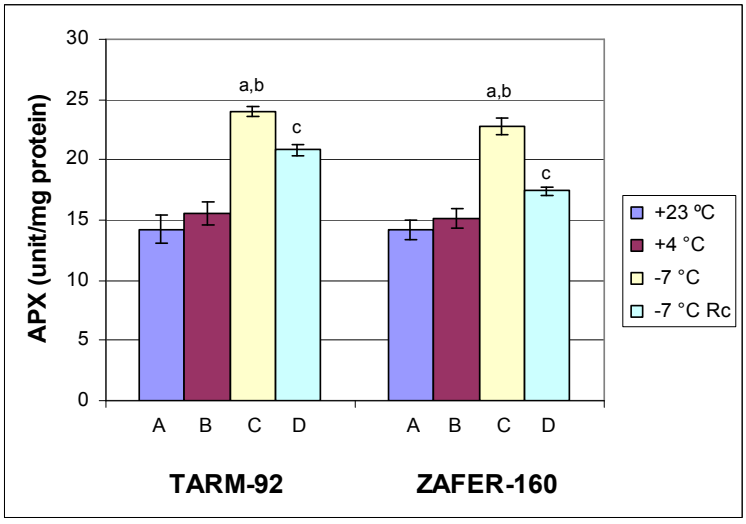

Figure 3.61. Root APX activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [b] shows significant difference ($p < 0.05$) with respect to acclimated control. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

3.10 GR Activity under Cold Stress

Effect of cold treatment on leaf and root GR activity of winter type, Tarm-92 and spring type cultivar, Zafer-160 were determined after 1, 3 and 7 days of +4 ºC cold stress, and -3 ºC and -7 ºC cold stress application.

3.10.1 Effect of +4 ºC cold stress treatment

1, 3 and 7 days of +4 ºC cold stress treatment elevated leaf GR activity in both Tarm and Zafer cultivars (Figure 3.62). Elevation in GR activity was significant after 3 days of +4 ºC application in leaves of Zafer. The increase on days 3 and 7 was significant and higher in Tarm leaves than in Zafer leaves.

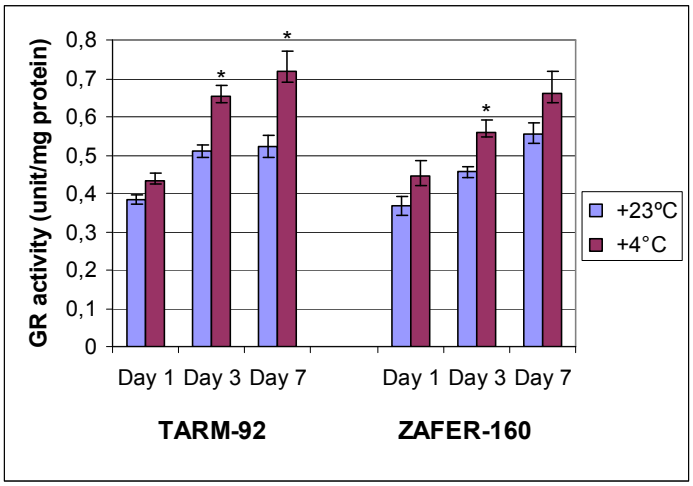

Figure 3.62. Leaf GR activity of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

GR activity in Tarm roots increased after +4 ºC cold stress treatment (Figure 3.63). The increase was significant after 7 days of stress application. In Zafer roots, there was no change in the GR activity after 1 day of + 4 ºC stress treatment, but the activity slightly increased after 3 and 7 days of + 4 ºC stress application.

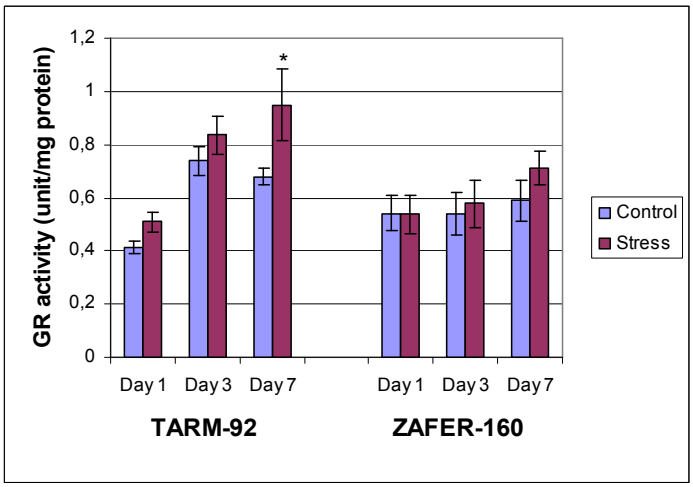

Figure 3.63. Root GR activity of control and 1, 3 and 7 days of +4 ºC cold stress treated two barley cultivars (winter type Tarm-92 and spring type Zafer-160). Vertical bars indicate ± SEM values. * Asterisk shows significant difference ($p < 0.05$) with respect to control.

3.10.2 Effect of -3 ºC and -7 ºC cold stress treatment

Compared to +23 ºC control, -3 ºC cold stress treatment significantly increased GR activity in leaf of Tarm, but it did not have any effect on GR activity in leaf of Zafer cultivar. -7 ºC cold stress application did not cause a change in leaf GR activity of Tarm and Zafer when compared with +23 ºC controls. There was also no meaningful change in leaf GR activity of -3 ºC treated Tarm and Zafer compared to acclimated controls. When compared with acclimated controls, -7 ºC cold stress application did not change leaf GR activity of Tarm, but it resulted in an insignificant decrease in leaf GR activity of Zafer cultivar. -3 ºC cold stress applied Tarm and Zafer cultivars significantly elevated leaf GR activity after recovery period. Similarly, -7 ºC cold stress treated Tarm cultivar significantly increased GR activity in leaf of Tarm. However, recovery period after -7 ºC stress application caused only a slight insignificant increase in root GR activity of Zafer. Effect of -3 ºC cold stress on leaf GR activity of Tarm and Zafer was shown in Figure 3.64 and effect of -7 ºC cold application on root GR activity of Tarm and Zafer was illustrated in Figure 3.65.

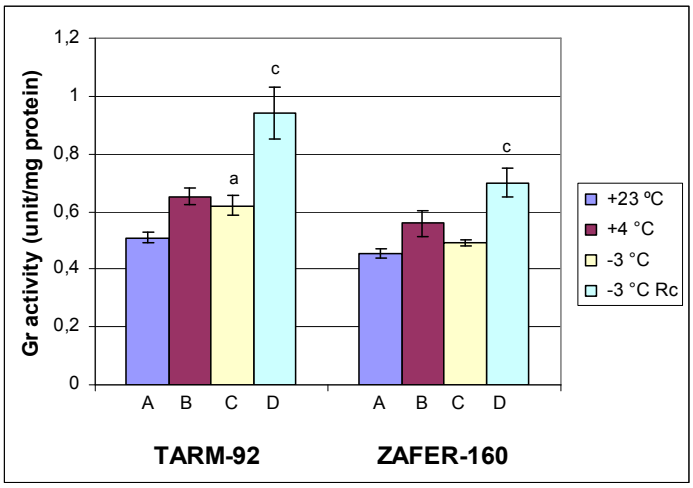

Figure 3.64. Leaf GR activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress applied plants treated with -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values. [a] shows significant difference ($p < 0.05$) with respect to +23 ºC control. [c] shows significant difference ($p < 0.05$) with respect to -3 ºC cold stress treated plants.

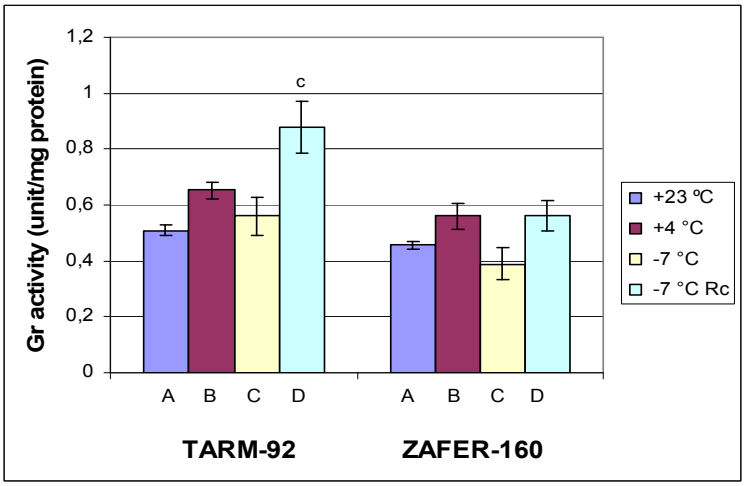

Figure 3.65. Leaf GR activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values. [c] shows significant difference ($p < 0.05$) with respect to -7 ºC cold stress treated plants.

GR activity in roots of Tarm and Zafer insignificantly decreased after -3 ºC stress treatment when compared to both 23 ºC and acclimated controls. The difference in root Gr activity between -3 ºC stress treated Tarm and acclimated control is more than the difference between -3 ºC stress treated Tarm and 23 ºC control. After -7 ºC cold stress application, no difference was observed in root GR activity of Tarm compared to 23 ºC control. Although Gr activity of Tarm root decreased after -7 ºC stress treatment, the decrease was lower compared to -3 ºC stress application. After application of -7 ºC stress, Gr activity of Zafer root reduced compared to both 23 ºC and acclimated controls. Recovery period after -3 ºC cold stress elevated root GR activity of Tarm and Zafer. The increase was more in Tarm cultivar than Zafer. Recovery period after -7 ºC cold stress increased root GR activity of Tarm and Zafer cultivars. In recovery period after -3 ºC cold stress, the increase was higher in recovery period after -7 ºC stress application. Root GR activity of Tarm and Zafer after -3 ºC cold stress application was shown in Figure 3.66 and GR activity in roots of Tarm and Zafer after -7 ºC stress treatment was illustrated in Figure 3.67.

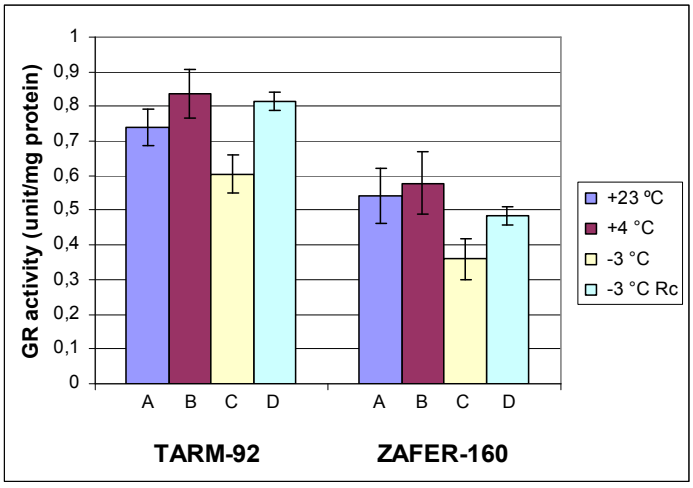

Figure 3.66. Root GR activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -3 ºC cold stress for 3 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -3 ºC cold stress. Vertical bars indicate ± SEM values.

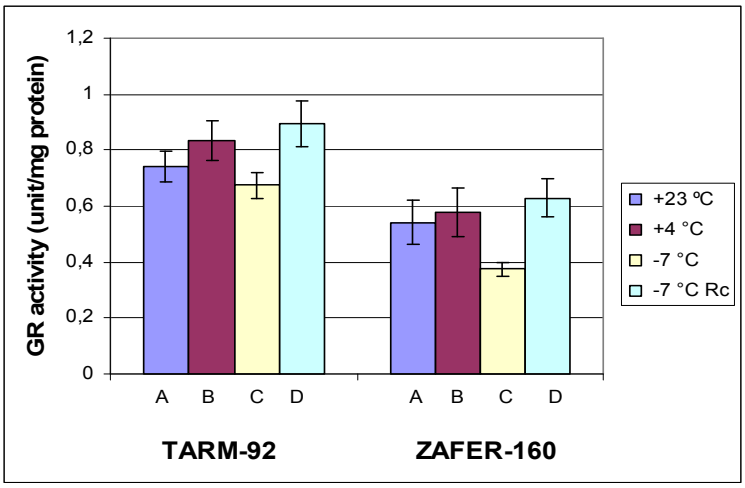

Figure 3.67. Root GR activity of two barley cultivars (winter type Tarm-92 and spring type Zafer-160), A; control plants grown at +23 ºC for 11 days, B; acclimated control plants grown at +23 ºC for 8 days and acclimated at +4 ºC for 3 days, C; -7 ºC cold stress applied plants first treated with -3 ºC cold stress for ½ hour and then with -7 ºC cold stress for 1,5 hours after acclimation D; recovery period applied plants for 4 days at +4 ºC after -7 ºC cold stress. Vertical bars indicate ± SEM values.

CHAPTER 4

DISCUSSION

Barley is the world's forth most important cereal after wheat, maize and rice. Althought it is known that barley is more tolerant than other cereals, extreme environmental stresses such as cold can adversely effect its growth and reduce crop production and quality (Stanca *et al.*, 1996). Cold stress has some similarities to drought stress, since in both situations water availability is reduced and plants suffer from dehydration (Ashraf and Foolad, 2007). Cold stress changes physiology and biochemistry of plants by slowing down metabolism (Foyer *et. al.*, 2002) reducing absorption of nutrients and water (Equiza *et al.*,1997), reduction of photosynthate flow through the phloem (Ercoli *et al.*, 2004), ROS production, dehydration, injury to membranes and photoinhibition (Xin and Browse, 2000). Hence, there are limited data in literature about the effect of cold on antioxidant mechanism of barley; we observed physiological changes, proline and MDA contents, electrolyte leakage, chlorophyll fluorescence (Fv/Fm), H_2O_2, levels, and antioxidant enzymes (CAT, APX,GR) in leaves and roots of winter typeTarm and spring type Zafer cultivars under chilling (+4 ºC cold stress) and freezing (-3 ºC and -7 ºC cold stresses) conditions.

4.1 Effect of cold stress on physiological parameters

In this study, both chilling and freezing stress treatments significantly decreased length, wet weight and dry weight of shoots and roots in winter type Tarm and spring type Zafer cultivars. In addition to growth retardation, freezing stress applications also caused wilting. The growth inhibition may be due to reducing absorption of nutrients and water (Equiza *et al.*, 1997). Although Tarm was able to recover, Zafer length, wet weight, and dry weight remained same in recovery period. Wilting symptom was more obvious after recovery period in Zafer than Tarm cultivar. These results may indicate a greater adaptation of the winter type Tarm compared to the spring type Zafer cultivar when exposed to cold stress.

Decreased shoot and root growth under cold stress has been shown in many different plant species. After 5 ºC chilling stress treatment, shoot fresh weight of spring wheat was found to be lower compared to control (Equiza *et. al.*, 1997). Chilling-sensitive maize cultivar decreased its root length while a chilling-tolerant cultivar maintained it during different low temperature treatments (Aroca *et. al.*, 2001). Short term cold application was observed to reduce leaf dry weight production in soybean (Wang *et al.*, 1997). In spring wheat, root dry weight accumulation was also decreased at 4 ºC compared to 20 ºC. On the other hand, winter wheat accumulated much the same way at 4 ºC compared to at 20 ºC (Karimzadeh *et al.*, 2000).Under water deficit, roots were found to be significantly shorter in barley, durum wheat, and rice and in young maize plant, it was observed that leaf elongation was significantly inhibited (Adda *et al.*, 2005). In this study, although Tarm cultivar was able to recover, length, wet weight, and dry weight of Zafer cultivar remained same in the recovery period. Wilting symptom was more obvious after recovery period in Zafer than Tarm cultivar. These results may indicate a greater adaptation of the winter barley compared with the spring cultivar when exposed to cold.

4.2 Effect of cold stress on proline content

Free proline is present in higher plants under normal conditions, but it accumulates in large quantities when exposed to extreme environmental conditions such as low temperatures. It is involved in osmotic adjustment, stabilization of membranes and proteins and scavenging free radicals (Ashraf and Foolad, 2007). Since proline offers protection against various environmental conditions, it has been extensively studied. Proline content was found to be rapidly increased in a barley cultivar exposed to +4 ºC chilling stress (Chu *et. al.*, 1973). Similarly, both winter type and spring type barley cultivars elevated proline content when exposed to low temperature stress (Murelli *et. al.*, 1995). On the other hand another, experiment with different barley cultivars showed that neither cold acclimation nor freezing exposure resulted in proline accumulation (Mazzucotelli *et. al.*, 2006). Accumulation of proline in winter wheat and potato increased tolerance against freezing. *A. thaliana* also accumulated proline in response to cold stress. (Xin and Browse, 2000). Proline accumulation in legumes indicated increased freezing

tolerance (Hekneby *et al.*, 2004). In wheat, drought tolerant cultivar accumulated and utilized more proline compared to senstitive cultivar when treated with drought stress (Ashraf and Fooland, 2007).

This study demonstrated that treatment of chilling, freezing and recovery period applications resulted in significant proline accumulation in leaves and roots of both winter type Tarm and spring type Zafer cultivars. Since there was no difference in leaf and total soluble protein content between stress treated and control barley cultivars, proline accumulation seems to be an indicator of chilling and freezing tolerance rather than a symptom of injury and protein degradation. Proline accumulation in shoots and roots may offer cold tolerance due to its involvement in cytosolic osmotic adjustment during water stress, preservation of protein structures and enzyme activities, scavenging of hydroxyl and singlet oxygen (Matysik *et.al.*, 2002).

4.3 Effect of cold stress on MDA content

ROS are produced as a result of extreme temperatures like chilling and freezing (Wang W. *et. al.*, 2003). They attack unsaturated double bonds of lipids in the biological membrane and results in lipid peroxidation and damage. Malondialdehyde (MDA) is one of the end products of lipid peroxidation and it indicates oxidative damage and cold sensitivity (Halliwell and Chirico, 1993).

According to our results, lipid peroxidation level and MDA content in leaves of winter type Tarm cultivar and spring type Zafer cultivar remained unchanged after +4 ºC chilling stress application. However, in literature application of chilling stress to rice and maize was found to increase lipid peroxidation and MDA levels in leaf tissue (Kuk *et al.*, 2003). Chilling sensitive *Coffea* plant also increased MDA content and electrolyte leakage after chilling treatment (Campos *et. al.*, 2003). In this study, both cultivars were able to protect their leaves against lipid peroxidation. Protection against lipid peroxidation in leaf tissue might be due to maintaining low levels of H_2O_2 and high levels of proline. Unlike chilling freezing stress resulted in serious lipid peroxidation and significantly increased MDA levels in leaves of both cultivars. In roots, all stresses caused significant increase in lipid peroxidation and MDA content in both winter and spring type barley. A study showed that freezing at

-8 ºC caused lipid peroxidation and increase in MDA content of cultured cells of alpine plant *Chorispora bungeana* at the early stages of stress treatment, but its level decreased at the later stages of stress exposure (Guo *et al.*, 2006). Chilling stress resulted in more severe membrane damage in roots than leaves of barley cultivars. Recovery period application did not have any effect on MDA levels in shoots and roots.

4.4 Effect of cold stress on electrolyte leakage

When plants are exposed to low temperatures, membrane permeability may increase due to decreased fluidity. Membrane fluidity decreases as a result of phase transition of membrane lipids from liquid to solid state. Membrane permeability may also increase due to peroxidation of membrane lipids. Increased permeability results in leakage of electrolytes from cells (Campos *et*. *al.,* 2003). The main cause of freeze-induced injury in plants is ice formation in the apoplast. Ice formation causes dehydration and distruption of cellular integrity (Mahajan and Tuteja, 2005).

Chilling sensitive *Coffea* plant increased electrolyte leakage and MDA content after chilling application (Campos *et*. *al.,* 2003). A study with barley showed that +5 ºC chilling stress treatment for 4 weeks caused an increase in electrolyte leakage in leaves (Plazek and Zur, 2003). On contrary, in this study chilling treatment did not cause a change in electrolyte leakage from leaves of Tarm and Zafer. This might be due to the fact that level of chilling injury is dependent on stress exposure time (Lee *et. al.*, 2002) and +4 ºC chilling stress for 7 days might not distrupt integrity of cell membranes. It is known that no obvious structural alteration and structural perturbation occur in cell membranes of chilling resistant plants (Lukatkin, 2003). In this study, the reason for the maintainance of integrity of membranes in leaf tissue after chilling stress can be also due to tolerance against chilling damage. Other than electrolyte leakage, the content of other stress indicators, MDA and hydrogen peroxide, remained same. Similarly electrolyte leakage was found to be only little after chilling application in chilling tolerant rice (Guo *et al.*, 2006).

Although electrolyte leakage in Tarm leaves was not different than control plants after application of -3 ºC freezing stress and recovery period, -7 ºC freezing stress severely damaged membranes of both Tarm and Zafer and significantly increased

electrolyte leakage from leaves. After recovery period, electrolyte leakage continued to increase insignificantly in both cultivars. So unlike chilling, freezing resulted in severe membrane damage in both cultivars. A study in literature also showed that freezing stress resulted in higher electrolyte leakage and membrane damage at -10 ºC than at -6 ºC in wheat seedlings. It is known that level of freezing injury is dependent on severity cold temperature (Lee *et. al.*, 2002). Another study with winter rape leaves and winter wheat tiller indicated that lowering the freezing temperature led to higher electrolyte leakage from cells (Prášil and Zámečík, 1998). This might be reason for higher electrolyte leakage and membrane damage in Tarm and Zafer leaves after -7 ºC stress compared to -3 ºC stress. It was observed that electrolyte leakage and membrane damage was more in spring type Zafer than winter type Tarm. Similar results were obtained with wheat cultivars. Winter type wheat showed higher tolerance than spring type wheat (Stupnikova *et. al.*, 2001).

4.5 Effect of cold stress on Chlorophyll Fluorescence

Photoinhibition is the reduction of photosynthesis in the presence of light. When plants suffer from chilling injury, photoinhibition of PS II takes place (Parkin *et al.*, 1988) The Fv/Fm ratio represents the maximum quantum yield of the primary photochemical reaction of PSII. It is an important parameter of the physiological state of the photosynthetic apparatus. PSII fluorescence by Fv/Fm method enables to observe photoinhibition and degree of chilling injury in plants (Kocheva *et al.*,2004). When +4 ºC chilling stress was exposed to one barley and two rice cultivars, all cultivars decreased Fv/Fm (Xu *et. al.*, 1999). Wheat seedlings also showed a decrease in Fv/Fm and photosynthetic activity under low temperature (Berova, 2002). In soybean, several cold treatments resulted in reduction in photosynthesis (Wang *et. al.*, 1997). Similarly in this study, +4 ºC chilling stress treatment led to significant reduction in Fv/Fm ratio which indicates photoinhibition in PS II in both Tarm and Zafer cultivars. However, reduction in Fv/Fm value was higher in spring type Zafer than winter type Tarm. This result showed that chilling injury to photosystem II was higher in spring type Zafer compared to winter type Tarm. The reduction in photosynthetic activity seems to be not related to H_2O_2 or MDA accumulation since their content remained unchanged after +4 ºC chilling stress application.

4.6 Effect of cold stress on H_2O_2 content

H_2O_2 is the most stable of the ROS and it can rapidly diffuse across cell membranes. At non-toxic concentrations, H_2O_2 can be a signaling molecule that mediates plant responses to a variety of biotic and adverse abiotic stress factors (Edreva, 2005). However, uncontrolled accumulation is toxic and can trigger pertubration of cellular redox state, damage to membrane lipids and DNA, finally cause cell death. In order to avoid damage under stressfull conditions, it is nessesary for plants remove H_2O_2 by APX and CAT (Guo, 2006).

Treatment of +4 ºC chilling stress did not result in significant leaf H_2O_2 accumulation in both cultivers. Leaf H_2O_2 content was significantly higher in Zafer after -3 ºC freezing stress. However, the increase was significant at -7 ºC in leaves of both cultivars. There was no significant difference in leaf H_2O_2 levels after recovery period. Accumulation of H_2O_2 after cold treatment was also detected in many plants like wheat (Janda et. al., 2003), *Arabidopsis* (Iba, 2002), and maize (Pastori *et.al.*, 2000). H_2O_2 accumulation in response to freezing was most probably due to decrease in leaf CAT activity in both Tarm and Zafer. It was observed that spring type Zafer accumulated more leaf H_2O_2 than Tarm. This result is consistent with higher electrolyte leakage in leaves of Zafer compared to Tarm.

Treatment of +4 ºC chilling stress did not cause a meaningful change in H_2O_2 content in roots of winter type Tarm. However, Zafer roots significantly elevated H_2O_2 level after 7 days of +4 ºC stress treatment. It indicates that while winter type Tarm roots showed tolerance to H_2O_2 accumulation, spring type Zafer could not avoid H_2O_2 accumulation when exposed to prolonged chilling conditions. Similar to leaves, roots increased H_2O_2 content after application of -3 ºC and -7 ºC freezing stress in both cultivars. Except -3 ºC stress treated Tarm cultivar, recovery period significantly increased leaf H_2O_2 in both cultivars. It was the situation in cucumber leaves and a marked increase in H_2O_2 content occurred after chilling stress and H_2O_2 level continued to increase after also post stress period (Lee and Lee, 2000). After freezing application, H_2O_2 accumulation was most probably due to decrease in leaf CAT activity in both cultivars. This might indicate higher tolerance of Tarm to cold. It is known that H_2O_2 increases more in sensitive seedlings than tolerant

cultivars. Chilling tolerant rice (Guo *et.al.*, 2006) and drought tolerant wheat (Sairam and Srivasta, 2001) increased more H_2O_2 than sensitive cultivars. For that reason, in this study accumulation of higher H_2O_2 in leaves and roots of Tarm compared to Zafer might indicate higher tolerance of cold in winter type Tarm.

4.8 Effect of cold stress on CAT activity

In plants, catalase (CAT) is found predominantly in peroxisomes (and also in glyoxysomes) where it is involved in the removal of H_2O_2 formed during photorespiration or during β-oxidation of fatty acids in glyoxysomes. Increase in CAT activity offers protection during extreme environmental conditions by reducing toxic levels of hydrogen peroxide produced.

In many studies it was observed that CAT activity is sensitive to extreme temperatures and generally, its activity is inhibited under low temperatures (Mittler, 2002). CAT activity in the leaves of a winter type barley was found to be lower after low temperature exposure (Janda *et. al.*, 2003). Similarly, in +5 ºC cold treated barley and rape significant decrease in CAT activity was obtained in leaves (Plazek and Zur, 2003). Chilling stress application gave rise to the inhibition in catalase activity in rice and cucumber (Lee and Lee, 2000). Freezing at -8 ºC decreased CAT activity at the early stages of stress application but thereafter elevated CAT activity of cultured cells of alpine plant *Chorispora bungeana* (Guo *et al.*, 2006).

We also observed both chilling and freezing stress treatment caused a significant decrease in leaf catalase activity in winter type Tarm and spring type Zafer cultivars After recovery period, -3 ºC stress applied Tarm seedlings managed to significantly increase CAT activity in leaf tissues (Figure 3.52). On the other hand, recovery period after -7 ºC stress treatment, it was observed that leaf CAT activity significantly decreased in both cultivars (Figure 3.53). These results suggest freezing treatment affected spring type Zafer more than winter type Tarm and lower freezing stress application led to more severe inhibition of CAT activity in both cultivars. Inactivation of leaf CAT after freezing and recovery period might be the reason for H_2O_2 accumulation. Inactivation of CAT may be due to the fact that CAT is light sensitive and suffers from photoinactivation with subsequent degradation in leaf tissue. In order to maintain a constant catalase level under light, its loss has to

be continuously compensated for by an adequate resynthesis of the enzyme. When, however, light degradation exceeds the capacity for repair or when resynthesis is impaired by inhibitors or by stress conditions such as low temperature, an obvious loss of leaf catalase is observed (Feierabend *et.al.*, 1992).

Treatment of +4 ºC chilling stress did not cause a meaningful difference in root catalase activity in both winter type Tarm and spring type cultivar Zafer cultivar Except the significant decrease after -7 ºC stress treatment neither freezing stress nor recovery period application caused a significant difference in root Cat activity. It indicates that root CAT activity was not affected from cold stress and maintained enough activity. It can be because photoinactivation of CAT occurs in leaf tissue (Feierabend, 1996), low temperature might not affect root CAT activity.

4.9 Effect of cold stress on APX activity

APX is an enzyme of ascorbate-glutathione cycle and is involved in H_2O_2 removal. Although APX is found in all cellular components, is most predominant in cytosol, and chloroplasts. It utilizes ascorbate as an electron donor in the scavenging of H_2O_2. APX has a smaller Km value than CAT and for that reason it has a higher affinity for H_2O_2 (Palatnik *et al.*, 2002).

Low temperature exposure resulted in different responses in APX activity with different plants. For instance cold application caused an enhancement in APX activity in leaves of winter type barley (Janda, 2003). CAT deficient barley mutant showed higher expression of APX (Palatnik *et. al.*, 2002). Cold treatment at 5 ºC under normal light intensity induced APX activity in a winter wheat (Janda *et al.*, 2007). The expression level of APX gene was increased after 1 week of 2 ºC chilling exposure in both winter type and spring type wheat cultivars (Baek and Skinner, 2003). It was also observed that chilling stress elevated APX activity in *Arabidopsis* and its subcucumber (Lee and Lee, 2000). When exposed to chilling or drought, APX activity increased in both drought and chilling tolerant rice cultivars. On the contrary, it decreased in both sensitive cultivars under chilling or drought stress (Guo *et. al.*, 2006). Freezing treatment at -8 ºC resulted in higher APX activity at the early stages of stress application, but thereafter decreased APX activity of cultured cells in alpine plant *Chorispora bungeana* (Guo *et al.*, 2006).

There was no significant change in leaf APX activity after stress and recovery applications in this study. However, after treatment -3 ºC (Figure 3.60) and -7 ºC freezing stresses (Figure 3.61), root APX activity showed a significant increase in both Tarm and Zafer cultivars. Although no significant change in root APX activity was observed in recovery period after -3 ºC stress application, recovery period after -7 ºC freezing stress caused significant decrease in root APX activity in both cultivars recovery period cold stress treatment in both cultivars. The increase in root APX activity in our study may be due to H_2O_2 accumulation since that high cellular level of H_2O_2 can increase APX activity (Lee and Lee, 2000). Since root CAT activity remained constant, H_2O_2 removal from roots seems to be achieved by APX. It was reported that induction of APX may have a more dramatic effect on the protection of plants against cold stress when compared with catalase, since H_2O_2 generated at the intercellular space appears diffuse first into the cytosol in which cytosolic APX is localized and only then into peroxysome in which catalase is located and in addition to these APX has a higher affinity for H_2O_2 compared to CAT (Lee and Lee, 2000).

4.10 Effect of on GR activity

Glutathione reductase (GR) is found in chloroplasts, mitochondria and cytoplasm and catalyzes rate-limiting last step of ascorbate-glutathione cycle and necessary for ascorbic acid regeneration. Under stress conditions the ratio of GSH/GSSG decreases, so GR is important for the maintenance of reduced form of glutathione in the cell at high levels (Arora *et al.,*2002).

There are several studies with GR under cold and drought stress. Low temperature treatment increased leaf GR activity of a winter type barley (Janda, 2003). The expression level of GR gene was increased after 2 ºC chilling exposure in both winter type and spring type wheat (Baek and Skinner, 2003). It has been also suggested that chilling stress increased GR activity in *Arabidopsis* and cucumber (Lee and Lee, 2000). Aluminium induced drought increased GR gene expression in barley roots (Támas *et. al.*, 2006). Application of freezing at -8 ºC elevated GR activity at the early stages of stress treatment, however then decreased GR activity of cultured cells of alpine plant *Chorispora bungeana* (Guo *et al.*, 2006). Wheat

cultivars increased GR activity in leaves after drought treatment, but the highest activities were observed in drought tolerant cultivars (Sairam and Srivastsva, 2001).

After +4 ºC chilling stress treatment, elevation in leaf GR activity was significant and higher in winter type Tarm leaves than in spring type Zafer leaves (Figure 3.62). After freezing stress application, except the significant increase in -3 ºC treated Tarm (Figure 3.64), there was no significant change in leaf GR activity. Except -7 ºC treated Zafer leaf (Figure 3.65), GR activity significantly increased after recovery period. GR activity in Tarm roots significantly increased after +4 ºC chilling stress treatment. There was no significant change in root GR activity of chilling stress treated Zafer and freezing stress applied Tarm and Zafer. These results suggest that, after cold stress exposure GR activity in leaves increased more than GR activity in roots. The GR activity may be induced due to H_2O_2 accumulation (Lee and Lee, 2000). Glutathione is not only important in ascorbate-glutathione cycle but also for stabilizing enzymes and scavenging of free radicals (Wise and Naylor, 1987). In literature, Higher GR activities were observed in the leaves of the relatively chilling tolerant *Zea diploperennis* than in the relatively chilling sensitive Z. mays (Pastori *et.al.*, 2000). Similarly, after chilling treatment, GR activity increased in and chilling tolerant rice cultivars. However, it decreased in both sensitive cultivars under chilling or drought stress (Guo *et. al.*, 2006). For that reason having a higher GR activity in winter type Tarm than spring type Zafer may indicate that winter type Tarm was more successful in maintaining reduced form of glutathione and protection against cold stress.

After +4 ºC chilling stress treatment, elevation in leaf and root GR activity was significant in winter type Tarm. After -3 ºC freezing treatment, Tarm significantly increased leaf GR activity Tarm. Except -7 ºC treated Zafer leaf, GR activity significantly increased after recovery period. There was no significant change in root GR activity in neither chilling stress treated Zafer nor in freezing and recovery period applied Tarm and Zafer.

CHAPTER 5

CONCLUSION

In this study, physiology and biochemistry of two Turkish barley cultivars, winter type Tarm-92 and spring type Zafer-160, were investigated under different cold stress applications. For this purpose, physiological parameters, oxidative stress evaluating parameters and antioxidant enzymes were analyzed.

The physiological parameters suggested that shoot and root of both cultivars exhibited growth retardation. While winter type Tarm was able to recover after freezing stress, spring type Zafer was not. Cold severely inhibited PS II in both cultivars.

It is known that cold stress causes osmotic stress in cells. Both cultivars accumulated high levels of osmoprotectant in leaves and roots. This may prevent dehydration and moreover help free radical scavenging and protect cell integrity.

There was no effect of chilling stress on membranes of leaves. On contrary, lipid peroxidation and damage was higher in roots. After freezing stress application, membranes in both leaves and roots were damaged. Membrane injury seems to be higher in spring type Zafer cultivar.

Cold stress affected antioxidant enzyme activities. H_2O_2 removal after freezing stress seems to be better performed in roots than leaves. High GR activity in leaves maintains reduced GSSG pool and it may offer protection against cold.

This study indicated that effect of cold on barley cultivars was more severe at freezing temperatures than at chilling temperature. Cold dependent injury was more obvious as the duration of chilling temperature increased. It was also

observed that when exposed to cold temperatures growth retardation, membrane damage, leaf CAT deactivation were more obvious and increase in leaf GR activity was less in spring type cultivar Zafer than in winter type Tarm. The resultes indicated that the winter type barley cultivar, Tarm-92 exhibited greater tolerance against cold stress than the spring type barley cultivar, Zafer-160.

The results in this study may be used in screening of cold stress tolerant barley cultivars.

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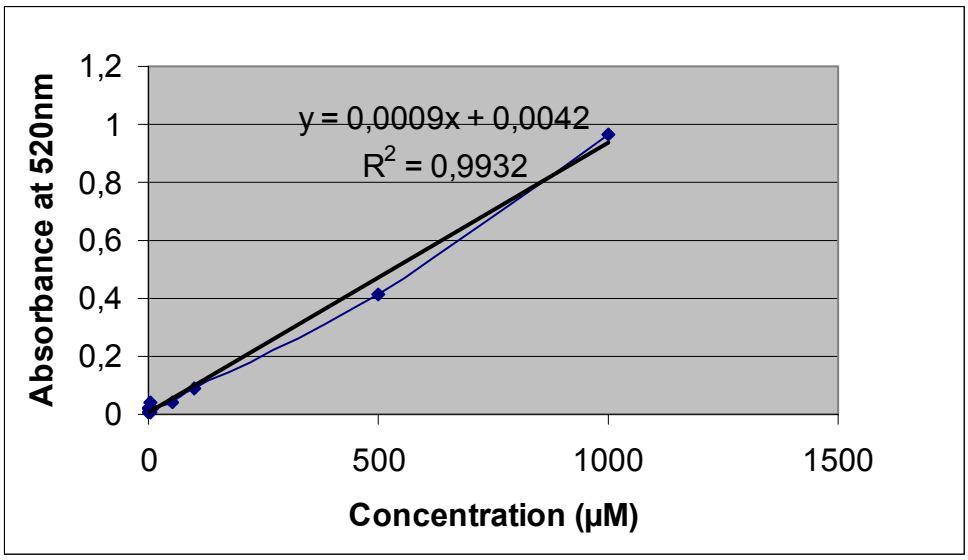
APPENDIX A

PROLINE STANDARD CURVE



APPENDIX B

HYDROGEN PEROXIDE (H_2O_2) STANDARD CURVE