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# Effects of low temperatures on the anti-oxidants activity of root in sensitive and tolerant genotypes of Barley (*Hordeum vulgare* L.)

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**Key words:** Catalase (CAT), $H_2O_2$, MDA, Peroxidase (POX), Proline.

**Abstract**

Cold stress causes a disturbance in micro-organelles, transport of lipids in the plasma membrane, and production of reactive oxygen species (ROS) in plants, as well as growth and development prohibition, which subsequently, reduce yield and production. To investigate the effect of cold stress on the activity of anti-oxidants and the following damages in two sensitive (aths-38) and tolerant (EC83-1215) barley genotypes, a factorial experiment based on randomized complete block design with three replicates was conducted under controlled conditions. At seedling stage, the cold stress of 4°C was applied for 48 h. After sampling, the anti-oxidants enzymes involved in the defense mechanisms were analyzed in roots. The results indicated that stress has significantly increased the quantity of proline, hydrogen peroxide ($H_2O_2$), malondyaldehid (MDA) and catalase whereas cold stress was significantly reduced peroxidase (POX). Amount of $H_2O_2$ and POX was lower in the roots of sensitive than the tolerant genotypes. In contrast, catalase was synthesized more in sensitive plants than of the tolerant counterparts.

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**Introduction**

Cold stress induces the overproduction of ROS whenever plant encounters low temperatures and freezing conditions. Over-production of ROS including Superoxide anion radical (O-2), $H_2O_2$ and Hydroxiyle radical (OH-) carries with it lipid peroxidation, enzyme inactivation and damage to nucleic acid molecules and cell membrane collapse (Bailly, 2004), imposing a second type of stress namely axidative stress (Desikan *et al.,* 2004).

Barley (*Hordeum vulgare* L.) is considered important for being used in feeding livestock, human nutrition and its use in brewery. It is mostly cultivated in marginal and non-fertile soils, where abiotic stresses including cold might confine its growth and development (Baum *et al.,* 2004). Roots constitute an important part of plant. They not only are responsible for water uptake, synthesis, food transfer and storage, but also influence the plans vegetative growth. Roots also form the first sensory system in plant and are damaged first under different abiotic stresses. Susceptibility of roots to stress jeopardizes the life of the whole plant (Steppuhn and Asay, 2005). Generally at early stages of growth, a very strong, well-built root system ensures the later growth of the plant (Richner *et al.,* 1997), hence important.

Anti-oxidant enzymes play vital roles in mounting plant tolerance to cold (Li and Zhang, 2012), especially peroxidase (POX) and catalase (CAT) protect plasma membrane against critical ROS damage, by scavenging $H_2O_2$ molecules (Bowler *et al.,* 1992). It is now generally accepted that the degree of peroxidation of the plasma membrane's lipids by free radicals reflects the severity of the damage on the cellular level. Accordingly, the amount of MDA, as the result of peroxidation of the lipids in plasma membrane, has been used as an indication of the size of the oxidative damage (Jain *et al.,* 2001). Besides osmoregulation properties, anti-oxidative role of proline in mitigating the damage has also been established (Matysik *et al.,* 2002). Most plants stock high levels of proline in response to a myriad biotic and abiotic stresses.

Proline also functions as a signal/regulating factor which triggers several molecular and physiological mechanisms (Aghaee *et al.,* 2013).

So far, the majority of researches done in plants concern leaves. Information on behavior pattern of roots, especially on oxidative stress imposed by cold (regarding pathways, mechanisms of the damages and treating the damages), is scares.

Although leaves and roots possess alike enzymatic systems, cooperation between the enzymes, anti-oxidative pathways and their expression in roots differ from those in leaves, especially information is scares on cytoplasmic anti-oxidant mechanisms and other root cellular organelles regarding cold stress-induced oxidative stress.

The objective of this study was to investigate oxidative defense system, lipid peroxidation and amount of protein in roots of two contrasting (sensitive and tolerant) barley genotypes under cold stress.

**Materials and methods**

Two genotypes-cold tolerant (EC83-1215) and cold sensitive (aths-38) were chosen as genetics materials after preliminary evaluations (data not shown) in greenhouse. Cold stress along with control plants were studied through a factorial experiment based on complete randomized block design with three replicates.

After the initial germination on filter paper, seeds were planted in PVC tubes of 15 cm diameter and 50 cm length containing fine sand and perlite (ratio 10:1) under 70% humidity, 16 hours daylight and 25 °C and irrigated regularly up to three-leaf stage. Afterwards, cold stress at 4 °C was imposed to a number of randomly chosen PVC tubes in a freezing test machine for 48 hours. At this point, the roots were sampled and immediately were frozen in liquid nitrogen and after transfer to laboratory stored at -80 °C.

*Chemical variables measurement*

*$H_2O_2$*

Amount of $H_2O_2$ was measured according to (Gong *et al.,* 2005). 0.2 gr of the specimens were homogenized in liquid nitrogen and a solution of 0.1 trichloroacetic acid (TCA) (V/W). The homogenous solution was centrifuged at 12,000 g for 15 minutes. 0.5 mL of 10 mol potassium phosphate buffer and 1 mL of 10 mol potassium iodide was added to 0.5 mL of the supernatant. Absorbance of the solution was read ant 390 nm. Amount of hydrogen peroxide was determined using a standard curve for pure hydrogen peroxide.

*MDA*

Level of MDA was measured based on reactive thiobarbituoric acid's amount. 0.5 gr from root specimen was homogenized in 0.1 TCA. The solution was centrifuged at 15,000 g for 5 minutes. 4 mL of 0.5% thiobarbituoric acid was added to 20% TCA (W/V). The mixture was heated at 95 ℃ for 3000 min. Then, it was immediately cooled on ice. After being centrifuged at 10,000g for 10 min, absorbance of the supernatant was read at 532 nm, from which, non-specific, general absorbance at 600 was subtracted. The total amount of thiobarbituoric acid was calculated using absorbance coefficient 155 $cm^{-1}$ $mmol^{-1}$ (Stepien and Klobus, 2005).

*CAT activity measurement*

Activity of CAT was measured according to (Aebi, 1984). 0.5gr of the root specimen was homogenized in cold potassium phosphate 0.1 mol (PH=7.5) containing 0.5 mmol Ethylenediaminetetra acetic acid (EDTA). Homogenized samples were centrifuged at 15,000g for 15 min. 0.5 mL from the supernatant was added to a solution of 1.5 mL potassium phosphate 0.1 mol (PH=7) and 1.45 mL double distilled water. The reaction started by adding 0.5 mL of $H_2O_2$ 75 mol. The reduction in absorbance was read at 240 nm for a minute.

*POX activity measurement*

For POX, activity was evaluated according to (MacAdam *et al.,* 1992). 0.5 gr of the root specimen was homogenized in cold potassium phosphate buffer 0.1 mol (PH=7.5) containing 0.5 mmol EDTA. Homogenized samples were centrifuged at 15,000g for 15 min. 20 µL of the homogenized supernatant was added to 0.81 mL potassium phosphate buffer 0.1 mol (PH=6.6). 90 µL of 1% guaiacol was added to the result. The solution was poured in a cuvett. And, 90 µL of 3% $H_2O_2$, as the electron receptor, was added just before measuring the reaction speed. Absorbance was measured at 470 nm at 25 ℃ for 60 seconds.

*Proline measurement*

The proline quantity in roots was measured according to (Bates *et al.*, 1973). 0.2 gr from the specimen was grinded to a fine powder, then, homogenized in 5 mL of 3% sulfosalicylic acid. The extract was, then, centrifuged at 6,000g at 25 ℃ for 7 min. The liquid phase was separated. 1 mL of the supernatant was mixed with 1 mL of ninhydrin acid and 1 mL of glacial acetic acid. The samples were placed in a 100 ℃ bath with gentle strokes for an hour. They were allowed to dry in an ice bath for 5 min. 2 mL toluene was added to the samples. Then, the amount of absorbance was read at 520 nm. Data were analyzed using MSTATC software.

**Results and discussion**

Analysis of variance (ANOVA) regarding investigated traits in the two sensitive and tolerant barley genotypes is provided in Table 1.

Stresses as a whole carry variety of changes in reactions in plants, from modifying gene expression and cellular metabolism to shift the pace of growth and development. Upon stress, increased production of ROS damages the very principle cellular macromolecules- proteins, lipids, nucleic acids- and disrupts cellular plasma membrane. Likewise, abiotic stresses cause the over-production of ROS, which in high concentrations are toxic to the cell (Bailly, 2004). Hurt is inevitable when capacity of anti-oxidant enzymes and detoxification mechanisms is much lower than the amount of ROS being produced (Abavisani *et al.,* 2013). Plants employ many defense barriers including scavengers and non-enzyme anti-oxidunts. Such mechanisms lower the pace of bio-molecular

oxidation or may even stop its development, by blocking oxidative cyclic reaction (Sgherri *et al.,* 2003). Cold tolerant plants possess more efficient anti-oxidative mechanisms than sensitive ones, which enables them to synthesize anti-oxidants able to offset harmful effects of ROS (Dionisio-Sese and Tobita, 1998).

**Table 1.** Analysis of variance for Proline, $H_2O_2$, MDA, CAT and POX.

| S.O.V. | d.f. | $H_2O_2$ (%) | CAT (mmol/minute) | POX (mmol/minute) | MDA (mmol/g fresh weight) | Proline |
|---|---|---|---|---|---|---|
| Repetition (R) | 2 | $0.063 \times 10^{-2}$ ns | $45.38 \times 10^{6}$ ns | $16.492 \times 10^{6}$ ** | 1.55ns | 0.746ns |
| Genotype (G) | 1 | $2.746 \times 10^{-2}$ ** | $11.370 \times 10^{8}$** | $22.461 \times 10^{8}$ ** | 0.196 ns | 3.730ns |
| Treatment(T) | 1 | $3.020 \times 10^{-2}$ ** | $26.937 \times 10^{8}$ ** | $11.915 \times 10^{8}$ ** | 25.076** | 6.194* |
| T × G | 1 | $1.116 \times 10^{-2}$ ** | $31.508 \times 10^{7}$ ** | $44.713 \times 10^{7}$ ns | 2.484* | 1.170ns |
| Error | 6 | $0.019 \times 10^{-2}$ | $13.139 \times 10^{7}$ * | $10.04 \times 10^{7}$ | 0.405 | 0.728 |
| C.V. | - | % 12.86 | % 30.14 | % 20.17 | % 24.66 | % 58.62 |

*,** and ns represent significant at 5%, significant at 1% and not significant, respectively. C.V. represents coefficient of variation. S.O.V. represents source of variation. d.f. is digree of freedom.

$H_2O_2$ is one very destructive and affinitive molecule, which, in addition to causing toxic consequences, causes irreparable damages to plasma membrane's lipids, proteins and nucleic acids, as the result of defense system dysfunction. Cold stress triggers $H_2O_2$ over-synthesis in many tissues, which induces oxidative stress via lipid peroxidation and other harms on plasma membrane (Jung *et al.,* 2003).

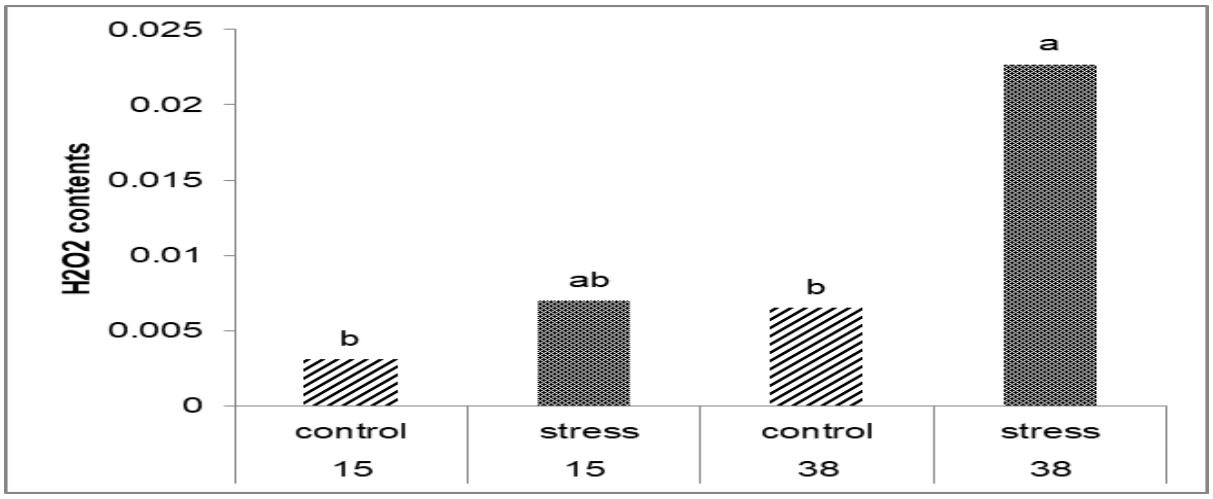


**Fig. 1.** Comparison of the means for $H_2O_2$ percentage in the root under both treatment and control conditions.

A significant difference concerning $H_2O_2$ existed between genotypes, the two thermal treatments, as well as the interaction of genotype ×temperature (Table 1). As such, comparison of the means for the interaction of genotype ×temperature showed that, at 4 ℃, the sensitive genotype contained the biggest amount of the substance, making a significant contrast with the rest (Fig. 1). No significant difference existed for other treatments. However, the tolerant genotype produced more $H_2O_2$ under stress than control.

In rice seedlings grown under cold stress, a significant difference was observed between genotypes and their interaction with temperature, as $H_2O_2$ content in sensitive genotype surpassed its amount in tolerant counterparts. Simultaneous cold and water deficit could also increase $H_2O_2$ production in sensitive rice genotypes (Gong *et al.,* 2005), which are congruent with our results.

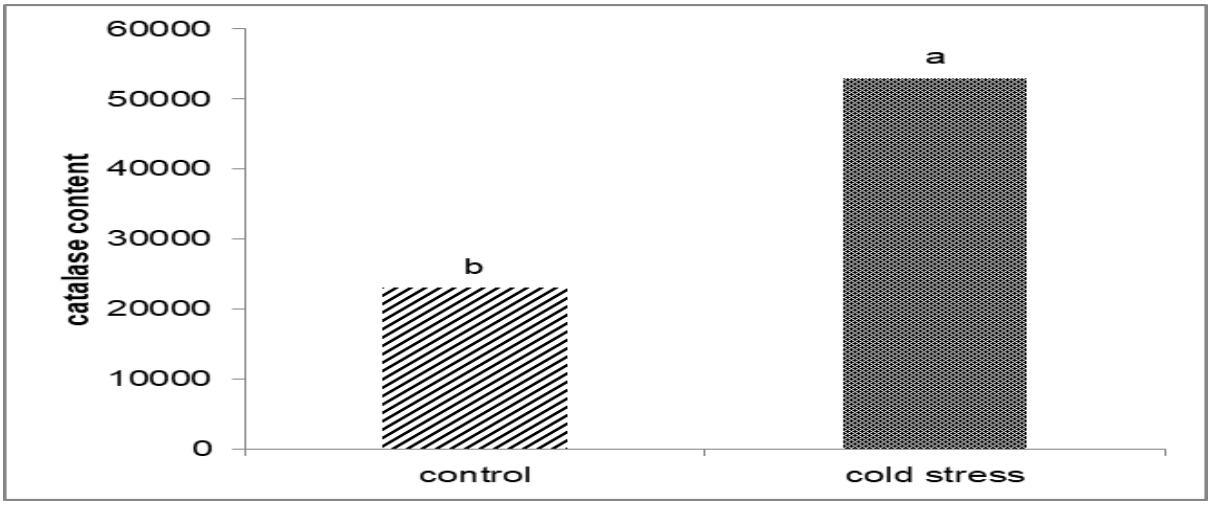


**Fig. 2.** Histogram representation CAT in two thermal treatment.

It has also been reported that cold stress can impose $H_2O_2$ over-production in wheat (Apostolova *et al.,* 2008), cucumber (Omran, 1980), strawberry (Yong *et al.,* 2008), and lentil (Öktem *et al.,* 2008). In our study, $H_2O_2$'s production increased in roots in respond to cold-induced oxidative stress. A growing body of evidence suggests the functioning of $H_2O_2$ as a molecular signal which elicits a vast physiological, biochemical and molecular reactions in plants (İşeri *et al.,* 2013). Although the role of $H_2O_2$ as a signaling molecule in response to abiotic and biotic stresses has been well established, its molecular network yet to be completely understood (İşeri *et al.,* 2013).

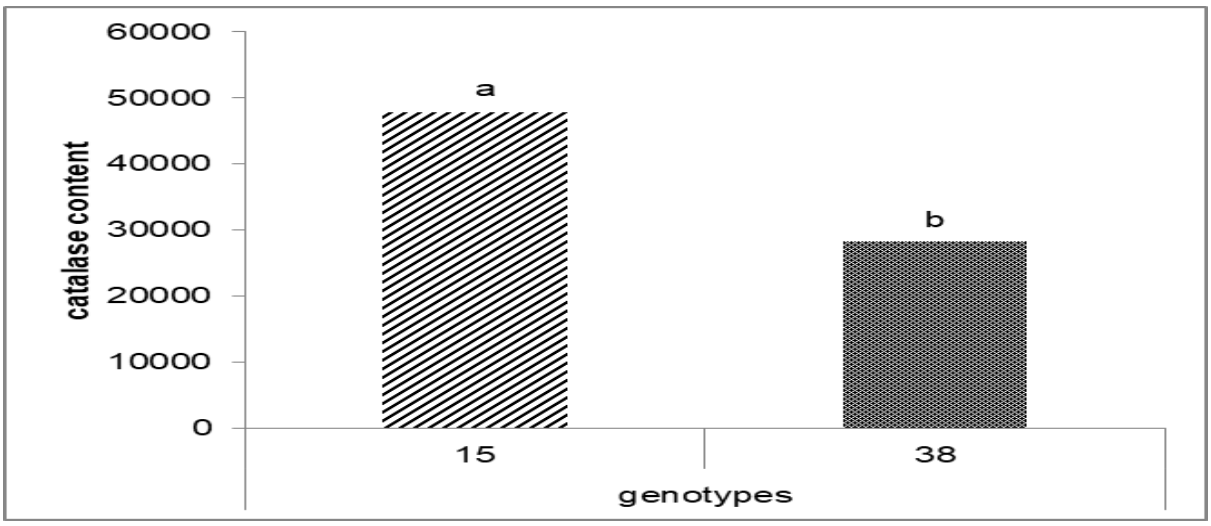


**Fig. 3.** CAT average in both sensitive and tolerant genotypes.

CAT and POX in association with other relevant enzymes work toward $H_2O_2$ elimination (Foyer *et al.,* 1994). Despite being located in peroxisomes and glyoxisomes, CAT can play an important role in fighting against oxidative stress, due to the fact that $H_2O_2$ can easily and immediately emits from one side of plasma membrane to the other side (Bowler *et al.,* 1992).

POX uses electron-donor-phenolic substances as a means to destroy $H_2O_2$ (Asada *et al.,* 1994). It is said that POX embody many functions in plant pathways, including lignification, oxidation of phenols, regulating cell elongation, as well as detoxification of toxic compounds such as $H_2O_2$ produced as a consequence of oxidative stress (Asada *et al.,* 1994).

The change in enzyme's activity- CAT and POX's for example- may be linked with the capacity of the plant to mitigate negative effects of abiotic stress in any morphological and physiological process.

ANOVA for CAT and POX revealed that between thermal treatment (stress and control), and between genotypes the difference is significant.

However, genotype ×temperature interaction was not significant.

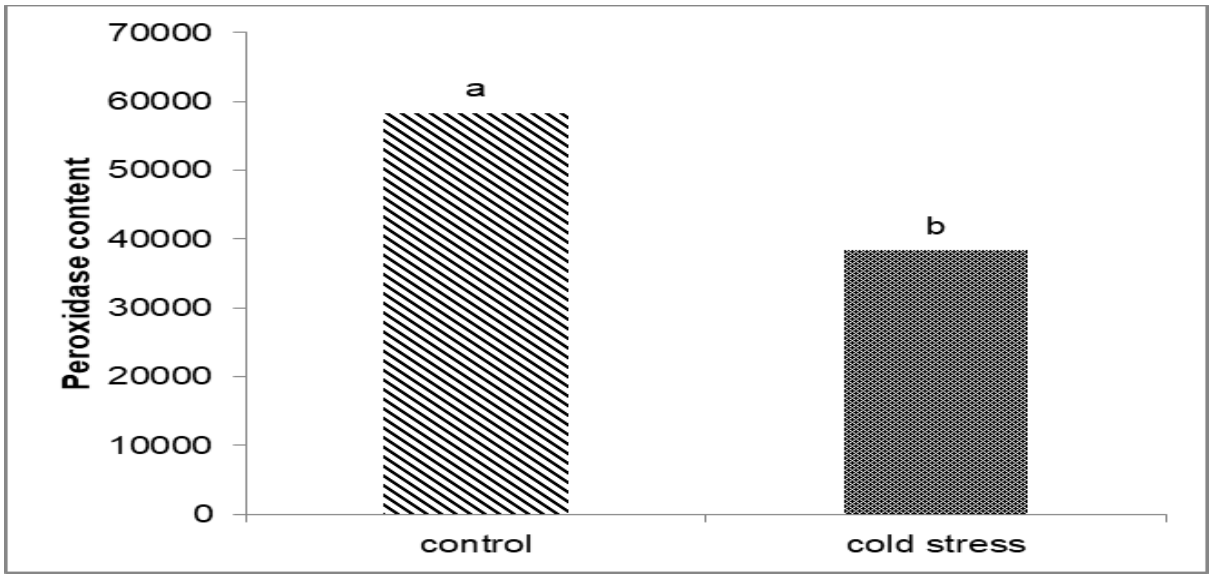


**Fig. 4.** Histogram representation of POX average in two temperatures.

The highest amount of CAT was found under stress, meaning CAT synthesis had significantly scaled up at 4℃ compared to the control.

Among the genotypes, the tolerant produced maximum CAT (Fig. 2 and 3), while the sensitive produced maximum POX. The POX content experienced significant drop at 4 ℃ compared to the control (Fig. 4 and Fig. 5).

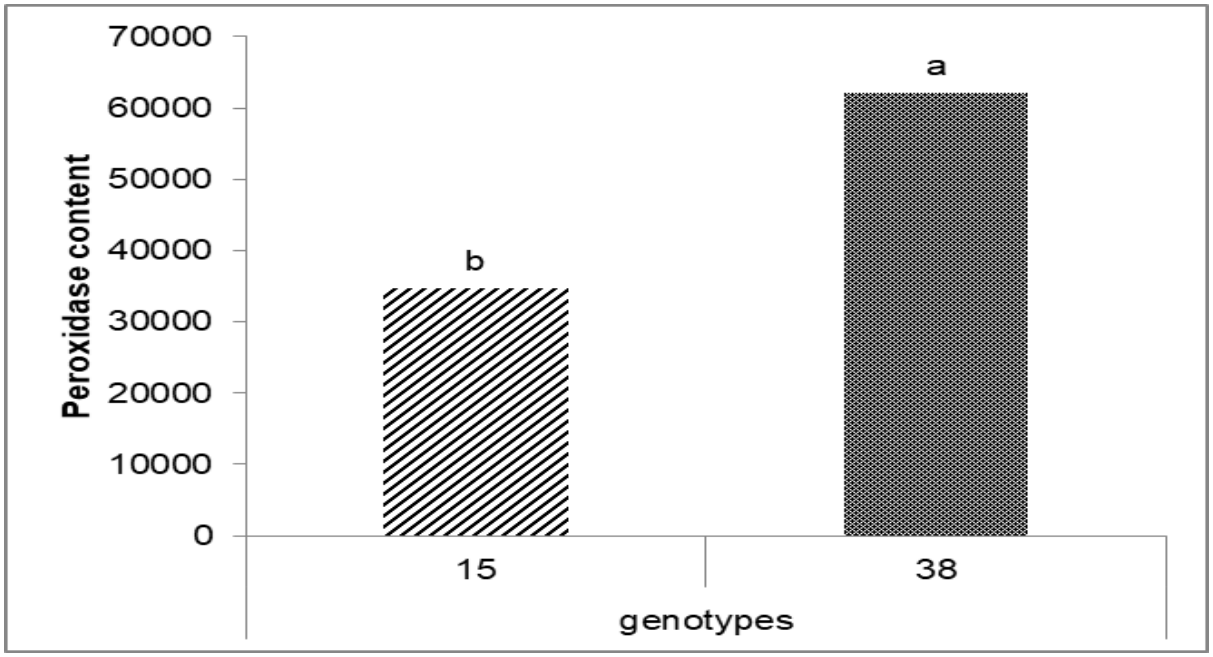


**Fig. 5.** POX average in both sensitive and tolerant genotypes.

In the present study, the quantity of CAT increased in both tolerant and sensitive genotypes, compared to the control, which, considering the obtained results, the amount was bigger in tolerant than sensitive**.** In other researches the significant increase of this enzyme has been reported in barley (Dai *et al.,* 2009), strawberry (Yong *et al.,* 2008), tomato (İşeri *et al.,* 2013) affected by cold stress chick pea (Eyidogan and Öz, 2007) under salinity conditions.

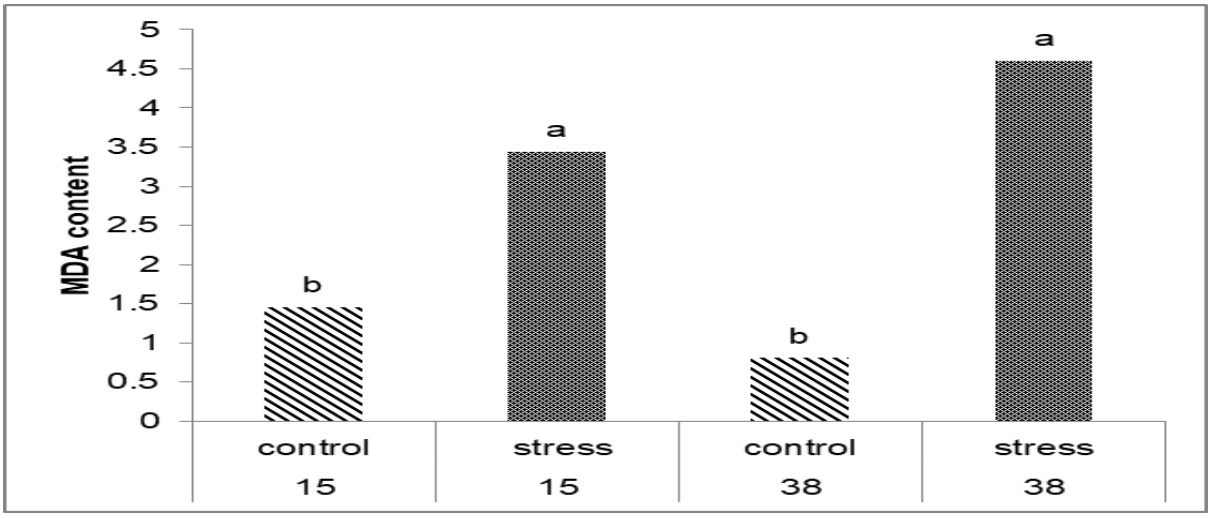


**Fig. 6.** Comparison of the means for MDA in roots in both control and treated plants.

According to the results, the quantity of POX has plummeted significantly compared to the control, and the scale of reduction was bigger in tolerant genotype than the sensitive one. Previously, the fall in amount of POX has been reported in wheat roots (Scebba *et al.,* 1998). Accordingly, it is safe to say that CAT is more involved in cold tolerant than POX.

ROS-mediated peroxidation in plasma membrane lipids induced by cold stress brings interruption in membrane permeability and stability index, which eventually give birth to membrane damage and disruption (Dhindsa, 1991).

Therefore, modification in lipid peroxidation, when integrity of plasma membrane is compromised, is used as an indication of magnitude of the damage imposed by oxidative stress in living things, when undergoing stressful conditions (Borsani *et al.,* 2001). In the same token, MDA, a byproduct of lipid peroxidation, is used as an indication of oxidative stress scale. In rice seedlings, for example, stressed by cold temperatures researchers linked MDA production to lipids' oxidative damage; increased production of MDA means significant increase in lipid peroxidation (Jain *et al.,* 2001).

For MDA content in roots, effect of thermal treatment was significant, so was the genotype ×temperature interaction. Considering the comparison of the means for genotype ×temperature interaction, MDA content in both genotypes had increased in response to cold stress at 5%. However, the figure seems bigger in sensitive than tolerant genotype, as the discrepancy in the MDA content under both stress and control treatments was bigger in the former (Fig. 6).

Its overproduction has also been reported in Lemon balm (Abavisani *et al.,* 2013), chick pea roots (Kazemi-Shahandashti *et al.,* 2014), wheat seedlings (Apostolova *et al.,* 2008), strawberry (Yong *et al.,* 2008).

It is now known that proline accumulates in plants in response to oxidative stress as a means to strengthen defense system (Banu *et al.,* 2009). Proline accumulation can happen by denovo synthesis or reduction in degradation or both. It can be produced during ornithine or glutamate cycles, and

accumulated in different parts of the cell, which involves mechanisms greatly dependent on species, developmental stage and cause of stress (Szabados and Savouré, 2010).

During stress, proline acts as a *cryptotectant*, carbon and nitrogen accumulator, PH stabilizer, cell redox normalizer and a stress-dependent growth organizer signal, which all help plant withstand abiotic stresses (Rai and Penna, 2013). Additionally, it has been established that proline by counteracting ROS under stress conditions works as a protein and membrane and sub-cellular structures stabilizer and cell-activity protectant (Kaur *et al.,* 2011).

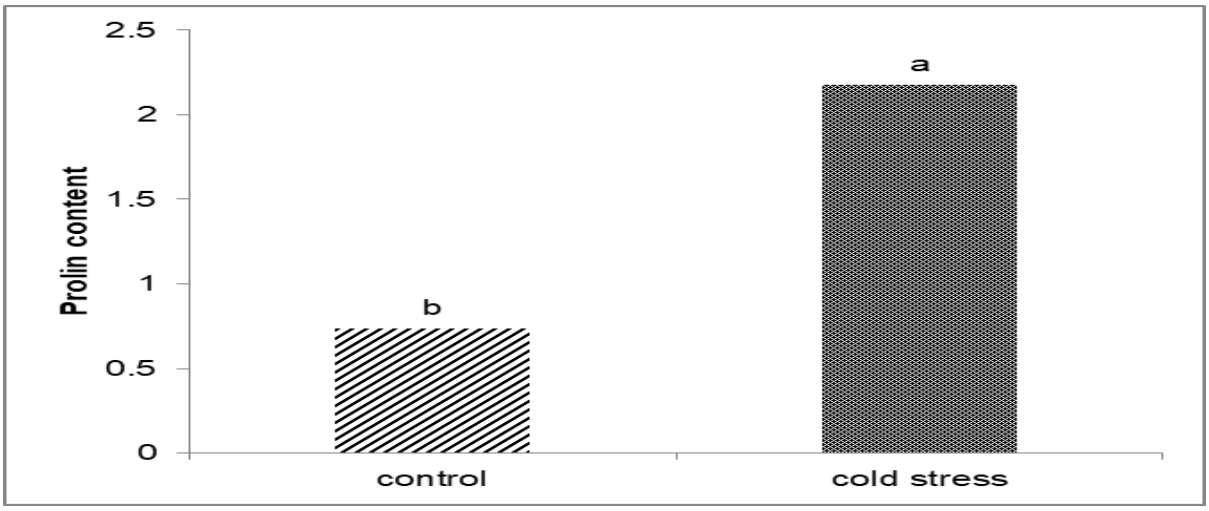


**Fig. 7.** The histogram representation of proline content in two temperatures.

Proline quantity in roots and the interaction of temperature × genotype were not significant, while a significant difference existed between 0 and 4 ˚C at a level of 5% (Table 1). Comparison of the means for this trait, in both temperatures, indicated that the trait has significantly scaled up in amount compared with the control (Fig. 7).

Congruent with our results, (Öktem *et al.,* 2008) reported that the quantity of proline had increase in response to cold stress. It is said that temperature reduction may trigger a boom in proline accumulation across the plant for as long as stress exists. On the other hand, proline accumulation is considered as an important indication of tolerance to stress in plants. Under cold or freezing stress, tolerant and sensitive plants tend to accumulate proline, but the amount of accumulation is bigger and lasting in the former, owing to the phonologic effects of growth. High levels of proline enable the stressed plant to adjust osmotic stress in its cytoplasm and, hence prevent water deficit (Aghaee *et al.,* 2013).

Based on obtained results, the experiment had been done accurately, fluctuating between 12.86% (minimum) and 58.62% for proline.

**Conclusion**

To investigate the effect of cold stress on the activity of anti-oxidants and the following damages in two sensitive (aths-38) and tolerant (EC83-1215) barley genotypes, a factorial experiment based on randomized complete block design with three replicates was conducted under controlled conditions. At seedling stage, the cold stress of 4°C was applied for 48 h. After sampling, the anti-oxidants enzymes involved in the defense mechanisms were analyzed in roots. Based on the results, researchers draw the conclusion that cold stress increases the activity of anti-oxidant enzymes, which is higher in tolerant genotypes than sensitive ones. Meaning, sensitive genotypes also experience increase in anti-oxidant enzyme activity in response to cold stress, but not as much as tolerant ones. This is probably associated with genetic composition, different space-time expression, as well as differential adaptation to cold temperatures.

Therefore, better understanding of underlying mechanisms is pre-requisite to any improvements towards cold tolerance, which includes a vast majority of molecular and cellular reactions all over the plant involving production of adjusting osmolyes and mechanisms to suppress radicals, which calls for more investigations.

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