COLD STRESS RESISTANCE AND THE ANTIOXIDANT ENZYME SYSTEM IN *PISUM SATIVUM*

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ABSTRACT

Pea (*Pisum sativum*) is one of the major commercial forage all over the world, but its yield is restricted by cold stress due to cold sensitivity. In this study, the response of 31 pea genotypes with different cold resistance was tested under cold stress. Pea plants were cultivated from seeds in a temperature-controlled greenhouse (25/15°C) for 10 days prior to experiments. Cold-resistant and cold-sensitive lines and cultivars were selected using values of different thermal analysis. In addition, alterations in the activity of antioxidant enzymes (ascorbate peroxidase, catalase and superoxide dismutase), free proline content and their relation to LT_{50} with cold resistance were investigated, and three breeding lines were selected at cold acclimation and non-acclimationconditions. Proline content gradually increased at cold acclimation compared to those leaves at non-acclimated. Cold acclimation improved the activities of APX, SOD and CAT. The highest correlation between enzyme activities and cold resistance was observed in the case of SOD, APX and CAT of 19 breeding lines' leaves. Our results indicated LT_{50} as closely related to proline content and antioxidant enzyme activities at cold acclimation.

Key words: pea, cold resistant, lethal temperature, enzyme activity.

INTRODUCTION

Abiotic stress is identified as environmental factors that reduce yield and productivity below optimum levels. Low temperature is one of the important abiotic stress factors influencing plant vegetative and reproductive development in many areas of the world (Janda *et al.,* 2003). Plants are complex organisms, which vary widely in their ability resist against chilling and freezing temperatures. It is hard to find out a precise estimate of the effects of cold stress on plant growth (Levitt, 1980). Freezing is defined as the death of the plant or damage to growth or differentiation of most of the living cells of plants as a result of cold stress. However, supercooling, a state where liquids do not solidify even below their normal freezing points is an avoidance mechanism against cold damage in plants, which are exposed to low temperatures. The degree of plant supercooling in normal environment conditions is primarily dependent on the ice-nucleating ability of the plant tissue and its immediate environment. Supercooling is particularly important in plants subjected to frosts during periods of high metabolic or growth activities (Reyes-Diaz *et al.,* 2006). When the extracellular supercooled water freezes, it creates a high temperature exotherm (HTE) and does not injure the plant. On the contrary, when the intracellular supercooled water freezes, it creates a low temperature exotherm (LTE), which is mostly lethal for organs. Differential thermal analysis is a method, which is utilized to predict the critical temperatures for plant tissues (Burke *et al.,* 1976). Freezing-resistant plants experience damages only at temperatures lower than the temperature at which extracellular ice formation begins. Many plant species develop freezing resistance when subject to low non-freezing temperatures, a physiological process known as cold acclimation. Cold acclimation is a dynamic process in which plants exposed to low but non-freezing temperatures acquire tolerance to sub-zero temperatures (Levitt, 1980). Plants respond to acclimation through a number of biochemical and physiological alterations including changes in carbohydrates, proline and protein content as well as enzymatic activities. Soluble carbohydrates and free proline may inhibit water loss during acclimation (Kovacz *et al.,* 2011). Cold acclimation increases the level of proline via changes in enzyme activities in the proline metabolism pathways, which enhances cold-resistance (Ruiz *et al.,* 2002). Proline is also strongly associated with plants' cold stress as free proline increase during acquisition of cold resistance in plant species. The antioxidant enzymes in the plants, when they are exposed to environmental stresses, have been known to play a main role in the regulation against stress (Chaitanya *et al.,* 2002; Turk *et al.,* 2014). To reduce the stress enhanced oxidative effects, plant species produce various types of antioxidants such as peroxidase, catalase and superoxide dismutase (Shahid *et al.,* 2012).

Pea (*Pisum sativum* L.) is a major legume crop in the world. The crops display an important role in the sustainable agricultural systems. However, frost damage

in early spring and late fall to the pea crop can restrict the growing season to a limited time period (Tan *et al.,* 2012). It is documented that the stress excited influences on physiological and biochemical attributes of pea, although the relative importance of cold stresses affecting its production has been poorly understood (Noreen and Ashraf 2009; Shahid *et al.,* 2012). The present study was carried out to evaluate the cold resistance of different pea breeding lines and cultivars under controlled conditions and to determine the lethal temperature values for commonly used pea breeding lines and to identify the relationship of alterations in enzymatic activities to cold resistance in pea plants.

MATERİAL AND METHODS

Plant material and growth conditions: Pea (*Pisum sativum* L.) seeds were obtained from East Anatolia Agricultural Research Institue, Erzurum, Turkey. Prior to experiments, seeds were surface-sterilized for 10 min with water/bleach (10:1, commercial NaOCl) solution and then washed three times with distilled water. Seeds were cultivated in plastic pots filled with a 1:1:1 mixture of soil: sand: peat. They were propagated ina greenhouse with day/night temperature of 25/15°C. Plants were watered and fertilized regularly. After 10 days, Plants of pea breeding lines and cultivars were subjected to differential thermal analysis and cold acclimation.

Determination of cold-resistance: A total of 31 pea breeding lines and cultivars were chosen from previous year's study. Three pea plants from each breeding lines and cultivars were carry out to determine the cold resistance. Leaf samples were obtained as uniform pieces from 25 breeding lines and 6 cultivars. One TEM in the top tray was left empty as a control for signal noise that was common to all TEMs. Foam insulation pads (4 cm x 4 cm x9 mm thick) were placed on top of leaf in each well to ensure adequate contact between the tissues and TEM. The chamber lid was then tightened and chambers were placed in the freezer. Up to four chambers were stacked in the freezer for a maximum of 35 TEMs loaded per run. The freezer was programmed to hold at 4°C for 1 hrs, and drop to -20°C in following 11 hrs (a cooling rate of 4°C/hr), hold at -20 °C for 1 hr (a warming rate of 4.4 °C/hr). The DAS recorded signals from each TEM at 15-sec intervals and downloaded voltage output directly to Excel. Exotherms were identified manually from a plot of thermistor output (x axis) versus loaded-TEM output minus empty-TEM output (y axis). Lethal temperatures for leaves were reported at which 10%, 50% and 90% of the leaves were killed, respectively (Andrews *et al.,* 1984) . These values were determined from the leaf exotherm range and distribution that were clearly visible for leaves on each TEM. For pea, lethal temperatures were reported as leaf tissue LTE_{10}.

Cold acclimation treatment: Three pea plants from each breeding lines and cultivars were carry out to determine the cold resistance. For cold acclimation, 10-day-old seedlings were incubated in a growth chamber set at 4°C to induce cold treatment for 21 days, with light condition (day/night 16/8 h photoperiod, 50µmol m^{-2} s^{-1} light) and relative humidity (65%). Leaf samples were obtained at 7, 14 and 21 days after cold treatment for proline contents and enzyme activity evaluation.

Proline assay: Three pea plants from each breeding lines were carry out to determine the proline content.To determine the proline content of leaves the acid ninhydrin method was used (Bates *et al.,* 1973). Leaf samples were crushed in a mortar and pestle with 3% (w/v) sulfosalicylic acid aqueous solutions and the homogenate was filtered through Whatman No. 1filter paper, then 2 ml of filtered extract was mixed with 2 ml glacial acetic acid and 2 ml acid ninhydrin. The reaction mixture was incubated at 100 °C for 1h and then left on ice for 20 min. Four milliliter of toluene was added to the reaction mixture. After centrifugation, the organic phase was extracted into quartz cuvette and absorbance was measured at 520 nm against toluene as blank by UV-visible spectrophotometer.

Antioxidant enzyme activities: Three pea plants from each breeding lines and cultivars were carry out to determine the antioxidant enzyme activities. Leaf samples (0.5 g) were crushed with a mortar and homogenized in ice-cold 0.2 M phosphate buffer (pH 7) containing 0.1m methylenedinitrilotetra acetic acid (EDTA). Extract was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was utilized to measure the activity of APX, SOD and CAT enzymes. CAT activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) including 20 mM H_2O_2. One unit of CAT activity was defined as the amount of enzyme that used 1 µM H_2O_2 per min (Upadhyaya *et al.,* 1985) . APX was analyzed by recording the decrease in absorbance at 290 nm in 3 ml sample mixture including 50mM potassium phosphate buffer (pH 7.0), 0,5mM sodium ascorbate, 0,1 mM EDTA, 0,2 ml supernatant and 6mM H_2O_2 (Nakano & Asada, 1981). SOD activity was determined by monitoring the reduction in absorbance of nitro-blue tetrazolium (NBT) dye (Dhindsa et al., 1981). Sample mixture included 13 mM methionine, 2 µM riboflavin, 75 µM NBT, 0.1 mM EDTA, 50 mM sodium carbonate, 50 mM phosphate buffer (pH 7.8), and 0.1 mL of the extract. Riboflavin was added at the end and the tubes were shaken and placed 30 cm below a light bank containing two fluorescent tubes. After 20 min, The reaction was finished by covering the tubes with a black cloth. The absorbance was recorded spectrophotometrically at 560 nm.

Statistical Analysis: All experiments were conducted in three replicates. Data were evaluated by analysis of variance (ANOVA), and means were compared by Duncan's multiple range test at $P < 0.05$.

RESULTS

Cold-resistance estimation among breeding lines and cultivars: Initially, differential thermal analysis profile of pea leaves was determined, which indicated only one ice-forming event at different temperatures. Temperature ranges associated with 10%, 50% and 90% injury were identified in the pea genotypes tested. According to our results, as represented in Figure 1, the cultivars and breeding lines can be categorized into several groups. In the leaves, LT_{10}, LT_{50} and LT_{90} (temperatures causing lethal freezing), the cultivars and breeding lines studied were classified as sensitive (35, 47, 43, 11, 9, 24, 8, 111, 171, 116 (Töre), 46, 27) tolerant (45, 26, 19, 54, 13, 113 (Ürünlü), 22, 16, 21, 40, 161) and resistant (115 (Taşkent), 14, 112 (Özkaynak), 114 (Kirazlı), 34, 117 (Ulubatlı), 6, 10). The LT_{50} for leaves ranged from -2.68 to -6.95°C. Among the breeding lines, the leaves of 10 and 35 had the highest and lowest supercooling points, respectively. Among commercial cultivars, Özkaynak and Ulubatlı had the highest supercooling points and Töre and Ürünlü had the lowest ones.

Cold-acclimation and proline assay: Plants grown for 10 days in the greenhouse were transferred into the 4°C environment for 21 days to investigate the potential of plants for cold resistance. Plants grown in the normal greenhouse served as controls. Cold-acclimation was assessed with three breeding lines which were selected based on their sensitivity (9) tolerance (19) or resistance (10) to temperature of -20°C. Proline accumulation was proven after 7 days of cold acclimation ($P \leq 0.05$) in leaves of three breeding lines (9, 10, and 19). Proline accumulation in 19 was higher than that in 9 and 10 breeding lines prior to cold acclimation. Cold acclimation gradually increased proline content, which reached the highest level at 21 days. Compared with the control, proline accumulation of 9, 10, and 19 breeding lines showed a 3.90-6.30, 4.10-5.50, and 4.10-7.20-fold rise after 7 days acclimation, respectively. At the day of 14, proline accumulation in the leaves of all investigated breeding lines increased consistently. During 21 days of acclimation, the most intensive proline accumulation was observed in the leaves of '19' breeding line, and after 7, 14 and 21 days of acclimation, the amount of proline in the leaves of this breeding line was significantly higher than that in leaves of non-acclimated ones (Table 1).

Antioxidant enzyme activity: Analysis of variance of data for antioxidant enzymes (APX, SOD, and CAT) showed that 7,14 and 21 days of acclimation had a significant ($P \leq 0.05$) improvement on the activities of SOD, APX and CAT of the three tested pea breeding lines. SOD activity was different among cultivars in both cold acclimation and non-acclimated stage. The highest SOD activity was on 7 days at cold acclimation; 19 (12.900 nmol/g FW), 10 (12.700 nmol/g FW), 9 (11.800 nmol/g FW). Whereas the lowest SOD activity was for 21 days acclimation; 10 (3.893 nmol/g FW), 9 (3.800 nmol/g FW) and 19 (4.300 nmol/g FW). On the contrary, the lowest SOD activity was on 7 days at non-cold acclimation; 9 (2.600 nmol/g FW), 10 (3.200 nmol/g FW) and 19 (3.700 nmol/g FW). Cold acclimated 19 breeding line indicated a significant increase in SOD activity in their leaves compared with the control for 7 days (Table 2). APX activity was also different among cultivars in both cold acclimation and non-acclimated stages. The highest APX activity was for 21 days at cold acclimation; 19 (7.80 nmol/mg protein), 9 (5.40 nmol/mg protein), 10 (5.00 nmol/mg protein), whereas the lowest APX activity was obtained for 21 days; 10 (1.200 nmol/mg protein), 9 (1.700 nmol/mg protein) and 19 (2.200 nmol/mg protein). In contrast, the highest APX activity was observed for 7 days at non-cold acclimation; 19 (1.400 nmol/g FW), 9 (1.200 nmol/g FW) and 19 (0.500 nmol/g FW). Similarly, 19 breeding line showed the highest APX activity in terms of cold acclimation (Table 3). No significant differences in the level of CAT were obtained in the leaves of pea among breeding lines subjected to cold acclimation. However, CAT activity was different among breeding lines at non-acclimation. At cold acclimation, the highest CAT activity was obtained in the breeding line 19 (15.300 µmol mg^{-1} protein), whereas the lowest CAT activity was obtained in the breeding line 9 (14.800 µmol mg^{-1} protein) for 21 day at cold acclimation. Similarly, the breeding line 19 (2.600µmol mg^{-1} protein) had the highest CAT activity at the non-acclimation (Table 4).

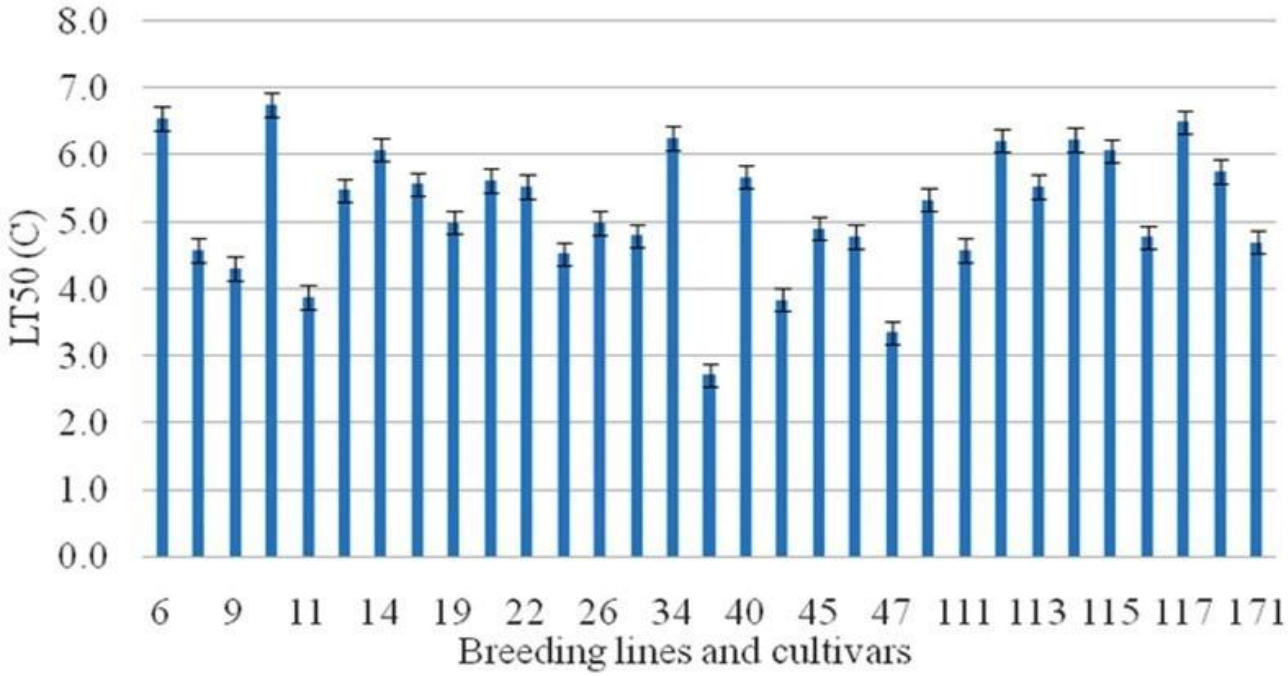

Fig 1.Changes in LT_{50} of 25 breeding lines and 6 cultivars. Vertical bars indicate standart errors from means (n=3).

Table 1. Changes in prolinecontent of three pea breeding lines.

Plant lines	7 days (CO)	7 days	14 days	21 days
9	3.9	6.3cB	14.6bA	22.10aB
19	4.1	7.2cA	14.3bA	28.00aA
10	4.1	5.5cC	10.4bB	21.3AB

*Values with the different supercript in the same column are different at the P<0.05 level based on Duncan test.

Table 2. Changes in SOD activities of three pea breeding lines.

Plant lines	7 days (CO)	7 days	14 days	21 days
9	2.6^{c}	11.8aB	6.1bA	3.9cB
19	3.7^{a}	12.9aA	8.2bc	4.3cA
10	3.2^{b}	12.7aA	7.1bB	3.8CB

*Values with the different supercript in the same column are significantly different at the P<0.05 level based on Duncan test.

Table 3. Changes in APX activities of three pea breeding lines.

Plant lines	7 days (CO)	7 days	14 days	21 days
9	1.2^{b}	1.7cB	2.4bB	5.4aA
19	1.4^{a}	2.2cA	4.0bB	7.8aA
10	0.5^{c}	1.2cC	2.3bB	5.0AC

*Values with the different supercript in the same column are significantly different at the P<0.05 level based on Duncan test.

Table 4. Changes in CAT activities of three pea breeding lines.

Plant lines	7 days	7 days	14 days	21 days
9	2.0^{c}	4.7	9.9	14.9
19	2.6^{a}	4.9	9.9	15.3
10	1.9^{b}	4.8	9.8	14.8

*Values with the different supercript in the same column are significantly different at the P<0.05 level based on Duncan test.

DISCUSSION

Cold stress interferes with all cellular processes due to induction of morphological and biochemical changes in plant tissues and physiological alterations in plasma membrane, changes in enzymatic reactions and interactions among macromolecules (Taşgın *et al.,* 2006). The combination of different thermal analysis and LT has been commonly used to identify cold tolerance mechanisms in species (Pearce, 2001). Determination of LT temperatures is basic to evaluate whether plant tissues can resist ice crystals without significant freezing tolerance. Pea genotypes might differ in freezing tolerance due to differential survival of their variation in the environmental conditions (Shereena *et al.,* 2006). Therefore, improving cold resistant cultivars and recognition of strategies of cold tolerance could greatly progress cold resistance for pea plants. In this study, we determined cold resistance of 6 cultivars and 25 breeding lines as indicated by LT_{50} for various environmental conditions. Pea breeding lines and cultivars investigated in the present study displayed considerable differences in their response to subfreezing temperatures and in their acclimation potential against cold. The lethal temperatures (from LT_{10} to LT_{90}) for survival for most of the 31 samples ranged between -2.1 and -7.14 °C. Breeding line 10 was obviously the strongest of all genotypes studied; while 35 was much less strong. Our results also showed that 14, 34, 6 and 10 breeding lines can be considered as the most freezing tolerant cultivars among the other studied cultivars (Fig. 1). In terms of cultivars, Töre and Ürünlü could be considered as the most freezing-sensitive cultivars. The LT_{50} values were somewhat similar to those reported in the literatures for garden pea (Wade, 1941). Ulubatlı, Özkaynak, Taşkent and Kirazlı were reported as frost tolerant cultivars in a previous study (Acikgoz *et al.,* 2009). In this study, we also found that Ulubatlı, Özkaynak, Taşkent and Kirazlı breeding lines had freezing resistance values close to that of freezing resistant 14, 34, 6 and 10 breeding lines. In a previous study Balackova et al (1986). reported that no damage was observed in leaves of *Pisum fulvum* cultivars exposed to -6°C cold stress. Cold tolerance in the genotypes of *Pisum sativum* from 5 to 100%; it was highest in four conventional lines and five short-stemmed lines. In France, temperatures of -23°C and below are evaluated lethal to overwintering *Pisum sativum*, while temperatures of -6 to -14°C are considered as not damaging to totally resistant pea (Eteve, 1985). The results of this study are in accordance with previous studies reported in the literature. Increased proline content at low temperatures has been commonly reported in many plants species, including pea (Zhang *et al.,* 2012). Proline content obviously increased in plants exposed to the acclimation condition in the leaves of breeding lines and cultivars compared to the controls. Under normal conditions, different basal response contents were observed in three lines. Breeding line 19 displayed higher contents of proline in the acclimated state with respect to the non- acclimated state. Similarly, the freezing sensitive line (9) had promoted levels of proline in response to cold (Table 1). However, total proline content is dependent on the degree of cold resistance. Resistance cultivars and breeding lines had the highest concentration of proline. In cold treated plants, proline accumulation was higher than non-treated plants. Cold treated plants recovered faster than non-treated ones, therefore, due to proline's beneficial effects in plants in cold stress, cold acclimated plants could tolerate freezing temperatures much better than non-acclimated plants. Induced proline content may function in osmotic adjustment, ROS scavenging and protection of enzyme denaturation (Aslamarz *et al.,* 2011), which are important for well adaptation to cold stress. Therefore, proline levels could be considered as potential markers for screening the cold resistance of pea breeding lines. These results are in agreement with other researchers' findings (Nayyar *et al.,* 2005a) that demonstrated the contents of proline in chickpea during cold acclimation. Furthermore, Javadian *et al.,* 2010. reported that proline level in wheat seedlings was gradually promoted when exposed to cold stress. The results obtained in this study are in accordance with a previous report for walnut cultivars, where Aslamarz *et al.,* 2011. reported that freezing resistant walnut genotypes contain more free proline than the less resistant ones under low temperature conditions. Development of cold resistant in plants is strongly linked to the enrichment of antioxidant enzyme activity. Antioxidative enzymes are known to prevent cellular properties against ROS, which is produced in response to cold stress (Mutlu *et al.,* 2009). It has been shown that cold resistant species have higher antioxidant enzyme activities compared to cold sensitive species in many crops, including rice and maize (Anderson *et al.,* 1994; Guo *et al.,* 2005). Our results demonstrated that under cold stress conditions, a significant increase in SOD, CAT and APX activities was observed against oxidative stress in three breeding lines. SOD is the first step in defense mechanism against cold stress. Assessment of SOD activity in the leaves of three breeding lines under cold acclimation have showed an apparent increase and then decrease in parallel with the duration of cold treatment. Under cold stress, the increase in SOD activity was much higher in cold acclimated plants particularly when they were kept in 4°C for 7 days compared to non- acclimated ones and this alteration was observed mostly in 19 and 10 breeding lines. SOD activity in the cold tolerant and resistant breeding line 19 was the highest, whereas in the cold sensitive breeding line 9, its activity was lowest (Table 2). These results suggested a significant positive relationship between LT_{50} and SOD under both cold treatment and non-treatment conditions. Higher SOD

activity in cold resistant lines reflects better ROS scavenging capacity that helps detoxification of cells. In accordance with this study, Janmohammadi *et al.,* 2012 also documented that the SOD activity after cold acclimation was closely correlated to the decrease of LT_{50} in winter-wheat. Besides, APX activity significantly increased in pea cultivars during cold acclimation (Table 3). Increase in APX activity during cold acclimation has also been documented at pine and spruce (Tandy *et al.,* 1989). In our study, the highest APX activity was detected in cold tolerant breeding line19 on 21 days, however, the lowest ones were assayed in cold resistant breeding line10 on 14 day at cold acclimate. However, a difference increase was observed in non-acclimated pea cultivars. In addition, correlation between LT_{50} with APX activity under cold acclimated and non-acclimated conditions was statistically significant. Our results suggest that although it coulddiminish the damage of ROS, the activity of the APX enzyme does not have a direct role in the cold resistance of pea breeding lines. This is agreement with previous study that showed APX activity is not directly related to cold resistance (McKersie *et al.,* 1999). Cansev *et al.,* 2009, also reported that APX activity may not be related to the extent of cold resistance. In the meanwhile, CAT enzyme is found mainly in peroxisomes and also important in the protection of plants against cold stress (Sudhakar *et al.,* 2001; Mutlu *et al.,* 2011). CAT activity significantly increased in three pea breeding lines during cold acclimation but no significant difference between breeding lines was observed. The lowest CAT activity was found in the cold resistant line (10), during both cold treatment and non-treatment conditions, whereas the highest CAT activity was found in cold tolerant line (19). These findings are in agreement with previous reports, where CAT activity was found to be closely relation with the extent of cold resistance in olive leaf tissue (Hashempour *et al.,* 2014).

Based on the results, our study helped clarifying the cold resistance of pea and demonstrated that four breeding lines have the highest cold resistance among the 25 studied pea breeding lines. Moreover, ten breeding lines displayed tendency to be cold resistant. Ourresults also indicated that cold acclimation improved the activities of APX, SOD, CAT enzymes. Therefore, substantial activity of antioxidant enzymes and proline content may be proposed as selection criteria for obtaining resistant pea species for various environment conditions.

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