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The Impact of Cold Acclimatization on Antioxidant Enzyme Activity of *Vicia sativa* L.

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Abstract

Vicia sativa L. is one of most significant forages all over the world, but yield is decreased by cold stress. This study aimed to investigate the cold stress mechanism of *V. sativa* under tissue culture in response to some biochemical analysis. In this study, six *V. sativa* cultivars (Tarım Beyazı, Ankara Moru, 24 Çilli, Kansur and Aygün) were carried out to determine cold resistance *in vitro* conditions. Doğu Beyazı cultivar was used as a cold resistant plant. For the cold acclimation, two weeks old seedlings were incubated in the test chamber set at 4°C for 14 days to induce cold stress. Leaf samples were obtained at 14 days after cold treatment for physiological analysis evaluation. Cold resistant cultivars were chosen using values of thermal degrees. Moreover, antioxidant enzyme activities were determined at cold acclimated and non-acclimated seedlings. Peroxidase enzyme activities gradually increased at cold acclimation compared to those seedlings at non-acclimated. The highest POX activity was found at Ankara Moru seedlings, while the lowest activity was found at Aygün seedlings. SOD and APX activities were detected inversely at cold acclimation compared to those seedlings at non-acclimated. ChlorophyII A, chlorophyII B and carotenoid values were also determined at cold acclimated seedlings. The highest activities and pigment values were detected at Tarım Beyazı seedlings. Whereas, the lowest activities were found at Ankara Moru and Aygün cultivars. Our results displayed that cold acclimation linked to SOD, APX and POX activities and pigment estimation at in vitro conditions.

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1. INTRODUCTION

Agricultural production is significantly decreased by biotic and abiotic environmental factors. Cold stress is an abiotic stress that causes limiting crop cultivation and directly influences plant growth and yield. Crop reductions caused by cold stress are becoming a serious problem in North regions of the world (Mutlu *et al*., 2013; Turk *et al*., 2014). Many plant species are affected by these stress factors, therefore it is very important to improve cold resistant lines. Cold stress induces tissue damage, osmotic imbalance, cell membrane, antioxidant enzyme activities, photosynthesis and the amount of carbon production. As a result of this, crop productivity is decreased (Kovács *et al*., 2011). Plants block the stress damage through various techniques in the development medium. Adaptation to cold stress dissimilar between plant species throughout their growth period and are firstly detected in the phenological properties in the life cycle of development process (Tasgin *et al*., 2003; Tasgin *et al*., 2006). In addition, cold stress participates in changes in various physiological and biochemical pathways, adheres to

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impact and degree of the stress and finally limits plant development. Degree of the cold damage in crops is determined by reduced ionic content, decreased cell divisions, stomatal disorders, and differentiation. Excessive low temperatures are restricted water flow by the crops, influencing ionic instability leading to membrane injury and tissue damage. Acclimation is especially important in crops exposed to extreme temperature of cold during seasons of metabolic activities or growth process (Levitt, 1972). *Vicia sativa* L. is a widely cultivated plant for livestock producers of various agricultural areas because of its superior feeding rate. *V. sativa* is grown for its great yield and potential for high input nitrogen, N fixation, soil conservation quality as well as its role on wide pest resistance, and nutritional feeding. *V. sativa* is considered sensitive to cold. However, its productivity could be greatly affected by the cold stress and also its reduced quality of crops (Maqbool *et al*., 2010; Siddiqui, 2015). Therefore, development of *V. sativa* cultivars greatly resistant to cold stress is rapidly required. Even though traditional breeding methods have provided reasonable improvement eliminating cold stress, these techniques currently have some limitations, because low temperature tolerance is controlled by quantitative traits and participates in multiple biochemical and molecular mechanisms (Zhou *et al*., 2018). The current report was performed to define the cold resistance of different *V. sativa* cultivars under in vitro conditions and to detect the relationship of changes in enzymatic activities to cold tolerance in *V. sativa* seedlings.

2. MATERIAL and METHODS

2.1. Plant Material and Cold Acclimation Treatment

V. sativa seeds were obtained from Seed Gen Banc Field Crops Research Center, Ankara, Turkey. Prior to experiments, seeds were disinfected for 15 min with water/bleach (10:1, commercial NaOCl) solution and then sterilized three times with ddH_2O. Seeds were transferred in hormone free MS medium. Six *V. sativa* and control seedlings from cultivars were carried out to determine the cold tolerance. For cold acclimation, 14-day-old seedlings were incubated in a growth chamber set at 4°C to induce cold treatment for 14 days, with light condition (day/night 16/8 h photoperiod, 50µmol m) and relative humidity (65%). Leaf samples were obtained at 14 days after cold treatment for SOD, APX and POX activity and pigment estimation evaluation.

2.2. Antioxidant Enzyme Activities

Six cold acclimated (Tarım Beyazı, 24 Çilli, Ankara Moru, Aygün and Kansur one (Doğu Beyazı-cold resistant cultivar) non acclimated 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 0.2M phosphate buffer (pH 7.0) containing 0.1M methylenedinitrilotetra acetic acid (EDTA). Extract was centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was utilized to measure the activity of APX and SOD.

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, 0,1mM EDTA, 0.2 mL supernatant (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 13mM methionine, 2µM riboflavin, 75µM NBT, 0.1mM EDTA, 50mM sodium carbonate, 50mM 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.

The POX activity was measured by monitoring the increase in absorbance at 470 nm in 50mM phosphate buffer (pH 5.5) containing 1mM guaiacol and 0.5 mM H_2O_2 (Siddiqui *et al*., 2015).

2.3. Pigment Estimation

Seedling samples were isolated 80% acetone and obtained at 645 and 663 nm were determined chlorophyll A, chlorophyll B and total carotenoid amounts were then measured.

2.4. Statistical Analysis

Each experiment was repeated at three times. Analysis of variance was conducted using one- way ANOVA test using SPSS 13.0 and means were compared by Duncan test at the 0.05 level of confidence.

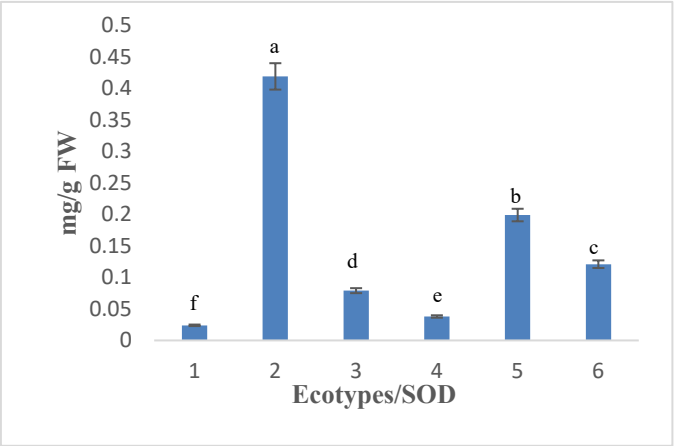
3. RESULTS and DISCUSSION

V. sativa is particularly susceptible to cold stress during seed germination, vegetative growth and flowering stages (Zhou *et al*., 2018). Cold stress in *V. sativa* is largely linked to the early germination phase. Therefore, selection and improvement of cold-resistance and remarkably productive varieties is needed to facilitate the increased *V. sativa* grown in Turkey. In present study, 6 cultivars of *V. sativa* were subjected to cold acclimation. As shown in Figures, all tested cultivars indicated various activities under normal and extreme cold conditions. Moreover, antioxidant enzyme activities displayed by cultivar Tarım Beyazı under cold acclimation showed that this cultivar had sufficient extent of cold resistance in the aspect of leaves. Improvement of cold tolerance in plants is remarkably linked to the enrichment of antioxidant enzyme activity. Several species have been well documented in terms of cold stress (Zhou and Guo, 2005). Earlier reports have indicated that antioxidant activities and chlorophyll content in *V. sativa* and *alfalfa* is remarkably associated with extreme cold resistance (Castonguay *et al*.,1995; Bertrand *et al*., 2017; Bezirganoglu *et al*., 2018). SOD activity was significantly different between *V. sativa* cultivars in cold acclimation and non-acclimated. The results of SOD activities alters between 0.024 and 0.419 nmol g^{-1} FW. The highest SOD value was found in 'Tarım Beyazı' (0.419 nmol g^{-1} FW) followed by '24 Çilli' (0.199 nmol g^{-1} FW), 'Aygün' (0.121 nmol g^{-1} FW). Whereas, the lowest was observed in the Ankara Moru (0.079 nmol g^{-1} FW) and Kansur (0.038 nmol g^{-1} FW) cultivars (Figure 1, Table 1). Peroxidase increased in seedlings of tested cultivar under cold acclimation. A continuous increase in peroxidase was determined in + 4 ^{0}C acclimated in all cultivars. Significant differences were observed between ecotypes compared to control. The highest peroxidase value was found in '24 Çilli' (1.191 nmol g^{-1} FW) whereas, the lowest value was found in Tarım Beyazı (0.126 nmol g^{-1} FW) (Figure 2, Table 1). APX value was also different between tested seedlings in both cold acclimation and non-acclimated conditions. The results of the APX analyzes ranged between 0.017 nmol g^{-1} FW and 3.100 nmol g^{-1} FW. Significant differences were observed between ecotypes compared to control. The lowest APX amount was found in 'Kansur' (0.017 nmol g^{-1} FW), followed by 'Aygün' (0.099 nmol g^{-1} FW), 'Ankara Moru' (0.170 nmol g^{-1} FW) (Figure 3, Table 1).

Table 1. Changes in SOD, APX and POD activity of six *V. sativa* cultivars.

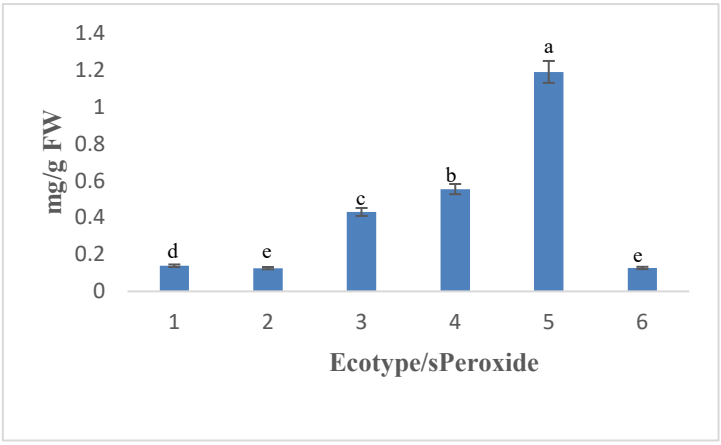
Ecotypes	SOD	APX	POD
Doğu Beyazı	0.024±0.001^{f}	1.237±0.166^{b}	0.139±0.001^{d}
Tarım Beyazı	0.419±0.001^{a}	3.100±1.000^{a}	0.126±0.001^{e}
Ankara Moru	0.079±0.001^{d}	0.170±0.001^{d}	0.431±0.001^{c}
Kansur	0.038±0.001^{e}	0.017±0.001^{d}	0.555±0.001^{b}
24 Çilli	0.199±0.001^{b}	0.937±0.001^{c}	1.191±0.010^{a}
Aygün	0.121±0.001^{c}	0.099±0.000^{d}	0.127±0.001^{e}

Figure 1. Changes in SOD activity of six *V. sativa* cultivars.



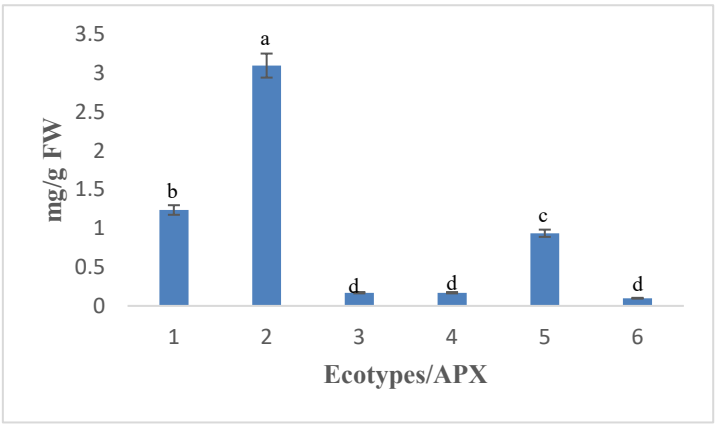
Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level b, c: SE; FW: Fresh weight (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 2. Changes in Peroxidase activity of six *V. sativa* cultivars.



Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e: SE; FW: Fresh weight (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 3. Changes in APX activity of six *V. sativa* cultivars.



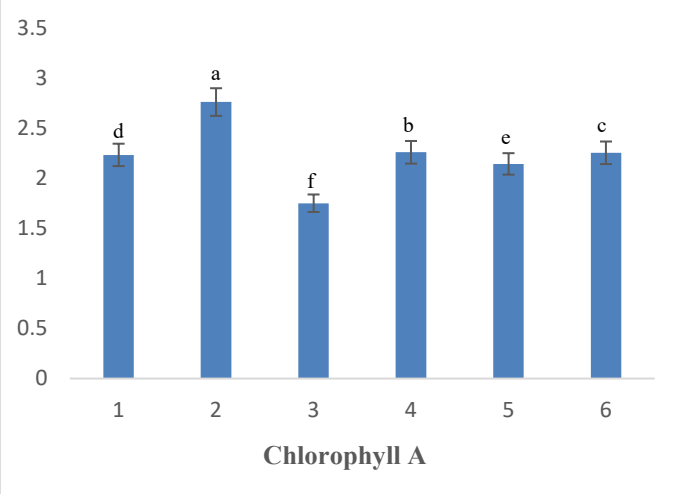
Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e SE; FW: Fresh weight (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

The findings of the current report are in agreement with earlier studies reported in the literature. Increased APX, POX and SOD content at extreme cold conditions has been commonly reported in several crops, including pea (Theocharis *et al*., 2012; Anower *et al*., 2016). The effects of stress severity on antioxidant enzymes are proven to be linked to the stress tolerant ability (Cunningham *et al*., 2003; Couée *et al*., 2006). Our results indicated that under cold acclimation, a considerable increase in APX, SOD and POX activities was detected against oxidative stress in six faba cultivars. These findings are in agreement with those published by Tasgin *et al*. (2003) in the study on wheat germinating seedlings cold acclimizated at 0.01, 0.1 and 1mM salicyclic acid during 15 days. Their results demonstrate that acclimization could induce against cold stress and promote quickly freezing tolerance in winter wheat leaves by decreasing in freezing damage 41% 0.01, 22% by 0.1 and 19% by 1mM SA treatments. Chlorophyll content was different among tested cultivars at cold acclimation. Chlorophyll A content was observed with very different results compared to non acclimated. The highest chlorophyll A content was obtained in Tarım Beyazı (2.763 nmol g^{-1} FW), whereas the lowest chlorophyll A Ankara Moru (1. 752 nmol g^{-1} FW) at cold acclimation (Figure 4, Table 2). Similarly, the same cultivars observed a similar trend at the cold acclimation in Chlorophyll B (Figure 5, Table 2). In terms of carotenoid, the highest content was obtained in Tarım Beyazı (2.269 nmol g^{-1} FW) and Kansur (2.115 nmol g^{-1} FW), whereas the lowest carotenoid Ankara Moru (0.985 nmol g^{-1} FW) and 24 Çilli (1.181 nmol g^{-1} FW) at cold acclimation (Figure 6, Table 2).

Table 2. Changes in Chlorophyll A, Chlorophyll B and Carotenoid activity of six *V. sativa* cultivars.

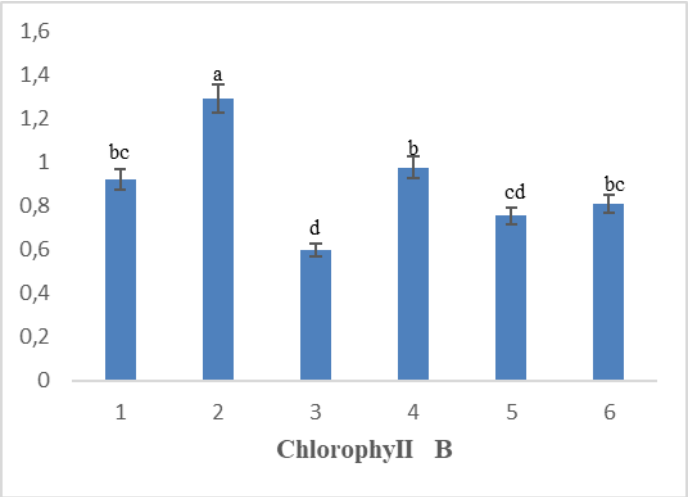
Ecotypes	Chlorophyll A	Chlorophyll B	Carotenoid
Doğu Beyazı	2.235±0.001^{d}	0.925±1.299^{bc}	1.910±0.010^{cd}
Tarım Beyazı	2.763±0.001^{a}	1.293±0.239^{a}	2.269±0.233^{a}
Ankara Moru	1.752±0.001^{f}	0.598±0.001^{d}	0.985±0.001^{e}
Kansur	2.261±0.001^{b}	0.978±0.001^{b}	2.115±0.001^{ab}
24 Çilli	2.2145±0.001^{e}	0.756±0.001^{cd}	1.181±0.001^{d}
Aygün	2.256±0.001^{c}	0.810±0.010^{bc}	2.038±0.001^{bc}

Figure 4. Changes in Chlorophyll A activity of six *V. sativa* cultivars.



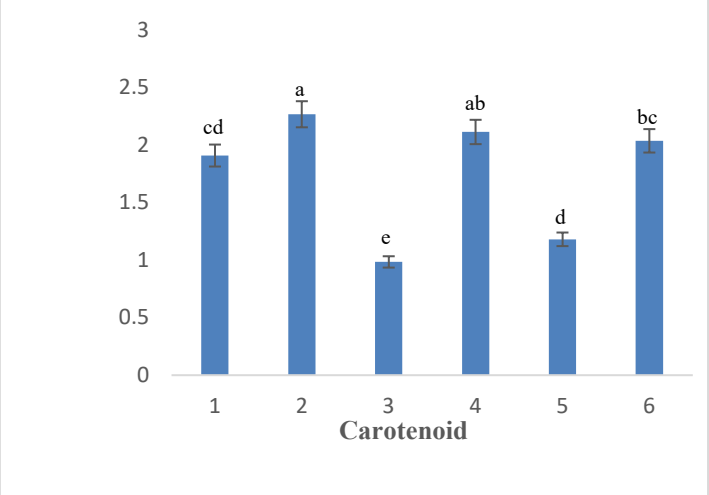
Differences between groups with different letters in a line are significant at the $P \leq 0.05$ significance level a, b, c, d, e SE. (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 5. Changes in Chlorophyll B activity of six *V. sativa* cultivars.



Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e SE. (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Figure 6. Changes in Carotenoid activity of six *V. sativa* cultivars.



Differences between groups with different letters in a line are significant at the *P ≤0.05* significance level a, b, c, d, e SE (1: Doğu Beyazı, 2: Tarım Beyazı, 3: Ankara Moru, 4: Kansur, 5: 24 Çilli, 6: Aygün).

Chlorophyll content is an efficient in vitro-selection way. However, it is significant to understand that more morphological and physiological stress factors must be explored for further screening and yield potential should be taken into account particularly for agricultural production. In our study, the highest chlorophyII A, B and carotenoid activity was observed in cold resistant cultivar Tarım Beyazı on 14 days, whereas, the lowest ones were assayed in cold tolerant Ankara Moru on 14 day at cold acclimate. However, a difference increase was detected in control cultivar. Moreover, chlorophyII content under non-acclimated and cold acclimation was statistically significant. This is in disagreement with an earlier report that indicated antioxidant activity is not linked to cold tolerance (Cansev *et al*., 2009). Siddique *et al*. (2018) indicated that Chl a and b values reduced the two cultivars (9 and 5) by 84% and 88% compared to controls. Carotenoid value also reduced in one cultivar (5) reduced by 54% compared to control. This outcome confirms that it could decrease the damage of antioxidant activity, at the

same time, the amounts of the chlorophyll enzyme have a direct role in the cold tolerance of *V. sativa* cultivars.

4. CONCLUSION

Cold acclimation protects plant tissues from injury under low temperature conditions and increases the productivity of crop species. Implement of cold acclimation in *V. sativa* is remarkably linked to the enrichment of antioxidant enzyme activity. *In vitro* selection methods implemented could be suggested for the cold resistance determination of seedlings in control conditions and important implications in selection breeding.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

KJ Senthil Kumar: Writing, Editing, Validation. **Ismail Bezirganoglu:** Experiment design, supervision. **Busra Yazicilar:** Laboratory work, statistical analysis. **Merve Simsek Geyik:** Laboratory work, statistical analysis. **Fatma Ozge Aslan:** Laboratory work, statistical analysis.

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