

## THE ROLE OF ANTIOXIDATIVE ENZYMES IN TOMATO LEAVES UNDER HEAT STRESS AT TWO DEVELOPMENTAL PHASES

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### Abstract

The role of antioxidative enzymes in response to heat stress tolerance (HST) of three tomato cultivars was investigated in this research. The leaves were obtained from tomato plants at the first bloom and yield stages and then were subjected to controlled high temperature treatments in water bath. The temperature in the water bath was increased 5 °C systematically for every half an hour from 35 to 60°C to generate a heat-stressed condition. The plants in yield stage exhibited an increase in HST when compared with the plants in first bloom stage and heat stress increased hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) level in leaves. Among the performed enzyme analysis, only catalase (CAT) involved in HST of tomato cultivars, however it may not be correlated with the degree of HST. In addition, two acidic and one basic isoperoxidases appeared in relatively heat tolerant cultivars in the yield stage, therefore isoperoxidases may be associated to HST in tomato plant.

Keywords: Developmental stage, high temperature, isoperoxidases, *Solanum lycopersicum* L., oxidative stress.

### 1. INTRODUCTION

Temperature is one of the critical environmental facts for survival of plants. Due to global climate change, heat stress is envisaged to be one of the contributing cause of the restrictions in future plant production. The temperature increase over the optimum, which can cause irrevocable injury to plant function and development, is called high temperature stress (Wahid et al., 2007). When temperature rises, cell damage and death may occur within a few minutes with increasing temperatures. This could be due to the deterioration of cellular homeostasis and discrete uncoupled physiological and biochemical actions (Awasthi et al., 2015).

Plants have evolved various mechanisms to combat stress factors as they occur in their environments. After high temperatures, plants have the ability to gain heat stress tolerance (HST) by being exposed to a gradual increase in temperature, resulting in stress acclimation. Plant acclimation to moderately high temperature has significant effect on inducement of tolerance to lethal high temperatures (Hasanuzzaman et al., 2013). Estimation of cell membrane stability under heat stress is used to express stress tolerance in various plant species including tomato (Inaba and Crandall, 1988; Cansev, 2012; Kesici et al., 2013; Din et al., 2015; Jahan et al., 2019). In addition, heat stress causes the production of highly reactive and toxic superoxide (O<sub>2</sub><sup>-</sup>), singlet oxygen (<sup>1</sup>O<sub>2</sub>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hydroxyl radicals (<sup>•</sup>OH) known as reactive oxygen species

(ROS) which causes oxidative stress by damaging proteins, lipids and carbohydrates (Suzuki and Katano, 2018; Cohen et al., 2021). During stress acclimation ROS act as signal transduction molecules and regulates different pathways and besides, they are toxic side products of stress metabolism (Choudhury et al., 2017). Plants have generated an effective antioxidative pattern composing of enzymatic and non-enzymatic antioxidants to minimize and repair the ROS-initiated damage. High temperatures stimulate and/or augment ROS-scavenging enzymes, which include superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), peroxidase (POX: EC 1.11.1.7), ascorbate peroxidase (APX: EC 1.11.1.11), glutathione reductase (GR: EC 1.6.4.2) and several antioxidants (Sharma et al., 2012).

According to the 2020 data, Turkey was the first in Europe and the fourth in the world rankings with 13.204.015 tonnes tomato production values (FAO, 2022). Despite the worldwide production potential tomato faces so many abiotic stress and heat stress is a pivotal problem currently (Golam et al., 2012). Daily average temperatures should be between 21-24°C optimum for tomato cultivation developmental period dependently (Geisenberg and Stewart, 1986) and only a few degrees higher than optimum temperature may diminish fruit production and seed set (Peet et al., 1997). Moreover heat stress has numerous specific effects depending on the cultivar (Sato et al., 2004; Almeselmani et al., 2006). Consequently, it is important to know the response of plant cultivars in stress conditions to breed for increased stress tolerance of plant. Besides some physiologic measurements, changes in activities of soluble POX and cell-well-bound POX were analyzed during heat stress in tomato cultivars in our previous work (Turhan et al., 2014). However, the role of antioxidative enzymes in thermal stress tolerance of tomato plant have not been fully clarified. Therefore the present paper discusses antioxidative enzymes reaction in response to heat stress in two different developmental stages (first bloom and yield stages) in three tomato cultivars with different levels of heat tolerance.

## 2. MATERIALS AND METHODS

Leaves of tomato hybrid cultivars ('Çaltı', 'Pembe' and 'Yaren'), widely grown throughout Turkey, were used in the study. The samples were collected from the plants grown in a field under favourable conditions for tomato production, in Eskisehir, Turkey (longitude: 39°45'38"N, latitude: 30°28'47"E) during first bloom stage (June) and yield stage (August). In June, the mean temperature was 21.7°C (range 7.0°C–35.2°C). In August, the mean temperature was also 21.7°C (range 4.8°C–35.4°C). The leaves, preferably from the third fully developed leaves from the apex, were sampled for heat stress treatments. The controlled heat tests were applied to the leaf samples as described previously by Arora et al. (1998), with some modifications. Briefly, leaves sampled at both developmental stages were collected into pyrex tubes and covered, then placed into water bath. After a 30-min acclimation of the leaf tissues containing tubes in water bath adjusted to 30°C, the water temperature was increased bit by bit, 5°C every half an hour from 35 to 60°C to inflict heat stress. Samples then that were obtained at each temperature divided into two groups: One group was used to detect the HST and leaf relative water content (RWC) of tomato leaves and the other group was immediately fixed in liquid nitrogen (N<sub>2</sub>) and stored at -80°C until H<sub>2</sub>O<sub>2</sub> and enzyme assays were performed.

At each treatment, HST of leaf tissues was evaluated by assaying cell membrane injury using the electrolyte-leakage method as described previously by Kesici et al. (2013) with minor modifications. In brief, leaf disks of 0.5 cm diameter from each treatment group were put into test tubes containing 10 mL of deionized water. The samples were then subjected to vacuum infiltrated

at -0.15 MPa for 5 min and incubated at room temperature ( $24 \pm 1^\circ\text{C}$ ) for 4 hours using an orbital shaker (Unimax 2010, Heidolph, Germany) at 250 rpm before the electrical conductivity of each solution was measured using a conductivity meter (YSI 3200, USA) then immediately autoclaved. Total conductivity was determined once more when the solution in test tubes cooled down to room temperature. Cell membrane injury was defined as the percentage of total ions present in the tissue (Gulen and Eris, 2003). In addition, HST ( $LT_{50}$ ) was calculated as the mid-point between the maximum injury and the control which expresses the temperature causing half maximum percentage injury.

Relative water content (RWC, %) of the leaves was measured as described by Gulen and Eris (2003). In short, 1 cm leaf discs were weighed for fresh weight (FW) and then placed in a petri dish containing deionized water for 4 h, then leaf discs were taken out from the petri dish, blotted and weighed, at room temperature for turgid weight (TW). For the dry weight (DW), leaf discs were oven-dried for 48 h at  $70^\circ\text{C}$ . Leaf RWC is calculated as follows:  $\text{RWC} = [(\text{FW}-\text{DW}) / (\text{TW}-\text{DW})] \times 100$ .

Hydrogen peroxide content of the leaves was determined according to Ngo and Lenhoff (1980). Briefly, liquid  $\text{N}_2$ -frozen leaf tissues of 1.0 g were homogenized in 4 mL of perchloric acid ( $\text{HClO}_4$ ) containing 1% (v/v) polyvinylpyrrolidone (PVPP) and sand. The homogenate was centrifuged at  $10\,000 \times g$  for 20 min at  $4^\circ\text{C}$ . Subsequently, the supernatant [adjusted to pH 7.5 using 4 M potassium hydroxide (KOH)] was centrifuged at  $1000 \times g$  for 1 min. A 400  $\mu\text{L}$  aliquot and 1.6 mL  $\text{dH}_2\text{O}$  were loaded to 2 mL columns of AG 1-X8 Resin 100–200 mesh chloride form,  $0.8 \times 4$  cm (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the eluent was used to determine  $\text{H}_2\text{O}_2$ . One mL of the reaction mixture contained 0.1 M sodium phosphate ( $\text{Na-PO}_4$ ), pH 6.5, 3.3  $\mu\text{moles}$  3-dimethylaminobenzoic acid (DMAB), 0.07  $\mu\text{moles}$  3-methyl-2-benzothiazolinone hydrazone (MBTH), and 10  $\mu\text{g}$  horseradish peroxidase (Sigma Chemical Co. Ltd.). The reaction was initiated by adding 1.0 mL of the tissue extract. The absorbance was recorded at 590 nm, and  $\text{H}_2\text{O}_2$  concentrations were calculated by using an  $\text{H}_2\text{O}_2$  standard curve.

To prepare the enzyme extracts, 0.5 g of liquid  $\text{N}_2$ -frozen leaf tissues were ground in a pre-chilled mortar and pestle with 5 mL of the following ice-cold extraction solutions containing 1.0% PVPP (Ergin et al., 2016): For SOD and CAT 100 mM potassium phosphate (K-phosphate) buffer, pH 7.0, containing 0.1 mM ethylenediamine-tetraacetic acid (EDTA), with addition 0.1% Triton; for APX and GR 50 mM K-phosphate buffer, pH 7.8, containing 50 mM ascorbic acid (AsA) and 50 mM K-phosphate, pH 7.6, containing 0.1 mM EDTA, respectively. The homogenate was centrifuged at  $15\,000 \times g$  for 20 min at  $4^\circ\text{C}$ . The supernatants were used to assay for the enzymatic activities and total soluble protein analysis. The assay procedure for the determination of total SOD activity in the leaf samples was carried out as described by McCord and Fridovich (1969). Briefly, 10  $\mu\text{g}$  of total protein was added to a mixture of 30  $\mu\text{M}$  cytochrome C, 100  $\mu\text{M}$  xanthine, and 0,0185  $\mu\text{M}$  xanthine oxidase. The reduction of cytochrome C was measured spectrophotometrically as the change in OD 550. One unit of SOD activity was defined as that which inhibited by 50% of the reaction rate of cytochrome C per mg of protein (McCord and Fridovich, 1969). The method of Rao et al. (1996) was employed for the assay of CAT by recording the decrease of absorption at 240 nm in a UV/VIS spectrophotometer (Perkin Elmer Lambda 25, USA) as  $\text{H}_2\text{O}_2$  ( $\epsilon = 39.4$  mM/cm) was consumed. The APX activity was carried out spectrophotometrically by monitoring the decrease in oxidized AsA ( $\epsilon = 2.8$  mM/cm) at 290 nm (Nakano and Asada, 1980). The GR activity was measured spectrophotometrically at 340 nm ( $\epsilon = 6.22$  mM/cm) by the method of Cakmak and

Marschner (1992), corresponding the oxidation of  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADPH).

The soluble protein content of the crude enzyme extracts was determined by the Bradford assay method using bovine serum albumin (BSA, Sigma) as standard (Bradford, 1976).

The method of Gulen and Eris (2004) was used for the POX extraction from leaf samples. Shortly, 0.1 g of ground leaf samples were homogenized with 0.6 mL 0.1M K-phosphate buffer, pH 7.5, containing 30 mM boric acid, 50 mM AsA, 17 mM sodium metabisulfite, 16 mM diethyldithiocarbamic acid (DIECA), 1mM EDTA, with addition PVP-40 as 4% (w/v) and sodium hydroxide (NaOH) was used to readjusted of the final pH to 7.5. The homogenate was centrifuged at  $21\ 000 \times g$  for 20 min at 4°C and the supernatant was used for POX electrophoresis. Samples were subjected to discontinuous polyacrylamide gel electrophoresis (PAGE) under nondenaturing, nonreducing conditions using a Mini-PROTEAN tetra cell electrophoresis system (Bio-Rad, Hercules, CA) as described by Davis (1964) and Reisfeld et al. (1962) for acidic and basic POX, respectively. Native gels, each consist of 10% separating gel and 5% stacking gel, were used for both systems. Then, samples were loaded onto the gel using micropipette, 20  $\mu$ L in each well. Running conditions were 30 min at 20 mA, afterwards 3 h at 40 mA. Staining procedure was taken from Wendel and Weeden (1989) then, the relative mobility (Rf) value of the bands on the gel was calculated by using  $Rf = \frac{\text{distance to the finishing point of the running}}{\text{distance to the starting point of the running}}$ , as the distance to the finishing point of the running and  $Rf = 0.0$ , as the starting point of the running (Manganaris and Alston, 1992). Optical density evaluation of POX bands on gel was performed using a Public Domain NIH Image program (U.S. National Institutes of Health, <http://rsb.info.nih.gov/nih-image/>). The areas under the clearance curves were quantified in arbitrary units.

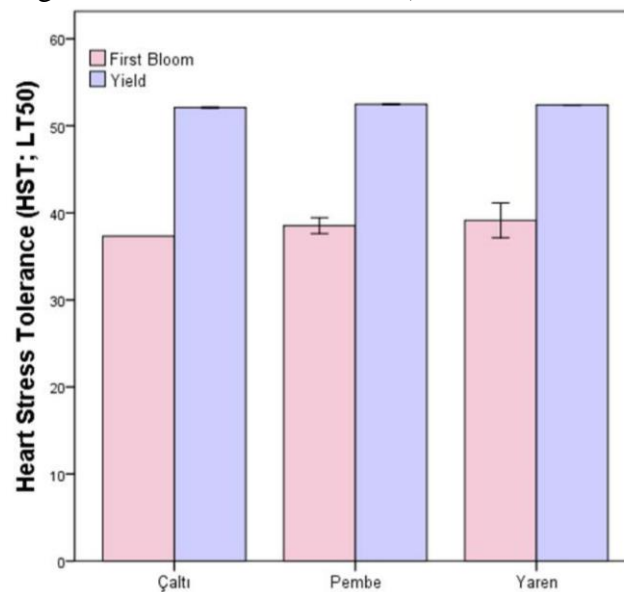
The experiment was set up using a randomized block design. Ten biological and 5 technical replications were used for all assays. The data were subjected to analysis of variance (ANOVA) and the means were evaluated using Duncan test at  $p < 0.05$  using the SPSS software (version 22., Chicago, IL, USA).

### 3. RESULTS AND DISCUSSIONS

Ion leakage is a common evaluation way for cell membrane thermostability and a valuable criterion for heat tolerance in selecting heat tolerant plants (Inaba and Crandall, 1988; Kesici et al., 2013; Din et al., 2015). Electrolyte leakage is affected by the age, sampling part, development period, growing season, hardening and species of plant (Wahid et al., 2007). The HST, denoted as the  $LT_{50}$  of leaf sample from three tomato cultivars, is indicated in Figure 1. Although the effect of sampling stage on HST was statistically significant, it has been found no significant effect on cultivar and interaction between sampling stage and cultivar (Table 1).

The average HST of three tomato cultivars showed an increase in the yield stage ( $LT_{50}$ ; 52.3°C) and decreased in the first bloom stage ( $LT_{50}$ ; 38.3°C). Based on the average values of data from the two stages, heat-acclimation provide an increment in HST of all studied cultivars (measured as an increase in  $LT_{50}$ ), which was lower in cv. 'Çaltı' ( $LT_{50}$ ; 44.7°C) than in cvs. 'Pembe' ( $LT_{50}$ ; 45.5°C) and 'Yaren' ( $LT_{50}$ ; 45.8°C). Generally reproductive phase in flowering plants is highly sensitive to temperature stresses. For tomato, pollen growth and fertilization appear to be particularly sensitive to heat stress (Zinn et al., 2010). The results of the current study showed that high temperature causes reduction in HST of the cultivars in the first bloom stage and the cultivars showing higher values of HST in the yield stage. Although it has been found no significant difference between HST degrees of the cultivars, it can be suggested that cv. 'Çaltı' is relatively more sensitive to high

temperatures than cvs. ‘Pembe’ and ‘Yaren’ because of the 1.1°C HST gap between relatively sensitive and tolerant cultivars in the current study. Similarly, Kesici et al. (2013) reported that, HST gap was found to be 1.09°C between relatively heat-sensitive and heat-tolerant strawberry cultivars. In addition, Din et al. (2015) reported that the tomato genotypes exhibiting high HST are likely to tolerate high temperature and thus avoid damage to leaf tissues. Higher values of HST indicate that less membrane damage, inhibition of electrolyte leakage, prevention of membrane protein denaturation, and higher tolerance to heat stress (Wahid et al., 2007).

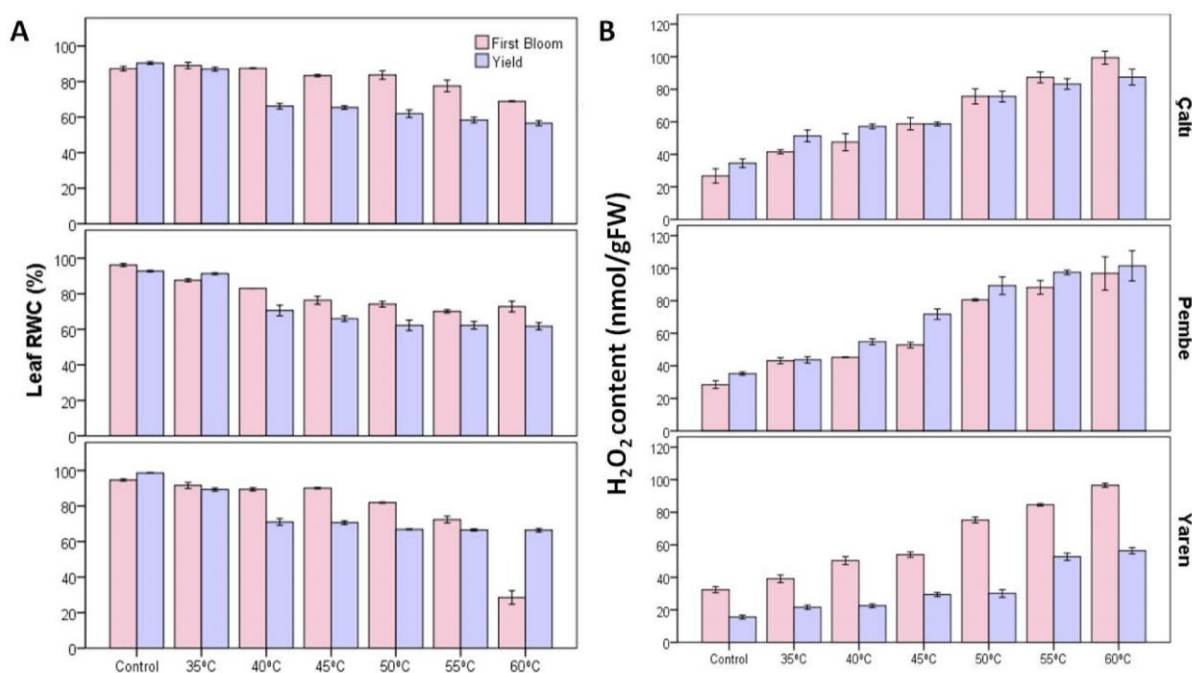


**Figure 1.** Heat stress tolerance (HST; LT<sub>50</sub>) of tomato cultivars in first bloom and yield stage. Vertical lines on bars indicate  $\pm$  standard errors (S.E.) of repetitions.

Relative water content could serve as indicator for plant water status which can indicate the balance between absorbed water by plant and consumed through transpiration (Arjenaki et al., 2012). The changes in leaf RWC values in the leaf samples are shown in Figure 2A. Data obtained from the current research revealed that leaf RWC generally decreased by heat treatments in both sampling stages in all cultivars. According to the average values, plants exhibited higher leaf RWC (80.3%) at first bloom stage than in the yield stage (72.5%). In addition the highest leaf RWC value in the first bloom stage was determined in cv. ‘Çaltı’ (82.5%) while the lowest leaf RWC value in the first bloom stage was detected in cv. ‘Yaren’ (78.3%). On the other hand, the highest (75.6%) and the lowest (69.4%) leaf RWC values in the yield stage were detected in cvs. ‘Yaren’ and ‘Çaltı’, respectively.

A decrease in RWC in response to high temperature was also reported in tomato (Morales et al., 2003; Zhou et al., 2019), strawberry (Gulen and Eris, 2003), olive (Cansev, 2012) and lablab bean (Myrene and Devaraj, 2013). First of all heat stress causes a decrease in leaf RWC and the loss of turgidity which is a consequence of elevated transpiration (Cansev, 2012; Turhan et al., 2014). However in this study, plants exhibited higher leaf RWC at first bloom stage than that in the yield stage (Figure 2A.) Besides, there was no significant difference in the value of leaf RWC with alterations in cultivars in response to heat stress (Table 1). It means that the higher leaf RWC was not associated with greater HST of the tomato cultivars.

Although ROS are produced in chloroplast, mitochondria and peroxysomes under optimal conditions, they may be generated by high temperature stress that induces lipid peroxidation and destruction which leads to membrane damage (Awasthi et al., 2015). Recently, H<sub>2</sub>O<sub>2</sub> has been suggested as a mobile signal in transcription factors associated with the induction of genes coding antioxidant enzymes (Exposito-Rodriguez et al., 2017). As shown in Figure 2B H<sub>2</sub>O<sub>2</sub> content of leaf tissues increased stepwise from control to 60°C in all cultivars. Based on the average values, H<sub>2</sub>O<sub>2</sub> content in the first bloom stage (62.1 nmol/gFW) was higher than those in yield stage (55.7 nmol/gFW) and H<sub>2</sub>O<sub>2</sub> content of the cultivars was almost same in the first bloom stage (~62.0 nmol/gFW). These results are parallel to HST results in which plants exhibited lower HST at the first bloom stage than that in the yield stage. In addition, the minimum H<sub>2</sub>O<sub>2</sub> activity occurred in cv. ‘Yaren’ which is relatively tolerant to high temperature stress in both the first bloom and the yield stages. It may be because of the activation of H<sub>2</sub>O<sub>2</sub> scavengers such as antioxidant enzymes (Lee et al., 2013) Similarly, Ergin et al. (2012) also reported that higher accumulation of H<sub>2</sub>O<sub>2</sub> in heat-sensitive cv. ‘CG3’ strawberry plants than heat tolerant cv. ‘Redlans Hope’ under heat stress. In addition H<sub>2</sub>O<sub>2</sub> content increased in both sampling stages at all high temperature treatments compared to the control plants. Likewise, H<sub>2</sub>O<sub>2</sub> content increased in chickpea (Ceylan et al., 2013) and rice (Lee et al., 2013) leaf tissues in response to heat stress. The effect of sampling stage, cultivar, heat stress treatment and their interaction on H<sub>2</sub>O<sub>2</sub> content was statistically significant (Table 1).



**Figure 2.** Effect of heat stress on leaf relative water content (RWC) (Panel A) and H<sub>2</sub>O<sub>2</sub> content (Panel B) of tomato cultivars in first bloom and yield stage. Vertical lines on bars indicate  $\pm$  S.E. of repetitions.

Various enzymes depended metabolic pathway are susceptible to different levels of heat stress (Hasanuzzaman et al., 2013). The growth period and season and variety of crops, depending upon their tolerance or sensitivity, also affects the activities of enzymes (Almeselmani et al., 2006). The

increased tolerance of plants to heat stress is related to the maintenance of high ROS scavenge ability due to high antioxidative enzyme capacity including SODs (Almeselmani et al., 2009). Superoxide dismutases have a significant role in plant physiology as a result of the dual role of ROS, as signals in numerous transduction pathways and as inducers of oxidative damage in the cellular components when ROS cellular overproduction. The elevated SOD activity may be a protective mechanism against generation of superoxide (del Río et al., 2018). The SOD activity of leaf tissues generally decreased from 45°C treatment to the highest temperature in both the first bloom and the yield stages, except cv. 'Pembe' in the yield stage (Figure 3A). In general, the mean SOD activity in the first bloom stage (~4.0 units/mg protein) was significantly lower than that in the yield stage (4.9 units/mg protein). The SOD activity was higher in cv. 'Pembe' than in cvs. 'Yaren' and 'Çaltı' at all sampling stages. The effect of sampling stage along with cultivar and heat stress treatment and their interaction on SOD activity was statistically significant except the interaction of sampling stage and heat treatment on SOD activity (Table 1). The results of the current study showed that leaf tissues of tomato cultivars increase SOD activity up to 45°C to activate the antioxidant systems. Similarly, tomato (Ogweno et al., 2008), mulberry (Chaitanya et al., 2002) and chickpea (Ceylan et al., 2013) showed an increment in SOD activity under high temperature stress. The SOD activity was constant in tolerant 'Eagleton' and reduced in sensitive 'Brilliant' during the high temperature in Kentucky bluegrass (He and Huang, 2010).

Catalase, found largely in peroxisomes, directly transforms H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and O<sub>2</sub> and it is more involved in detoxification of H<sub>2</sub>O<sub>2</sub> not as a signaling molecule (Sofu et al., 2015). Data from the current research showed that up to 40°C CAT activity of leaf tissues was almost stable, while it increased significantly at 45°C; the leaf CAT activity rose up to 50% of its basal values when the temperature was increased to 55°C (Figure 3B). Based on the average values, CAT enzyme activity was generally lower in the first bloom stage than that in the yield stage as 104.8 nmol/mg protein and 139.6 nmol/mg protein, respectively. Current research revealed that cvs. 'Pembe' and 'Çaltı' had higher CAT activity than cv. 'Yaren' in all sampling stages. Statistical analysis revealed significant effect of sampling stage, cultivar, heat stress treatment and their interaction, except the interaction of sampling stage and heat treatment on CAT activity (Table 1). It was evident from the results that, there was a notable increase in the CAT activity induced by high temperatures (Figure 3B). The cultivar variations were also significant (Table 1). Likewise, increments in CAT enzyme activity were associated to alterations in HST of Kentucky bluegrass cultivars (He and Huang, 2010) and wheat (Almeselmani et al., 2009). In addition, the CAT activity of tomato cultivar (cv. 9021) increased due to heat stress (Ogweno et al., 2008). Increments in the activity of CAT enzyme considered as a response to the elevated H<sub>2</sub>O<sub>2</sub> generation under stress and may be effective in detoxifying of high temperature induced H<sub>2</sub>O<sub>2</sub> (He and Huang, 2010). Interestingly, however, cv. 'Yaren', which has the highest HST in the current study, exhibited lower CAT activity than that of cv. 'Çaltı', having lower HST. According to the presented data, although related to HST of tomato cultivars, CAT may not be associated with the degree of HST. Similar results have been found in common bean plant subjected to heat stress (Aydogan et al., 2017).

In the AsA–glutathione cycle, APX reduces H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O utilizing AsA as an electron donor (Sofu et al., 2015). The APX activity of leaf tissues increased stepwise from control to 60°C in all cultivars in both sampling stages (Figure 3C). Parallel to this research, the APX enzyme activity of strawberry (Ergin et al., 2016) and tomato (Ogweno et al., 2008) enhanced with temperature and duration of heat stress. Higher APX activity might improve the H<sub>2</sub>O<sub>2</sub> scavenging system in chloroplasts and prevent the accumulation of H<sub>2</sub>O<sub>2</sub> (Chaitanya et al., 2002). According to the mean

values, the higher APX activity occurred in first bloom stage (~0.36  $\mu\text{mol}/\text{mg}$  protein) than that in yield stage (~0.16  $\mu\text{mol}/\text{mg}$  protein). In addition, APX enzyme activity was higher in cv. ‘Çaltı’, which had lower HST than cvs. ‘Pembe’ and ‘Yaren’ at all sampling stages. It means that APX may not be involved in determining cultivar sensitivity to heat stress and HST in tomato. Statistical tests revealed significant effect of sampling stage, cultivar, heat stress treatment and their interaction on APX activity (Table 1).

Glutathione reductase is a possible enzyme of the AsA-reduced glutathione (GSH) cycle and acts a part in the defence system against ROS by maintaining the reduced position of GSH (Noctor et al., 2012). According to the average values of data from both stages, heat treatments did not affect the GR activity of leaf tissues (Figure 3D). The GR activity was increased with as temperature increases in leaves of *Dolichos lablab* (Myrene and Deveraj, 2013) and *Phaseolus vulgaris* (Aydoğan et al., 2017). Generally, the mean GR activity in the first bloom stage (12.3 units/mg protein) was remarkably higher than that in the yield stage (4.8 units/mg protein). These results suggest that, higher GR activity was not related to higher HST of the tomato cultivars. The GR activity in the first bloom stage was lower in cv. ‘Pembe’ than in cvs. ‘Çaltı’ and ‘Yaren’ whereas, the values were almost the same in all cultivars in the yield stage (~4.8 units/mg protein). Statistical analysis revealed significant effect of sampling stage and cultivars and the interaction between sampling stage, cultivar and heat stress treatment on GR activity (Table 1).

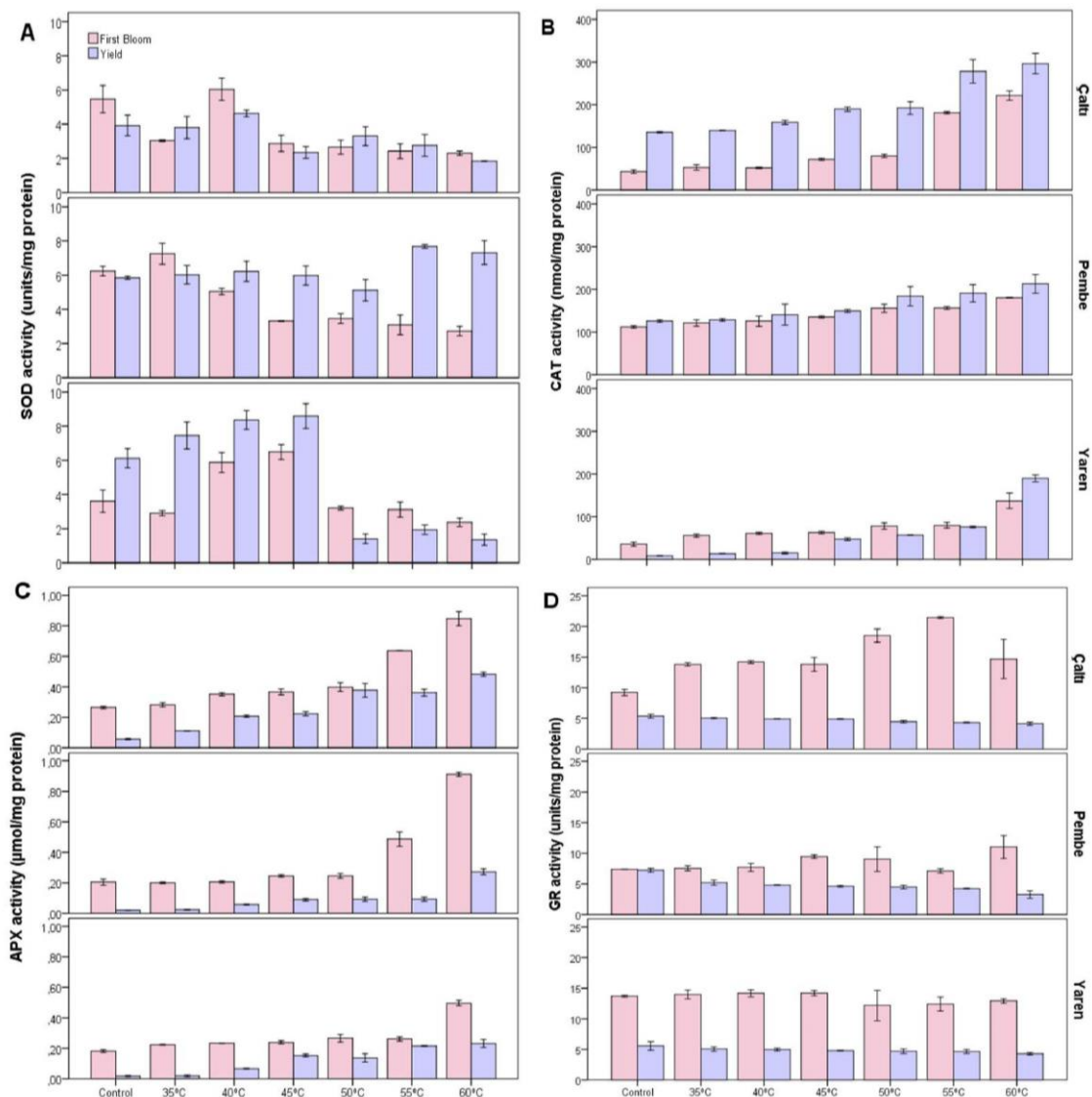
**Table 1. Results of analysis of variance (ANOVA) of stage (S), cultivar (Cv.) temperature (T) and their interactions with  $LT_{50}$ , RWC,  $H_2O_2$  content, SOD, CAT, APX and GR activity in leaf tissues. Numbers represent F values relative to a significance level of 0.05.**

Dependent Variable	Independent Variable						
	S	Cv.	T	S×Cv.	S×T	Cv.×T	S×Cv.×T
$LT_{50}$	201,496*	0,329 <sup>ns</sup>	-	0,183 <sup>ns</sup>	-	-	-
RWC	178,808*	1,164 <sup>ns</sup>	231,322*	26,745*	37,946*	16,739*	20,157*
$H_2O_2$ content	34,400*	117,837*	238,720*	110,993*	4,221*	3,054*	2,232*
SOD activity	29,316*	51,237*	30,757*	15,678*	1,545 <sup>ns</sup>	11,331*	10,025*
CAT activity	107,293*	291,730*	113,009*	99,599*	1,973 <sup>ns</sup>	9,466*	2,221*
APX activity	1424,598*	327,984*	422,623*	39,033*	57,416*	25,281*	15,252*
GR activity	1335,065*	90,217*	1,337 <sup>ns</sup>	95,023*	10,828*	7,753*	7,859*

\*, ns Significant and not significant at  $p < 0.05$

Peroxidases are a kind of antioxidant enzymes that catalyze the decomposition of  $H_2O_2$  in to water and molecular oxygen with the concomitant formation of broad variety of substrates (Pandey et al., 2017). Each POX group has a diversified function in the cell; such as lignification, suberization, stress protection and defense response (Wang et al., 2015). The POX enzyme has been associated to the occurrence of physiological damages occasioned by high temperature stress in plants, and its activity was escalated by heat stress (Gulen and Eris, 2004). Native PAGE of POX enzymes was performed to get acidic and basic isozyme bands in the leaf samples. The results from a single representative experiment repeated three times are presented herein. The acidic isoperoxidase profile showed the appearance of new isoforms in tomato leaves (Figure 4A), whereas basic POX isoform could not be observed in the first bloom stage.





**Figure 3.** Effect of heat treatments on the leaf superoxide dismutase (SOD) (Panel A), catalase (CAT) (Panel B), ascorbate peroxidase (APX) (Panel C) and glutathione reductase (GR) (Panel D) activities of tomato cultivars in first bloom and yield stage. Vertical lines on bars indicate  $\pm$  S.E. of repetitions.

In fact acidic POX bands were observed with different densities in all cultivars temperature-dependently in both first bloom and yield stages (Figure 4A and Figure 4B). While native PAGE of acidic POX isozymes yielded five bands in cv. ‘Çaltı’ (POX1: Rf=0.14, POX2: Rf=0.21, POX3: Rf=0.35, POX4: Rf=0.45 and POX5: Rf=0.63), there were no POX1 and POX3 acidic bands in cv. ‘Pembe’ and no POX1 band in cv. ‘Yaren’ in the first bloom stage (Figure 4A). The band intensity of acidic POX1 isoperoxidase of cv. ‘Çaltı’ was higher at 35°C, 40°C and 50°C treatments than control treatment. The band intensity of acidic POX2 isoperoxidases of three cultivars showed some alterations (dense and light), especially the band intensities of cvs. ‘Pembe’ and ‘Yaren’ showed a sharp increase at 35°C treatment for POX2. In addition, the band intensities of cvs. ‘Pembe’ and ‘Yaren’ were generally higher than cv. ‘Çaltı’ at all heat treatments. Besides, it was determined that

a very sharp increase in the POX3 band intensities of cv. 'Yaren' at 35°C and 55°C treatments. In addition, acidic POX4 and acidic POX5 bands intensities of three cultivars showed some fluctuations (dense and light) and generally denser in cv. 'Yaren' than in cvs. 'Pembe' and 'Çaltı' (Figure 4A). Regarding the results of yield stage, three acidic POX bands in cv. 'Çaltı' (POX3: Rf= 0.35, POX4: Rf= 0.45, POX5: Rf= 0.63), while five acidic POX bands in cvs. 'Pembe' and 'Yaren' (POX1: Rf=0.14; POX2: Rf=0.21, POX3: Rf=0.35, POX4: Rf=0.45, POX5: Rf=0.63) were determined (Figure 4B). The band intensity of acidic isoperoxidase POX1 of cvs. 'Pembe' and 'Yaren' was denser only at 50°C than control treatment. In addition, acidic POX2 band intensity of cv. 'Pembe' showed generally stepwise increase until 50°C. In cv. 'Yaren', it has found that similarly to acidic POX1 band the intensity of acidic POX2 band was higher only at 50°C treatment than control treatment. Moreover the band intensity of acidic POX3, POX4 and POX5 isoperoxidases of three cultivars showed some fluctuations (dense and light) (Figure 4B). Basic POX bands (POX1: Rf=0.42, POX2: Rf=0.54) were observed at different concentrations depending on the temperature only in the yield stage (Figure 5). It was determined that the band intensity of basic POX1 showed a sharp increase at 35°C in cvs. 'Çaltı' and 'Pembe'. This sharp increase was observed at 40°C in cv. 'Yaren'. Basic POX2 band was observed generally in 35°C temperatures in cv. 'Pembe' and the intensity of this band showed an increase at 40°C treatment in cv. 'Yaren' (Figure 5).

In the current study two acidic isoperoxidase (POX1; Rf=0.14 and POX2; Rf=0.21) and one basic isoperoxidase (POX2; Rf=0.54) may be related to heat tolerance in yield stage (Figure 4B, Figure 5). He and Huang (2010); detected the response of three isoperoxidases (Rf=0.06, Rf= 0.13, Rf= 0.21) in Kentucky bluegrass. The temporary escalation in isoperoxidase density may represent temporary H<sub>2</sub>O<sub>2</sub> generation during the early phase of heat stress and/or changes in the generation of secondary metabolites for stress defence (He and Huang, 2010). It was stated in the previous works of Gulen and Eris (2004) and Ergin et al. (2012) that thermal stress causes an increase at isoperoxidase activity in strawberry. In addition, our previous study also showed that especially cell wall-bound POX activity increased in tomato plant by high temperature treatment (Turhan et al., 2014). Five isoenzymes of POX were identified also in chickpea under heat stress (Ceylan et al., 2013). Aydoğan et al. (2017); have showed that POX isoenzyme activities notably differed depending on HST of common bean genotypes.

#### 4. CONCLUSIONS

This study indicates that high temperature stress disrupts the growth of tomato plant by damaging cellular membranes and increasing H<sub>2</sub>O<sub>2</sub> level in leaves and also reveals that the cultivars in the earlier developmental phase (first bloom) are much more sensitive to heat stress than those in the yield stage. The plants suffered heat stress when treated at 38.3°C and 52.3°C at first bloom and yield stage, respectively. The cultivars 'Yaren' and 'Pembe' were relatively heat-tolerant, while 'Çaltı' was relatively heat-sensitive. In addition, tomato leaf tissues were found to enhance the structural stability of cellular membranes under heat stress by increasing both the activity of such enzymes as SOD, APX, CAT, GR and POX to activate/induce the antioxidative systems. However, SOD, APX and GR activities were not related to higher HST of the tomato cultivars. Despite the fact that involved in HST of tomato plants, CAT may not be related to the level of HST. Besides, POX isozyme forms reacted differently to high temperatures in three cultivars of tomato and isoperoxidases could be opt in ascertaining cultivar susceptibility to thermal stress. Hence, further molecular biological researches are needed to clarify the HST of tomato plants and new strategies are required to improve the ability of tomato to adjust thermal stress.

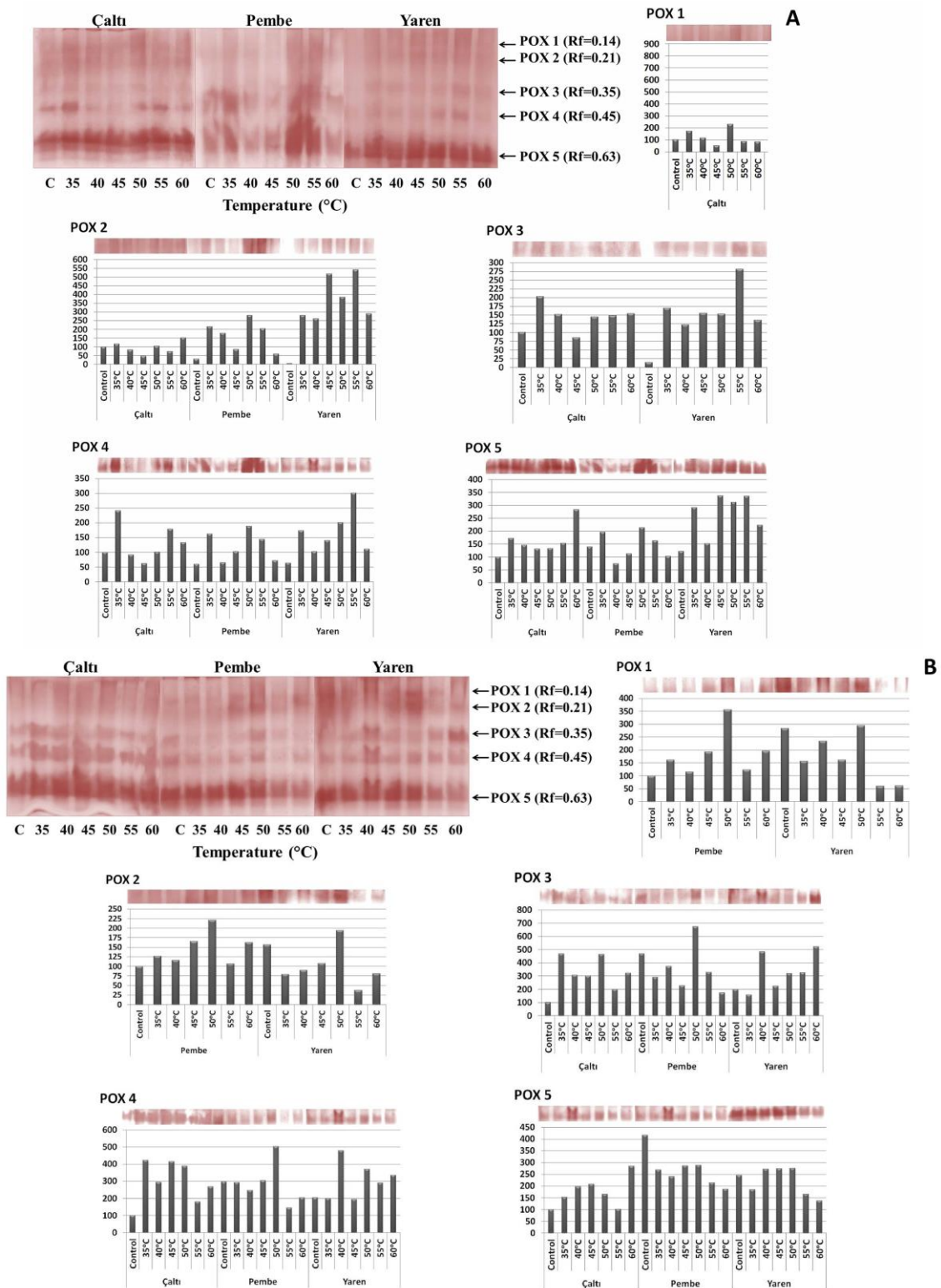


Figure 4. Effect of heat stress on acidic POX profiles of tomato cultivars. A: Acidic POX activity and band intensities of the acidic POX activity in first bloom stage; B: Acidic POX activity and band intensities of the acidic POX activity in yield stage. Equal volumes of the crude extracts, 20µL, were loaded in each lane.

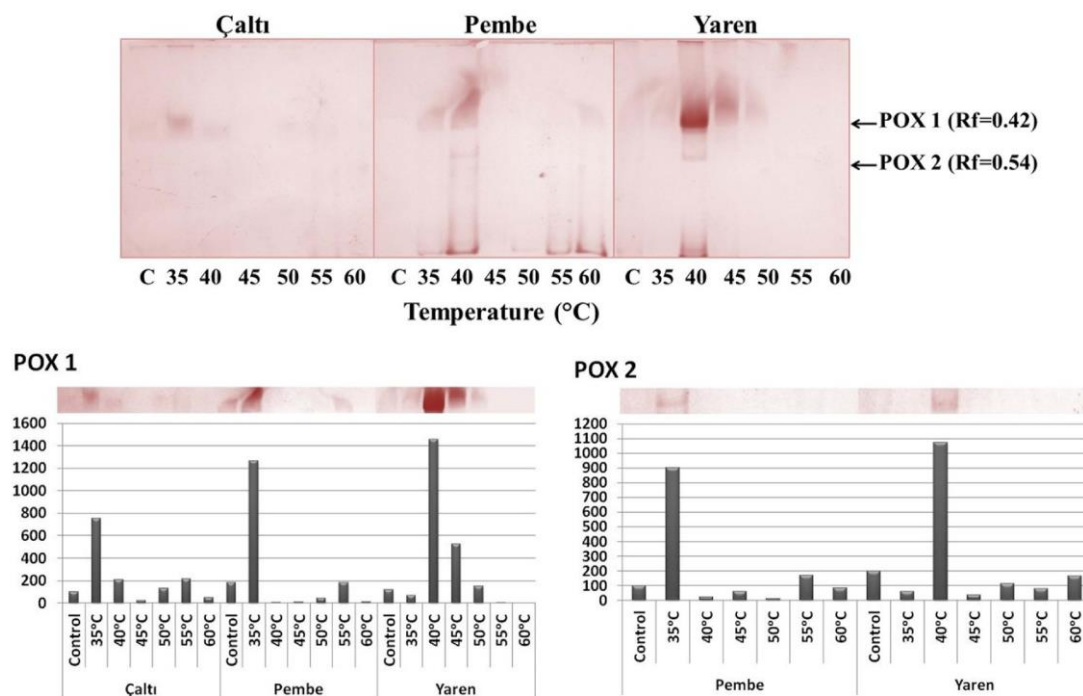


Figure 5. Effect of heat stress on basic POX profiles and band intensities of tomato cultivars in yield stage. Equal volumes of the crude extracts, 20 $\mu$ L, were loaded in each lane.

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