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EFFECTS OF HEAT STRESS ON SOME BIOCHEMICAL TRAITS OF SMALL REDDISH BEAN

Cigdem Aydogan[1]*, Ece Turhan[1]

[1]Eskisehir Osmangazi University, Faculty of Agriculture,
Department of Agricultural Biotechnology, Eskisehir, Turkey

Abstract
The effects of heat stress on malondialdehid (MDA) content, ascorbic acid (AsA), isoperoxidases, total soluble protein (TSP) and SDS-PAGE protein profiles of small reddish bean (Phaseolus vulgaris L. cv. Keklik) leaves were investigated. For this purpose, the collected leaf samples (preferably the third from the apex) were placed into pyrex tubes with caps closed and incubated in a water bath: after a 30-minutes habituation at 30ºC, to apply heat stress the leaves were subjected to 35, 40, 45, 50, 55 and 60°C with gradual increments every 30-minutes. According to the results, the MDA content increased with escalating temperatures, starting with 40°C. Although there was fluctuation, the AsA content generally decreased with high temperatures. Besides, TSP content decreased after 50°C. Moreover, three asidic and four basic isoperoxidase bands were identified. There was a marked difference at acidic isoperoxidase band intensities under 40 and 45°C heat treatment. Protein bands with molecu1ar weight of approximately 14.5-195 kDa were determined. In conclusion, especially 40°C is critical for small reddish bean plants, besides 40°C and above temperatures causes oxidative stress in small reddish bean plant. Additionally, isoperoxidases and proteins may be an effective mean on heat stress tolerance.

Keywords: Ascorbic Acid, high temperature stress, lipid peroxidation, peroxidase, Phaseolus vulgaris L., protein

1. INTRODUCTION

The global climate change sets forward the variance in temperature, rainfall and atmospheric conditions and impacts the plants by negatively affecting their morphological, developmental, cellular and molecular processes (Chaudhry and Sidhu, 2022). Exposure of plants to high temperature for long duration may cause serious alteration in the metabolic activeties and cellular disorganization that leads to reduced growth and development (Rai et al., 2018).

Heat stress induces the denaturation of cellular structures, disrupts protein stability, anf membrane disintegration which causes the over-abundant production of reactive oxygen species (ROS) and serious oxidative stress in plants (He et al., 2017) resulting in cell membrane damage and electrolyte leakage by lipid peroxidation (LPO) (Dongsansuk et al., 2021). The ROS are toxic and damage the bio-molecules such as lipids, and proteins (Sidhu et al., 2016). Heat stress enhances ROS generation in plants resulting in increased LPO, membrane dissociation, and eventually cell death (Hasanuzzaman et al., 2020). The scavenging mechanisms, including enzymatic and non-enzymatic antioxidants counteract on ROS and convert them into less harmful products in the cell under normal conditions (Sidhu et al., 2016). The enzymatic antioxidants [superoxide dismutase

*Corresponding author, E-mail address: ciaydogan@ogu.edu.tr

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(SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), peroxidases (POXs: EC 1.11.1.X), glutathione reductase (GR: EC 1.6.4.2)] work in phase with non-enzymatic antioxidants [ascorbic acid (AsA), glutathione (GSH), flavonoids, alkaloids and phenolic compounds] to suppress the excessive production of ROS in plants (Laxa et al., 2019). The POXs, as an enzymatic antioxidant, belong to the oxidoreductase enzyme class and their catalytic capacity is associated with peroxidative, oxidative, catalytic, and hydroxylation reactions (de Oliveira et al., 2021). There are many POXs in plant cells that perform important functions in cell wall modifications such as lignification, suberization and cross-linking of hydroxyproline-rich glycoproteins and ploisaccharides, phytohormone metabolism, aging and stress-related processes. These enzymes play a key role in the detoxification of ROS, as POXs exhibit approximately 1000 times greater affinity for hydrogen peroxide compared to the CAT enzyme, and their activities can be modified in the presence of different stress factors (Lüthje and Martinez-Cortes, 2018). In addition to enzymatic antioxidants, non-enzymatic antioxidants, especially AsA, known with its strong antioxidant capacity and low energy requirement for its biosynthesis, provides the initial support to plants against oxidative stress (Alayafi, 2020) caused by photosynthesis, aerobic metabolism and abiotic stresses such as heat stress (Kumar et al., 2011). Temperature increases to which plants are exposed causes alteration of membrane fluidity affects membrane functions such as disruption of photosynthesis and respiration (Raja et al., 2020), misfolding of proteins and accumulation of protein aggregates which create proteotoxic stress (Osman et al., 2020; Haider et al., 2021). Plants actuate the antioxidant defense system to relieve the injurious effects of oxidative stress (Chaudhry and Sidhu, 2022). Thus, heat stress tolerance of plants is correlated to their ability of ROS scavenging and maintenance of membrane thermostability (Hameed et al., 2012) due to a higher antioxidant capacity (Chakraborty and Pradhan, 2011).

The objective of this study was, to investigate the effects of heat stress on some of the biochemical traits of small reddish bean. The experiment was also conducted to examine the effect of heat stress on changes in cell membrane damage by determining the amount of LPO.

2. MATERIALS AND METHODS

The sampling was made from small reddish bean plants (*Phaseolus vulgaris* L. cv. Keklik) grown in Eskisehir, Turkey (longitude: 39°45'38"N, latitude: 30°28'47"E), preferably from fully developed trifoliate leaflet third from the apex, showing varietal characteristics.

The controlled heat tests were applied to the leaf samples as follows: the collected leaf samples (preferably the third from the apex) were placed into pyrex tubes with caps closed and incubated in a water bath: after a 30-minutes habituation at 30ºC, to apply heat stress the water temperature was escalated to 35, 40, 45, 50, 55 and 60 °C gradually with 30 min. interval (Arora et al., 1998). Half of the samples obtained at each treatment temperature, were used for LPO and AsA assays while the other half were immediately fixed in liquid nitrogen (N_2) and stored at -80 °C until further analysis.

Lipid peroxidation was determined, by measuring malondialdehyde (MDA) content in the leaf samples, according to Rajinder et al. (1981). Approximately 100mg of fresh leaf tissues were homogenized in 0.1% trichloroacetic acid and centrifuged (10 000 × g, 5 min, 4°C) (Beckman Coulter Allegra 64R, USA). Afterwards a mixture of the supernatant and thiobarbituric acid (0.5%) was incubated at 95°C for 30 minutes. The reaction was terminated by placing the tubes in an ice bath, then the mixtures were centrifuged (10 000 × g, 10 min, 25°C). The absorbance was recorded

*Corresponding author, E-mail address: ciaydogan@ogu.edu.tr

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spectrophotometrically (Perkin Elmer Lambda 25, USA) at 532 nm and non-specific absorbance at 600 nm. The MDA concentration was determined using an extinction coefficient of 155 mM^{-1} cm^{-1}.
Ascorbic acid concentrations were determined according to Schoner and Krause (1990). Briefly, 0.5 g fresh weight of leaf samples were homogenised in 5 mL ice-cold 4% (v/v) metaphosphoric acid, and centrifuged at 4 000 × g for 10 min. A mixture was prepared with equal volumes of 1 mL each from the supernatant, 50 mM Na citrate buffer (pH 2.6) and 10 mM dichlorophenolindophenol. The optical intensity of the mixture, which was incubated at 25°C for 1 min, was determined at 524 nm. The AsA of the samples were compared with those of standard AsA solutions.
A discontinuous polyacrylamide gel electrophoresis (PAGE) under nondenaturing, nonreducing conditions (Native PAGE) was performed for the detection of isoperoxidases. Briefly, 100mg of N_2-frozen leaf samples were homogenized with 0.6 mL 0.1M K-phosphate buffer (pH 7.5). The homogenate was centrifuged at 21 000 × g for 20 min at 4°C and the supernatant was used for POX electrophoresis (Gulen and Eris, 2004). Samples were subjected to PAGE using a Mini-PROTEAN tetra cell electrophoresis system (Bio-Rad, Hercules, CA) as described by Davis (1964) for acidic POX and Reisfeld et al. (1962) for basic POX. The gels were stained according to the method of Wendel and Weeden (1989). The results from a single representative experiment repeated three times are presented herein. The relative mobility (Rf) value of the bands on the gel was calculated by using Rf= 1.0, 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 (U.S. National Institutes of Health) Image program. The areas under the clearance curves were quantified in arbitrary units.
Total soluble protein (TSP) was extracted from leaf tissues using the methods described by Shen et al. (2003). Leaf tissues (0.25 g) were homogenized at 4°C in extraction buffer [25 mM Tris base, 275 mM sucrose, 2 mM ethylenediaminetetraaceticacid, 10 mM 1,4 dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride and 1% polyvinylpyrolidone, pH 7.8]. The homogenates were centrifugated (10 000 rpm, 10 min., 4°C) and TSP content of supernatant was quantified according to the Bradford assay using bovine serum albumin as standard (Bradford, 1976). Equal amounts (7.5 µg) of TSP aliquots of each sample were loaded into the wells, each of and separated by sodium dodecyl sulphate-PAGE (SDS-PAGE) and the gels were visualized via Coomassie stain (Arora et al., 1992).
The experiment was set up as a randomized block design. All of the assays were repeated three times. The mean values of the data were evaluated using Duncan test at $p<0.05$ using the SPSS software (version 20., Chicago, IL, USA).

3. RESULTS AND DISCUSSIONS

Lipid peroxidation is considered as one of the most harmful processes known to occur in every living organism. High temperature stress causes modification in the membrane function mainly due to the alteration of membrane fluidity (Asthir, 2015). The MDA content, as an indicator of LPO, of small reddish plants under heat stress conditions were shown in Figure 1. The results of the current study showed that high temperature induces LPO (MDA formation). The MDA content was almost constant until 45°C and increased after then, at 50°C it was doubled comparing to the control values. The effect of heat stress treatment on MDA content was statistically significant (Table 1). Similarly, Mansoor and Naqvi (2013) found that the content of MDA in mung bean seedlings treated with 50 °C was highest. In addition, Buttar et al. (2020) reported that, heat stress increased LPO to nearly twice of control plants within hours. Also heat stress increased LPO of pigeon pea

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genotypes (Kaur et al., 2019). The MDA contents of lettuce plants peaked rapidly after the heat stress application (Zhao et al., 2022). Plants exposed to different stress conditions resulted in the accumulation of ROS which degrade polyunsaturated lipids forming MDA. Thus MDA content is directly proportional to the amount of LPO and membrane damage (Raja et al., 2020).

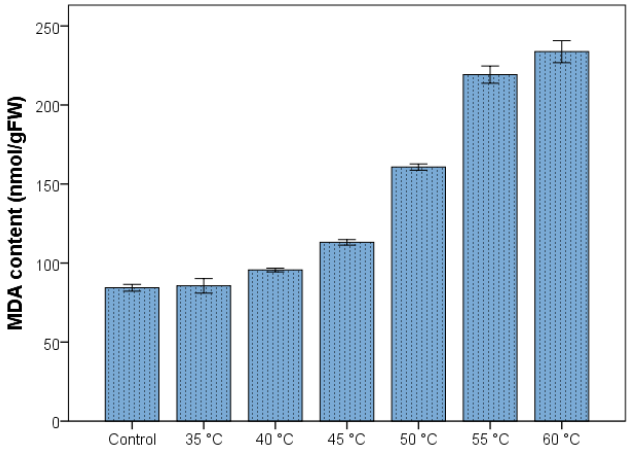

Figure 1. Effects of heat stress on malondialdehyde (MDA) content small reddish bean plants. Bars represent ±standard error (S.E.) of three replications.

Table 1. ***The MDA (nmol/gFW), AsA (mg/gFW) and TSP (mg protein/gFW) contents at different heat treatments. Values are mean ± SE (n = 3).***

Treatment	MDA content (nmol/gFW)	AsA content (mg/gFW)	TSP content (mg protein/gFW)
Control	84.37±4.01a	0.745±0.05b	11.884±0.301bc
35°C	85.60±4.01a	0.746±0.04b	11.948±0.301bc
40°C	95.59±4.01a	0.537±0.05a	11.758±0.301bc
45°C	113.09±4.01b	0.594±0.04ab	12.425±0.301c
50°C	160,74±4.01c	0.699±0.05b	12.642±0.301c
55°C	219.20±4.01d	0.622±0.04ab	11.263±0.301b
60°C	233.75±4.01e	0.530±0.05a	6.364±0.301a

Different letters indicate significant difference among various temperature treatments ($p < 0.05$).

Under stressful environments, plasma membrane oxidation is prevented by antioxidants acting as redox buffering agents such as AsA and GSH (Pandey et al., 2015). The changes in the AsA content in the leaf samples are shown in Figure 2. According to the average values, although there were increases and decreases due to the heat treatments, the leaf AsA content generally decreased by heat treatments. Data obtained from the current research revealed that plants exhibited higher AsA content (0.745 mg/gFW) at the control treatment than in the 60°C treatment (0.530 mg/gFW). Besides, the effect of heat stress treatment on AsA content was statistically significant (Table 1). A decrease in leaf AsA content in response to high temperature was also reported in tomato plants at

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first bloom stage (Turhan et al., 2021). The leaf AsA content of melon and watermelon showed fluctuation with increasing temperatures (Aydogan et al., 2021).

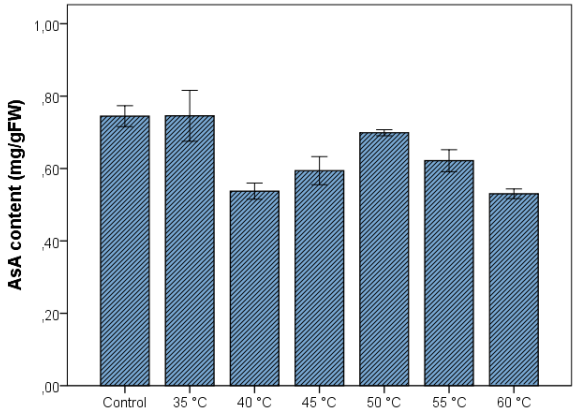

Figure 2. Effects of heat stress on ascorbic acid (AsA) content small reddish bean plants. Bars represent ± S.E. of three replications.

The POXs can be found in a broad array of organisms in various isoforms (de Oliveira et al., 2021) which have various function (stress protection and defense response) in the cell (Wang et al., 2015). Native PAGE was performed to get acidic and basic isoperoxidase bands in the leaf samples, both the acidic and basic isoperoxidase profiles showed appearance of new isoforms (Figure 3). Three acidic isoperoxidase bands with Rf of 0.14, 0.35, 0.47 were identified (Figure 3A). The acidic POX1 and POX2 were more intense at 45°C and 40°C treatment, respectively. Besides, acidic POX3 was observed only in the control treatment. Native PAGE of basic POX isozymes yielded four bands; with Rf of 0.28, 0.69, 0.81, 0.84 (Figure 3B). The basic POX1, POX3 and POX4 bands disappeared at temperatures higher than 40°C, 50°C and 45°C, respectively. It was determined that the band intensity of basic POX2 showed a sharp increase at 40°C however generally declined with heat stress.

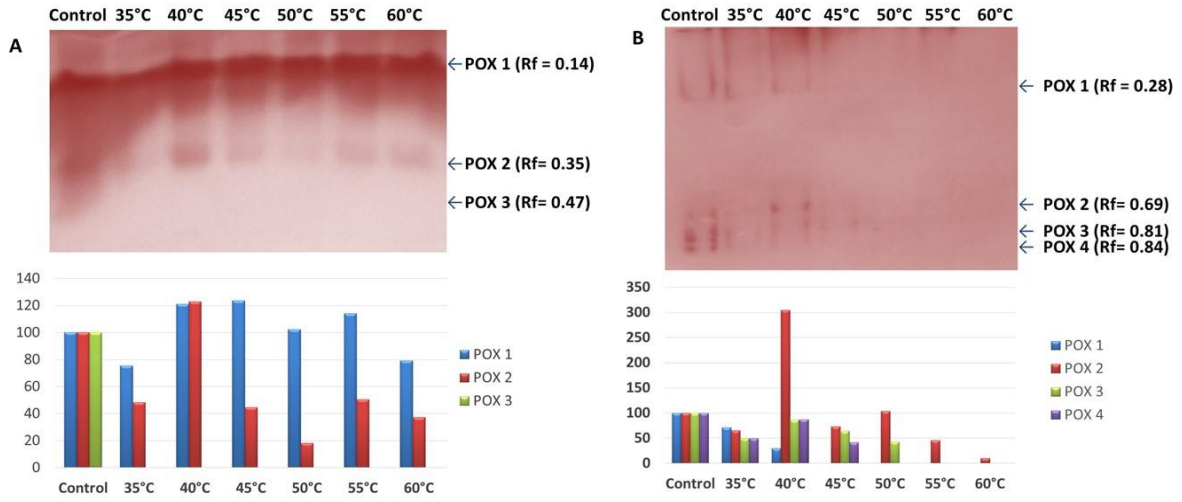

Figure 3. Effects of heat stress on acidic (Panel A) and basic (Panel B) isoperoxidase activity and band intensities. Band intensities varying depending on heat stress treatments were calculated for each band compared to its own control samples. Equal volumes of the crude extracts, 20µL, were loaded in each lane.

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The temporal increase in POX isozyme intensity may reflect transient H_2O_2 production during the early phase of heat stress and/or changes in the production of secondary metabolites for stress defense (He and Huang, 2010). Similarly, POX activity in the tolerant genotypes of cotton increased under 40 °C heat stress treatment (Mohamed and Abdel-Hamid, 2013). These results are parallel to Naji and Devaraj (2011) found that isoperoxidase activity in horse gram increase under heat stress. There are several researches with strawberry plants indicating increase in POX isozyme activities under high temperatures (Gulen and Eris, 2004; Ergin et al., 2012). Aydogan et al. (2017) observed one acidic and four basic isoperoxidases with differing intensities between green bean genotypes and high temperatures. Besides, one basic isoperoxidase band in zucchini and acidic isoperoxidase bands with different Rf values in melon, watermelon and zucchini plant with different heat-tolerance were determined by Aydogan et al. (2021).

The ROS generation due to heat stress induces oxidative degradation of proteins, DNA and lipids (Ul Hassan et al., 2021). Effects of heat stress on protein of small reddish bean plants were shown in Figure 4. The TSP content was almost constant until 40 °C and slightly increased at 45°C and 50°C (Figure 4A). However, the TSP content decreased after 55°C and almost by 50%, comparing to the control plants, at 60°C. The effect of heat stress treatment on TSP content was statistically significant (Table 1). Likewise, the TSP content of *Trigonella* plants was reduced 69.4% at 40°C comparing to control samples (Osman et al., 2020). Tokyol and Turhan (2019) found that the TSP content of green bean genotypes reduced and/or induced with heat stress. Besides, in tomato (Turhan et al., 2021) and cucumber (Ergin et al., 2021) plants fluctuations in the TSP content under high temperatures were reported. Besides, the TSP contents of zucchini plants were not affected by the temperature, while the TSP content of melon plants did not change up to 50°C and then decreased with the increase in temperature (Ergin et al., 2021).

Protein structure and activity is adversely affected by heat stress (Hasanuzzaman et al., 2013). The SDS-PAGE analysis yielded protein bands ranging in size between about 14.5-195 kDa with decreasing intensities due to increasing temperatures (Figure 4B). The band intensities began to decrease at 55 °C, and either decreased or disappeared at 60 °C significantly. Besides, a new protein band with a molecular weight of 44 kDa was observed only at 50°C high temperature treatment.

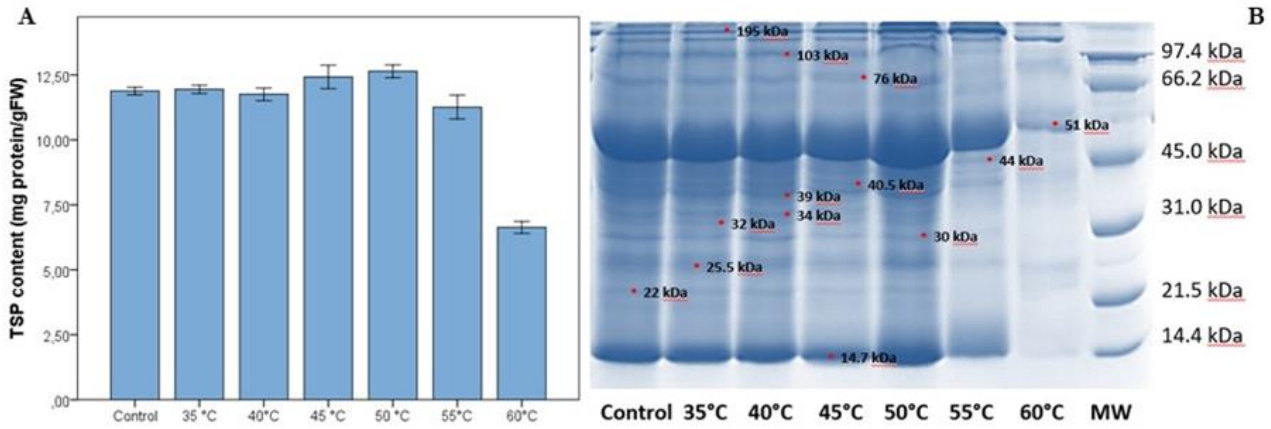

Figure 4. Effects of heat stress on total soluble protein (TSP) content Bars represent ± SE of three replications (Panel A). Total protein profiles (Panel B) of small reddish bean plants. 7.5µg protein were loaded in each lane. MW; molecular weight standard.

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Parallel to current findings Turhan et al. (2015) determined bands in the range of 7-54 kDa in pepper plants due to heat stress and pointed out that 40 kDa HSP protein may be associated with thermal tolerance. Likewise, in tomato plants protein bands ranging between 17-89 kDa (at first bloom stage) and 13-89 kDa (at yield stage) were detected, of which a protein band of 43 kDa was found to be only in cv. 'Yaren' (relatively tolerant) at yield stage (Turhan et al., 2021). Besides, Ergin et al. (2016) indicated that heat stress resulted in new protein bands and decreases/disappearance of some proteins in strawberry plants. In addition, protein bands with molecular weights ranging from 211.0 and 18.9 kDa in watermelon, 158.2 and 21.4 kDa in melon, 84.1 and 16.4 kDa in cucumber, 121.3 and 6.5 kDa in zucchini were detected, however the synthesis of these proteins was lost with increment of temperature (Ergin et al., 2021).

4. CONCLUSIONS

This study indicates that high temperature stress disrupts the growth of small reddish plant by damaging cellular membranes. 40°C found to be critical and especially at 50°C above temperatures causes oxidative stress in small reddish bean plant. Additionally, isoperoxidases and proteins may be an effective mean on heat stress tolerance of small reddish bean plants. In order to clarify the temperature stress tolerance of small reddish bean plants, further studies on the molecular behavior of these plants are needed.

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*Corresponding author, E-mail address: ciaydogan@ogu.edu.tr

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*Corresponding author, E-mail address: ciaydogan@ogu.edu.tr