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Effect of fungicide pretreatment on lipid peroxidation, antioxidant enzyme systems and proline accumulation in tomato (*Lycopersicon esculentum* Mill.) leaves under high temperature stress

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Abstract: The aim of the present study was to determine the effect of high temperature stress (HT) and to evaluate the protective role of fungicide pretreatment against HT stress in tomato leaves. The oxidative stress was triggered under HT stress through an increase in the content of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). Also, it caused a decline in photosynthetic pigments and enhanced the proline in tomato. However, the activities of catalase (CAT; EC 1.11.1.6), peroxidase (POX; EC 1.11.1.7), and glutathione S-transferase (GST; EC.2.5.1.18), as well as ascorbate-glutathione cycle enzymes (APX, EC 1.11.1.11, DHAR, EC 1.8.5.1, MDHAR EC 1.6.5.4, GR; EC 1.6.4.2) were found to be increased after heat treatment. In addition, the transcript level of *LeCAT1, LeAPX1, LeAPX6, LecGR, LeDHAR* genes was up-regulated under HT stress. Foliar pretreatment of fungicide improved the physiological process that decrease H_2O_2 and MDA content, enhance pigment content, increase proline accumulation and CAT, POX, and GST activity, as well as *LeCAT1* and *LeGST2* transcript levels compared to nonpretreated leaves under control temperature. When tomato leaves exposed to high temperature stress, fungicide pretreatment accelerated the antioxidant enzyme system by enhancing activities and gene expression levels of CAT, POX, GST, and ascorbate-glutathione enzyme, resulting in the decrement of MDA and H_2O_2 contents. Further, the protective effect of fungicide pretreatment improved the efficiency of the photosynthetic pigment under HT stress. Consequently, the study was to indicate the evidence for the ability of potential use of fungicide as a protective agent against HT stress in tomato.

Key words: Chlorophyll, gene expression, heat stress, lipid peroxidation, thiram

1. Introduction

Plants have been influenced by extreme changes in their natural environment, which was known as abiotic stress such as high temperature, salinity, presence of heavy metal, and paraquat toxicity. Among all the factors causing stress, the extreme temperature has become a major problem for agricultural production due to global warming in the worldwide (Sheikh–Mohamadi et al., 2018). The increasing temperature over the optimal level can cause a reduction of plant growth and yield loss of agricultural production; therefore, it will be a great limiting factor for sustainable crop production in the world (Fahad et al., 2017).

The tomato (*Lycopersicon esculentum* Mill.), a member of the Solanaceae family, is widely cultivated in different parts of the world. Tomato is both a member of important cultivated dicotyledonous plants and a model system for the elucidation of classical genetics, cytogenetics, molecular genetics, and molecular biology. Many studies have focused on the physiological and biochemical responses of tomatoes to abiotic stresses such as heat, drought, and xenobiotic (Gerszberg and Hnatuszko-Konka, 2017). Tomato cultivation requires a high-water-demand to increase its productivity, and its optimum temperature is around 26 °C (day) and 20 °C (night). When the temperature is above 31 °C, tomato is negatively affected in terms of plant development, reproductive stage, fruit quality, and yield (Camejo et al., 2006).

High temperature stress has a wide range of negative effects on normal cellular, physiological, biochemical, and development processes such as photosynthesis, mitochondrial respiration, and membrane stability (Hemantaranjan et al., 2014). Photosynthesis is one of the most heat-sensitive processes due to the change of the redox equilibrium of the chloroplast in a plant cell. This result causes the overproduction of superoxide radical ($O_2^{\cdot-}$), which transforms to the highly reactive and toxic formation of reactive oxygen species (ROS) such as hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) (Gill and Tuteja, 2010). H_2O_2 is a reactive and long half-life (1 ms) molecule. It can easily diffuse in

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biological membranes because of not having unpaired electrons (Sharma et al., 2012). The excessive production of H_2O_2 can lead to increase in peroxidation of membrane lipids, denaturation of protein, damage of nucleic acid, inactivation of enzymes, and destruction of pigment in the plant cell (Awasthi et al., 2015). However, plants have developed an enzymatic scavenging system to reduce the toxicity of H_2O_2. The enzymatic mechanism consists of multiple antioxidant enzymes such as catalase (CAT), peroxidase (POD), glutathione S-transferase (GST), and the ascorbate–glutathione cycle enzymes: ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) (Ergin et al., 2016). Among the antioxidant enzymes, catalase (CAT) directly converts H_2O_2 to water and molecular oxygen (Gill and Tuteja, 2010). CAT has the fastest turnover rates in all antioxidant enzymes. Peroxidase (POX) is a heme-containing glycoprotein in plants, catalyzes the aromatic electron donor for use up H_2O_2 (Gill and Tuteja, 2010). The other H_2O_2-scavenging enzyme system is the ascorbate-glutathione cycle also named as Foyer-Halliwell-Asada pathway which includes APX, MDHAR, DHAR, and GR enzymes (Gill and Tuteja, 2010). In the first step of the cycle, H_2O_2 is converted to monodehydroascorbate radical (MDHA) by APX using peroxidation of ascorbate as a specific electron donor. MDHA spontaneously turn into ascorbate (AsA) and dehydroascorbate (DHA) or enzymatically transform to AsA and DHA by the action of monodehydroascorbate reductase (MDHAR). In the next step of the cycle, dehydroascorbate reductase (DHAR) reduces DHA to AsA using glutathione (GSH) as the reductant, with the consequent production of oxidized glutathione (GSSG). Finally, GSSG is reduced to GSH, and, at the same time, the free-electron transmits to NADPH by the activity of NADPH-dependent GR. The AsA-GSH cycle localizes in cytosol, chloroplast, mitochondria, peroxisome, and it plays a major role in improving heat tolerance in many plant species (Sheikh–Mohamadi et al., 2018).

During the lifespan, plants have naturally been exposed to abiotic stress either individually or in combination, so they have developed a variety of survival and adaptation mechanisms. Amongst them, pretreatment of chemical agent is caused naturally defense response that enhanced resistance to the future stress factor. These agents can improve plant tolerance through activation of basal resistance and control of the oxidative stress against abiotic stress in plants (González–Bosch, 2018). Plants resistance can be triggered by natural or synthetic chemical compounds such as hormones, sodium nitroprusside, hydrogen peroxide, and fungicides, which are named as chemical priming agents (Savvides et al., 2016; Balestrini et al., 2018). Recently, many studies suggest that fungicides have a potential role as a priming agent against various abiotic stress (Filippou et al., 2016). Low-dose application of synthetic fungicide kresoxim-methyl (KM) has a protectant role as an acting priming agent for the diminishing of salt and drought stress in *Medicago truncatula* plants (Filippou et al., 2016). The other strobilurin fungicide, trifloxystrobin, triggered systemic tolerance against high and low temperature, drought, and freezing in red pepper plants (Han et al., 2012). However, there is limited information about the effect of fungicide against abiotic stress in plants. Hence, this study has aimed to investigate how fungicide pretreatment affected tolerance to high temperature stress in tomato leaves. To prove this hypothesis, the experimental results determined the effect of fungicide pretreatment on the oxidative stress, photosynthetic pigments, proline accumulation, and enzymatic antioxidant system in tomato leaves exposed to high temperature.

2. Materials and methods

2.1. Plant material and experimental design

The seeds of tomato (*Lycopersicon esculentum* Mill. Narcan-8) were purchased from Balıkesir Küçükçiftlik Seed Corporation in Turkey. Seed sterilization was done in 5% sodium hypochlorite and sterile seeds were imbibed in deionized water for 24 h at room temperature. Plants were grown in plastic pots containing perlite watered by ¼ Hoagland solution (Caisson Labs, USA) during 21 days in a growth chamber at 16–8 h photoperiod, 25/20 °C, and 60% relative humidity. The 21-days-old seedlings were divided into two groups. The control group stayed in a growth chamber-1 at 16-8h photoperiod, 25/20 °C, and 60% relative humidity. The heat-treated group performed in the growth chamber-2 at 16–8 h photoperiod, 36/30 °C, and 60% relative humidity (VB 0714, Bioline, Vötsch Industrietechnik, Germany; internal dimensions: 970 × 750 × 1400 cm; lighting intensity: 450 μmol $m^{-2}s^{-1}$ @ 200 mm). Initially, thiram fungicide (Sigma–Aldrich, 45689, Sigma-Aldrich Corp., St. Louis, MO, USA) was sprayed in different concentrations as 40, 80, 160, and 240 μM for determining the optimal dose of thiram for tomato leaves under 25 and 36 °C during 7 days. 80 μM thiram was chosen according to MDA and H_2O_2 level as the optimal dose in stressed plants. In the experimental design, 21-days-old tomato seedlings were sprayed with deionized water or 80 μM thiram on two groups; 24 h after thiram treatment, a group of plants was exposed to heat stress at 36 °C for 7 days in growth chamber-2; the other group was maintained at 25 °C for 7 days in growth chamber-1 as a control. The tomato leaves were harvested 1, 3, and 7 days from three independent plants after the heat treatment. The leaves collected from the three independent plants were

randomly pooled for experimental analyses. Analyses were performed at least three independent biological replicates. Epoch 2 microplate spectrophotometer (Winooski, USA) was used for all spectrophotometric analyses.

2.2. Determination of chlorophyll and carotenoid pigments

Leaves were homogenized in 100% acetone and then centrifuged at 3000 × g 4 °C for 15 min. The supernatant was measured spectrophotometrically with a wavelength of 661.6, 644.8, and 470 nm. The amount of chlorophyll a, b, total chlorophyll, and carotenoid were calculated as µg/mL, by employing Lichtenthaler and Buschmann (2001) method.

2.3. Determination of hydrogen peroxide (H_2O_2)

The determination of H_2O_2 content was conducted on fresh leaves materials (0.5 g) according to Velikova et al. (2000). The sample was homogenized with 0.1% (w/v) TCA (Trichloroacetic acid) buffer and the extract was centrifuged at 12,000 for 15 min. Absorbance at 390 nm was recorded in a reaction buffer (10 µM potassium phosphate buffer (pH 7.0), 1 M potassium iodide), and supernatant. H_2O_2 level was calculated using a standard curve.

2.4. Determination of lipid peroxidation

Lipid peroxidation was determined by measuring malondialdehyde (MDA) content according to Jiang and Zhang (2001) method. The sample of fresh leaves (0.5 g) was homogenized in 10 mL extraction buffer containing 0.25% TBA and 10% TCA and then was boiled at 95 °C for 30 min. The reaction mixture was cooled in ice after centrifugated at 5000 × g for 10 min. The absorbance was measured at 532 and 600 nm wavelengths. Lipid peroxidation was expressed using the extinction coefficient of 155 μM^{-1} cm^{-1} as µmol g^{-1}.

2.5. Determination of proline content

The proline method was conducted spectrophotometrically following the ninhydrin method described by Bates et al. (1973), using pure proline as a standard. The sample leaves were homogenized in 3% sulphosalicylic acid and filtered in Whatman No.2 filter paper. The supernatant was mixed with the equal volume glacial acetic acid and ninhydrin for boiling at 1 h in water bank tank. After the reaction mixture was cooled in ice, toluene was added to it, and the absorbance was measured at 520 nm.

2.6. Determination of antioxidant enzymes activities

The leaf samples were homogenated in 50 µM potassium phosphate buffer (pH 7.0) containing 1 µM EDTA and 1% PVPP. After the centrifugation at 13,000 × g, 4 °C for 40 min, the supernatant was used for measurement of the total protein and the enzyme activity. The total protein content was carried out using the Bradford (1976) method.

The activity of catalase (CAT) (EC 1.11.1.6) was performed based on Bergmeyer (1970). The CAT activity was indicated in 200 µL reaction mixture containing 50 µM sodium phosphate buffer (pH 7.0), 0.3% H_2O_2, 0.1 µM EDTA and 12 µL enzyme extract. The reaction was followed by monitoring the decomposition of H_2O_2 for 2 min at 240 nm extinction coefficient (extinction coefficient 39.4 μM^{-1} cm^{-1}).

The activity of peroxidase (POX) (EC 1.11.1.7) was performed based on Herzog and Fahimi (1973). POX activity was indicated in 200 µL reaction mixture containing DAB solution (0.15 M sodium phosphate-citrate buffer (pH 4.4), 0.6% H_2O_2, 50 % (w/v) gelatin), and 5 µL enzyme extract. The reaction was followed by monitoring the increase in the absorbance at 465 nm (extinction coefficient 2.47 μM^{-1} cm^{-1}) for 3 min.

The activity of glutathione S-transferase (GST) (EC.2.5.1.18) activity was performed based on Habig and Jacoby (1981). GST activity was indicated in 200 µL reaction mixture containing 50 µM potassium phosphate buffer (pH 6.5), 1 µM CDNB, 1 µM EDTA, 5 µM GSH and 10 µL enzyme extract. The reaction was followed by monitoring the increase in the absorbance at 340 nm (extinction coefficient 9.6 μM^{-1} cm^{-1}) for 5 min.

2.7. Determination of ascorbate-glutathione cycle enzymes activities

Ascorbate-glutathione cycle enzymes activities were performed according to the previously published protocol as described by Murshed et al. (2008). Plant samples were extracted into 50 mM MES/KOH buffer (pH 6.0), containing 2 mM $CaCl_2$, 40 mM KCl, , and 1 µM L-ascorbic acid (AsA). AsA (1 M) is freshly prepared and added to the extraction medium. The extract is centrifuged at 14.000 × g, 10 min at 4 °C. All enzyme analyses are performed in a final volume of 0.2 mL in the Epoch 2 microplate spectrophotometer.

APX (EC 1.11.1.11) activity was measured by the decrease in the reaction rate at 290 nm for 5 min. The reaction mix included 50 µM potassium phosphate buffer (pH 7.0), 0.25 µM ascorbate, 5 µM H_2O_2, and enzyme extract in 200 µL. Specific activity is calculated using the 2.8 μM^{-1} cm^{-1} extinction coefficient.

DHAR (1.8.5.1) activity was measured by the increase in the reaction rate at 265 nm for 5 min. The reaction mix included 50 µM Hepes buffer (pH 7.0), 0.1 µM EDTA, 2.5 µM GSH, 0.2 µM DHA and enzyme extract in 200 µL. Specific activity is calculated using the 14 μM^{-1} cm^{-1} extinction coefficient.

MDHAR (EC 1.6.5.4) activity was measured by the decrease in the reaction rate at 340 nm for 5 min. The reaction mix included 50 µM Hepes buffer (pH 7.6), 2.5 µM Ascorbate, 0.25 mM NADH, 0.4 U/well ascorbate oxidase and enzyme extract in 200 µL. Specific activity is calculated using the 6.22 μM^{-1} cm^{-1} extinction coefficient.

GR (EC 1.6.4.2) activity was measured by the decrease in the reaction rate at 340 nm for 5 min. The reaction mix included 50 µM Hepes buffer (pH 8.0), 0.5 mM EDTA, 0.25 µM NADH, 0.5 µM GSSG, and enzyme extract in 200 µL. Specific activity is calculated using the 6.22 μM^{-1} cm^{-1} extinction coefficient.

2.8. The total RNA extraction, cDNA synthesis and quantitative real-time PCR

Analytik Jena Innuprep Plant RNA kit was used for total RNA isolation from tomato leaves based on the manufacturer's manual. The total RNA was digested with 10U RNAse-free DNaseI (Thermo Scientific) for 20 min at 37 °C for eliminating the genomic DNA. Bioline sensifast cDNA synthesis kit was used for the first-strand cDNA synthesis. The specific mRNA sequences were obtained from NCBI (http://www.ncbi.nih.nlm.gov/) and Sol Genomics Network (https://solgenomics.net/). Primers were designed using Primer 3 (Premier Biosoft, CA, USA) online software (Thornton and Basu, 2011). The detailed information of the primer sequences is shown in Table 1. Quantitative real-time PCR was performed with a Light Cycler 480 Real-Time PCR System (Roche Diagnostics, Mannheim, Germany). The reactions were applied by LightCycler® 480 SYBR Green I Master (Roche, Mannheim, Germany). Primer's specificity was checked with melting curve analysis. A comparative CT analysis method was done for the quantification of the mRNA level. ΔCt value was calculated according to the formula: $\Delta CT = CTgene - CT_{housekeeping}$. The relative expression level (ΔΔCt) was calculated according to the formula: $\Delta\Delta Ct = \Delta CT_{target} - \Delta CT_{control}$. The relative gene expression level was calculated as $2^{-\Delta\Delta Ct}$.

2.9. Statistical analysis

Analysis of variance was performed for all data sets using GraphPad Prism version 5.2 software (GraphPad Software, San Diego, CA, USA). The difference between the results was compared by one-way analysis of variance (ANOVA) followed by Bonferroni Post Hoc Test analysis at $P < 0.05$. All the samples were analyzed in five replicates and each experiment was repeated at least three different times.

3. Results

3.1. Effect of fungicide pretreatment on chlorophyll and carotenoid content under high temperature (HT) stress in tomato leaves

Chlorophyll a, chlorophyll b, and carotenoid contents were determined in fungicide-pretreated and nonpretreated leaves at 7 DAT under 36 °C temperature. Compared to control temperature (25 °C), high temperature led to a significant decrement in chlorophyll a, chlorophyll b, and carotenoid content. The pretreatment of thiram fungicide enhanced by 72%, 67%, and 62% respectively

Table 1. The primer sequences of antioxidant enzyme genes used in quantitative real-time PCR.

Gene	Accession no	Forward and reverse primer sequences (5' →3')	Product size (bp)
Catalase (cat1)	M93719	F: ATCAGGGACATTCGTGGTTT R:CAGGGAACGACTTAGCATCAC	112
Cytosolic ascorbate peroxidase 1 (APX1)	NM_001247853.2	F: AGCAGTTTCCCACCCTCTC R: TGGCTCTGGCTTGTCCTC	119
Cytosolic ascorbate peroxidase 2 (APX2)	NM_001247859.2	F: GAGCAGTTTCCCACTCTCTCC R: GGTGGTTCTGGTTTGTCCTCT	122
Thylakoid-bound ascorbate peroxidase 6 (APX6)	NM_001247702.2	F:CCCCTAACCCTTCATCTCATTT R: ACCACTACGCTCTGGTCTGG	119
Glutathione reductase chloroplastic (GR)	NM_001247314.2	F:GACAAAGAACGGAGCCATAGA R: CCTCCCTCCATCAAAGCA	120
Glutathione reductase (GR1)	NM_001321393.1	F: AGCAGAAGGTGGCATCAAAG R: CGACACCAACAGCATCCA	122
Dehydroascorbate reductase (DHAR)	NM_001247893.2	F: TTCTTGGAGGTGAACCCTGA R:AGCAATGAGAGAGGGATTTGG	123
Glutathione-S-transferase (gst2)	EF409975.1	F: TCTACTCGTTTTTGGGCTCGT R:GTAATCCCTCTACCACCGATTCA	118
Elongation factor 1 alpha (EF1) (housekeeping)	X14449.1	F:TCAGGTTTTCTTCGGTCTAGCGA R: CGCCTGTGTCTCCGTCAGTA	118

in chlorophyll a, chlorophyll b, and carotenoid contents in tomato leaves under control temperature (Table 2). In addition, fungicide-pretreated leaves' pigment content increased under high temperature stress compared to nonpretreated leaves. Chlorophyll a, chlorophyll b, and carotenoid contents were increased respectively by 19%, 23%, and 26% in fungicide-pretreated leaves at 36 °C temperature (Table 2).

3.2. Effect of fungicide pretreatment on oxidative stress under high temperature (HT) stress in tomato leaves

The oxidative stress was determined as hydrogen peroxide (H_2O_2) level and malondialdehyde (MDA) content. The results revealed that tomato leaves were negatively affected by temperature up to 36 °C. High temperature caused 8%, 47%, and 95% increase in H_2O_2 level respectively at 1, 3, and 7 DAT compared to control plants (Figure 1A). Under control temperature (25 °C), foliar application of thiram fungicide increased by 30% H_2O_2 level at 1 DAT; however, it decreased at 3 and 7 DAT compared to nonpretreated plants. With increased temperature up to 36 °C, H_2O_2 level clearly reduced by 14%, 16%, and 19% in fungicide-pretreated leaves respectively at 1, 3, and 7 DAT (Figure 1A).

MDA content increased by 13%, 20%, and 35% at 1, 3, and 7 DAT in tomato leaves under HT stress compared to the control temperature, respectively (Figure 1B). Under the control temperature, fungicide-pretreated leaves showed the trend of decline in MDA content compared to the non-pretreated leaves at 1, 3, and 7 DAT. Also, MDA content significantly reduced by 17%, 37%, and 9% respectively at 1, 3, and 7 DAT in fungicide-pretreated leaves compared with nonpretreated leaves under HT stress (Figure 1B).

3.3. Effect of fungicide pretreatment on proline accumulation under high temperature (HT) stress in tomato leaves

High temperature stress caused a significant increase in proline accumulation by 14%, 18%, 93% in tomato leaves respectively at 1, 3, and 7 DAT compared to the control temperature (Figure 2). For instance, the highest proline accumulation was observed at 7 DAT compared to the control treatment. In the normal temperature, there was no significant change in the proline content in fungicide pretreated leaves at 1 DAT; however, the proline accumulation increased by 18% and 40% in fungicide pretreated leaves at 3 and 7 DAT (Figure 2). Interestingly, there was no significant difference in proline content between fungicide-pretreated and nonpretreated leaves under high temperature stress at 1 and 3 DAT, but fungicide pretreatment caused a remarkable decrease in proline content exposed to high temperature stress at 7 DAT.

3.4. Effect of fungicide pretreatment on antioxidant enzyme activities under high temperature (HT) stress in tomato leaves

The CAT activity showed a similar pattern with control after one-day heat treatment. However, CAT activity significantly enhanced with the increase in exposure time to HT stress, and the highest level of CAT activity was observed at 7 DAT with a 27% increase in tomato leaves compared to the control temperature. In fungicide-pretreated leaves, CAT activity was higher than nonpretreated leaves under control temperature. In addition, fungicide pretreatment increased CAT activity by 13%, 10%, and 9% under HT stress respectively at 1, 3, and 7 DAT (Figure 3A).

The experiment examined the activity of total peroxidase (POX) in fungicide-pretreated and nonpretreated tomato leaves under HT stress. The POX activity initially declined at 1 DAT but subsequently increased by 50% at 3 and 7 DAT compared to control under heat stress. In the fungicide-pretreated leaves, POX activity increased by 33%, 73%, and 21% respectively at 1, 3, and 7 DAT under control temperature. Besides, POX activity clearly enhanced in the fungicide-pretreated

Table 2. The effects of 80 µM thiram pretreatment on photosynthetic pigments in control (25 °C) and high (36 °C) temperatures. Tomato leaves were harvested in the absence or presence of 80 µM thiram pretreatment 7 days after treatment. The different letters indicate the statistically significant differences ($P < 0.05$) according to one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test analysis. Data are means (±) standard deviations (SD).

Treatment	Chlorophyll a (µg/mL)	Chlorophyll b (µg/mL)	Carotenoid (µg/mL)
Control (25 °C)	344.06 ± 1.89[a]	126.78 ± 1.01[a]	165.95 ± 0.76[a]
80 µM Thiram (25 °C)	592.50 ± 2.36[b]	211.72 ± 1.35[b]	267.95 ± 1.61[b]
Control (36 °C)	289.52 ± 9.30[c]	99.33 ± 4.28[c]	139.35 ± 5.85[c]
80 µM Thiram (36 °C)	343.79 ± 1.43[a]	122.36 ± 7.04[a]	175.10 ± 3.48[d]

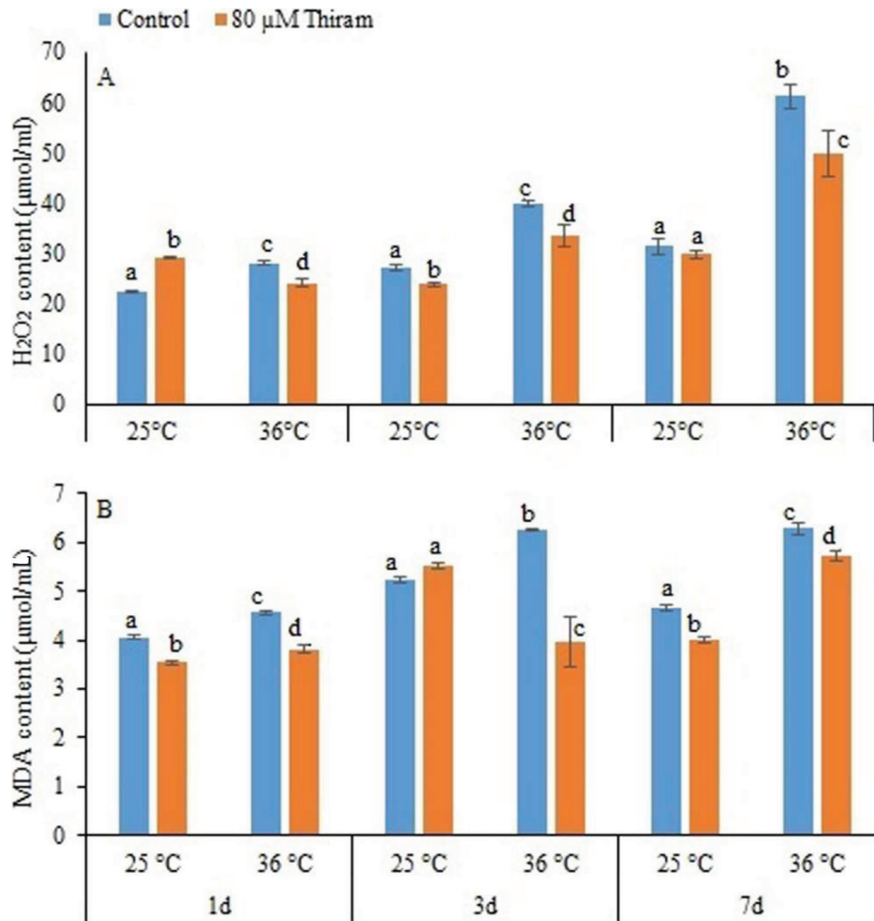

Figure 1. The effects of 80 µM thiram pretreatment on H_2O_2 and MDA contents in control (25 °C) and high (36 °C) temperatures. Tomato leaves were harvested in the absence or presence of 80 µM thiram pretreatment 1, 3 and 7 days after the treatment (DAT). The different letters indicate the statistically significant differences (P < 0.05) according to one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test analysis. Bars represent standard deviations (SD).

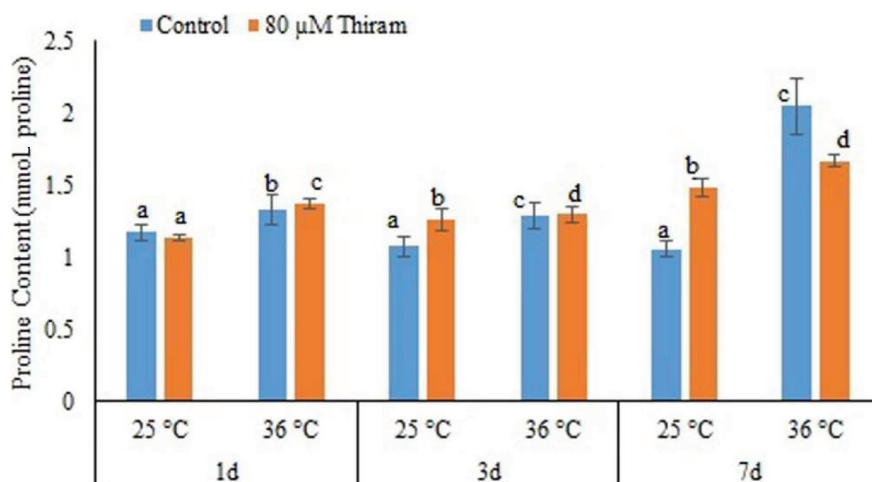

Figure 2. The effects of 80 µM thiram pretreatment on proline accumulation in control (25 °C) and high (36 °C) temperatures. Tomato leaves were harvested in the absence or presence of 80 µM thiram pretreatment 1, 3 and 7 days after the treatment. The different letters indicate the statistically significant differences (P<0.05) according to by one-way analysis of variance (ANOVA) followed by Bonferroni Post Hoc Test analysis. Bars represent standard deviations (SD).

leaves compared to nonpretreated leaves under HT stress. Especially, POX activity significantly increased by 41% and 25% respectively at 3 and 7 DAT in fungicide-pretreated leaves of tomato under HT stress (Figure 3B).

The GST activity showed a significant increment in tomato leaves under heat stress compared to the control temperature. High temperature stress stimulated 10%, 33%, and 20% increase respectively in GST activity at 1, 3, and 7 DAT. Compared to nonpretreated leaves under control temperature (25 °C), GST activity was clearly increased by 9%, 39%, and 20% in fungicide-pretreated leaves of tomato. Under HT stress (36 °C), GST activity decreased in fungicide-pretreated leaves at 1 DAT. However, fungicide-pretreated leaves displayed increment by 20% in GST activity 3 and 7 days after heat treatment (Figure 3C).

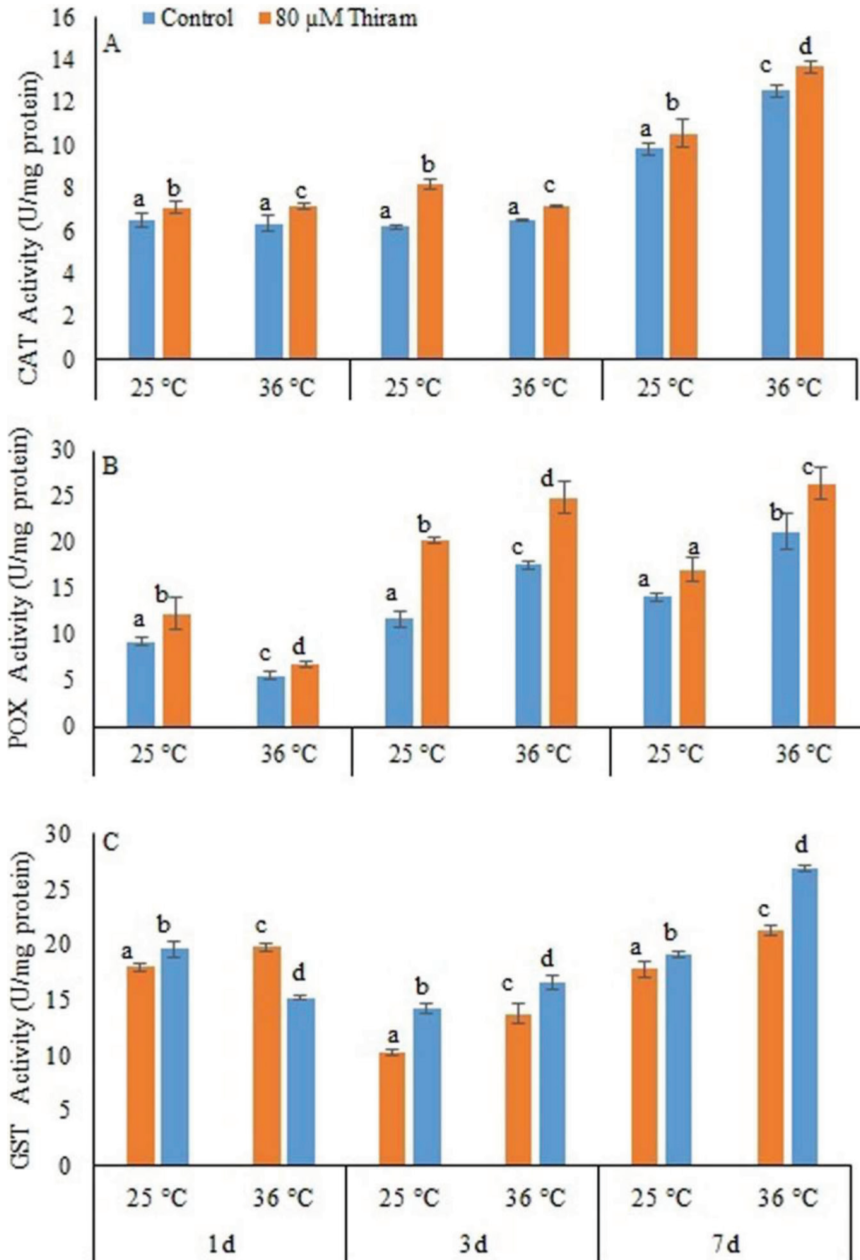

Figure 3. The effects of 80 µM thiram pretreatment on CAT, POX and GST enzymes activities in control (25 °C) and high (36 °C) temperatures. Tomato leaves were harvested in the absence or presence of 80 µM thiram pretreatment 1, 3 and 7 days after the treatment. The different letters indicate the statistically significant differences (P < 0.05) according to one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test analysis. Bars represent standard deviations (SD).

3.5. Effect of fungicide pretreatment on the expression of catalase (CAT) and glutathione S-transferase (GST) genes under high temperature (HT) stress in tomato leaves

The expression pattern of the *LeCAT1* gene was tested in fungicide-pretreated and nonpretreated leaves of tomato under 7 days after HT stress. *LeCAT1* gene expression was up-regulated by 2-fold exposed to 36 °C temperature, compared to 25 °C temperature. The fungicide pretreatment increased *LeCAT1* gene expression by 83% under control temperature, and the fungicide pretreatment enhanced CAT1 transcript abundance by 30% under HT stress in tomato leaves (Figure 4B).

LeGST2 gene transcript abundance was determined in fungicide-pretreated and nonpretreated leaves of tomato 7 days after HT stress. Fungicide pretreatment was highly induced by approximately 2.5-fold the *LeGST2* gene expression under control temperature. However, high temperature stress did not have a significant effect on *LeGST2* gene expression. In addition, *LeGST2* gene expression increased by 36% in fungicide-pretreated leaves compared to nonpretreated leaves under HT stress (Figure 4A).

3.6. Effect of fungicide pretreatment on ascorbate-glutathione cycle enzyme activities under high temperature (HT) stress in tomato leaves

Activities of the enzymes in the ascorbate-glutathione cycle, including APX, DHAR, MDHAR, and GR have shown different responses under HT stress in tomato leaves. Ascorbate peroxidase activity was not significantly changed at 1 and 3 DAT under HT stress. However, APX activity increased by 12% at 7 DAT after HT stress compared to control. APX activity did not change statistically in fungicide-pretreated leaves when compared to nonpretreated leaves under control temperature. But, its activity enhanced by 8% and 17% at 1 and 7 DAT in fungicide-pretreated leaves under temperature up to 36 °C compared to nonpretreated leaves in tomato (Figure 5A).

The activity of the MDHAR enzyme showed a similar trend with control temperature 1 and 3 days after HT stress treatment in tomato leaves. However, heat treatment caused an induction by 27% in MDHAR activity at 7 days. Besides, MDHAR activity decreased by 11%, 25%, and 10% in fungicide-pretreated leaves respectively at 1, 3, and 7 DAT under control temperature. Conversely, fungicide pretreatment increased by 17%, 21%, and 35% in MDHAR activity respectively at 1, 3, and 7 DAT under HT stress (Figure 5B).

Tomato leaves showed a significant enhancement as 14%, 7%, and 18% in DHAR activity respectively at 1, 3, and 7 DAT under HT stress compared to control. When compared to nonpretreated leaves at 25 °C, fungicide-pretreated leaves clearly decreased DHAR activity as 11% and 8% at 3 and 7 DAT. Under increased temperature up to 36 °C, fungicide-pretreated leaves significantly increased by 13% at 7 days after treatment compared to the nonpretreated leaves (Figure 5C).

Under high temperature, the GR activity decreased by 6% at 1 DAT, whereas it increased by 24% at 7 DAT under HT stress treatment in leaves. Also, GR activity increased by 12% and 28% in fungicide-pretreated leaves at 1 and 7 DAT compared to nonpretreated leaves in control temperature. In addition, GR activity clearly enhanced by 18%, 9%, and 26% respectively at 1, 3, and 7 DAT under 36 °C (Figure 5D).

3.7. Effect of fungicide pretreatment on the expression of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) genes under high temperature (HT) stress in tomato leaves

Three different APX enzymes genes including *LeAPX1*, *LeAPX2*, and *LeAPX6* were evaluated in fungicide-pretreated and nonpretreated leaves of tomato under 7 days after HT stress. HT stress caused an abundance of the *LeAPX1* and *LeAPX6* transcripts compared to the control temperature. Among all APX genes, *LeAPX1* was highly upregulated under HT stress in tomato leaves. All APX genes had similar expression levels in fungicide pretreated-leaves compared to nonpretreated leaves under control temperature. *LeAPX1* and *LeAPX6* genes were up-regulated by nearly 2-fold and 4-fold in fungicide pretreated leaves under HT stress. However, *LeAPX6* was down-regulated by almost 20% in fungicide pretreated leaves under HT stress (Figure 6A)

LecGR and *LeGR1* genes were evaluated for the expression level of GR enzymes. *LecGR* genes were up-regulated by 2,5 fold compared to control temperature after the exposure to HT stress in tomato leaves. In addition, *LecGR* genes were expressed by 60% in fungicide pretreated-leaves under control temperature. Further, fungicide pretreatment enhanced the *LecGR* transcript level under HT stress in tomato leaves. Interestingly, the *LeGR1* gene was down-regulated in all treatments at both high and control temperatures in tomato leaves (Figure 6B).

LeDHAR gene expression was up-regulated by 3-fold under high temperature compared to the control temperature in tomato leaves. There was no significant difference in the *LeDHAR* gene expression level in fungicide pretreated leaves under control temperature. Yet, fungicide pretreatment induced the upregulation of *LeDHAR* transcript under HT stress in tomato leaves (Figure 6C).

4. Discussion

In recent years, high temperature (HT) stress has become one of the most important abiotic stress factors limiting

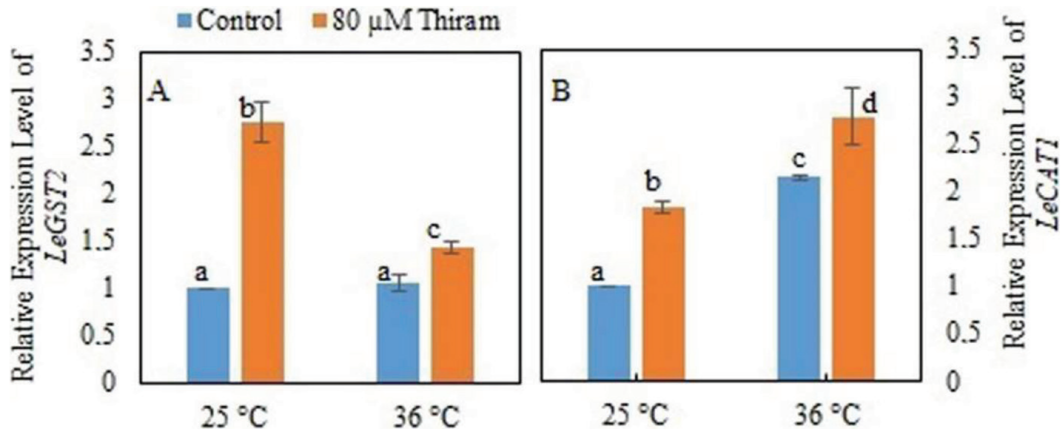

Figure 4. The effects of 80 μM thiram pretreatment on relative gene expression level of *LeCAT1* and *LeGST2* genes in control (25 °C) and high (36 °C) temperatures. Tomato leaves were harvested in the absence or presence of 80 μM thiram pretreatment 7 days after the treatment. The different letters indicate the statistically significant differences (P < 0.05) according to one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test analysis. Bars represent standard deviations (SD).

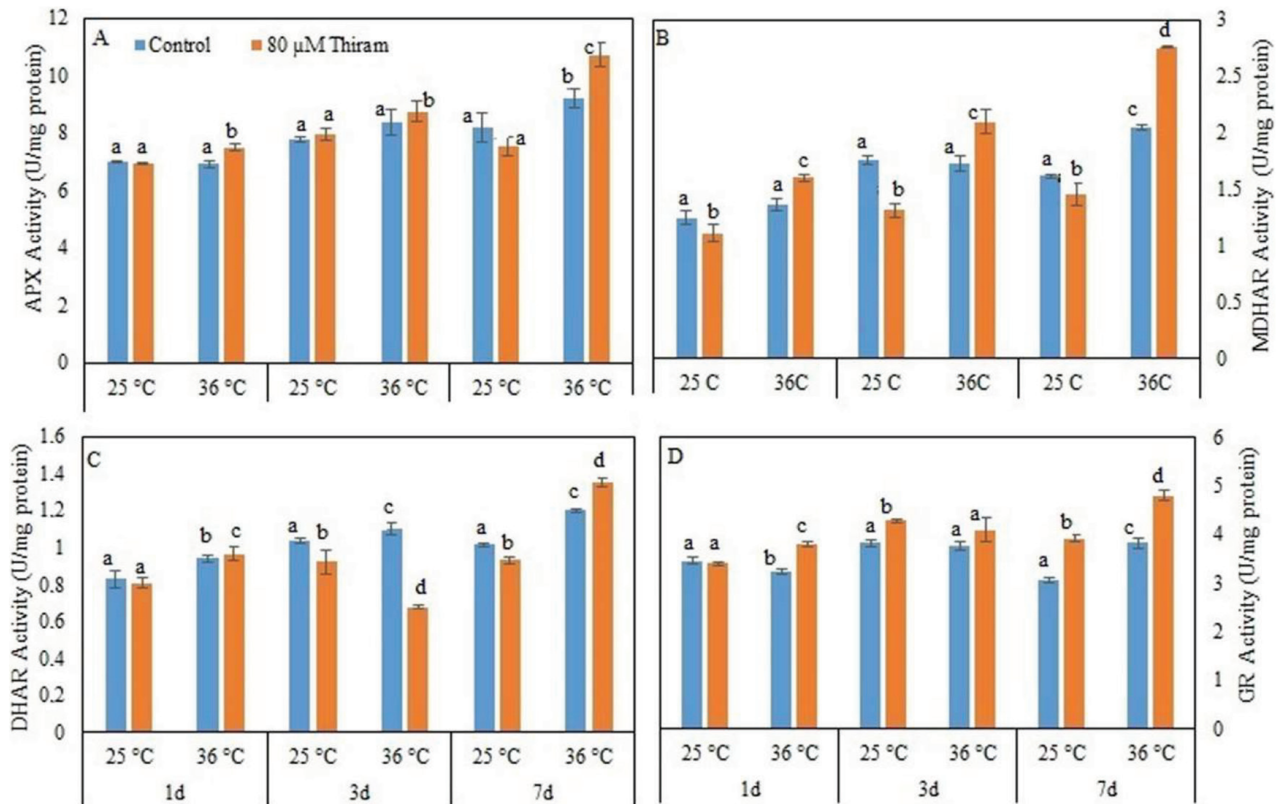

Figure 5. The effects of 80 μM thiram pretreatment on ascorbate-glutathione cycle enzyme activities in control (25 °C) and high (36 °C) temperatures. Tomato leaves were harvested in the absence or presence of 80 μM thiram pretreatment 1, 3 and 7 days after the treatment. The different letters indicate the statistically significant differences (P < 0.05) according to one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test analysis. Bars represent standard deviations (SD).

crop development and productivity due to the rising global temperature in the world. For this reason, it is critical to increase knowledge about the process of high temperature stress and to increase the heat tolerance of plants. Pretreatment strategy is a commonly known method against abiotic stresses factor using biological and chemical agents in plants. Exogenous application of chemical compounds has become an important

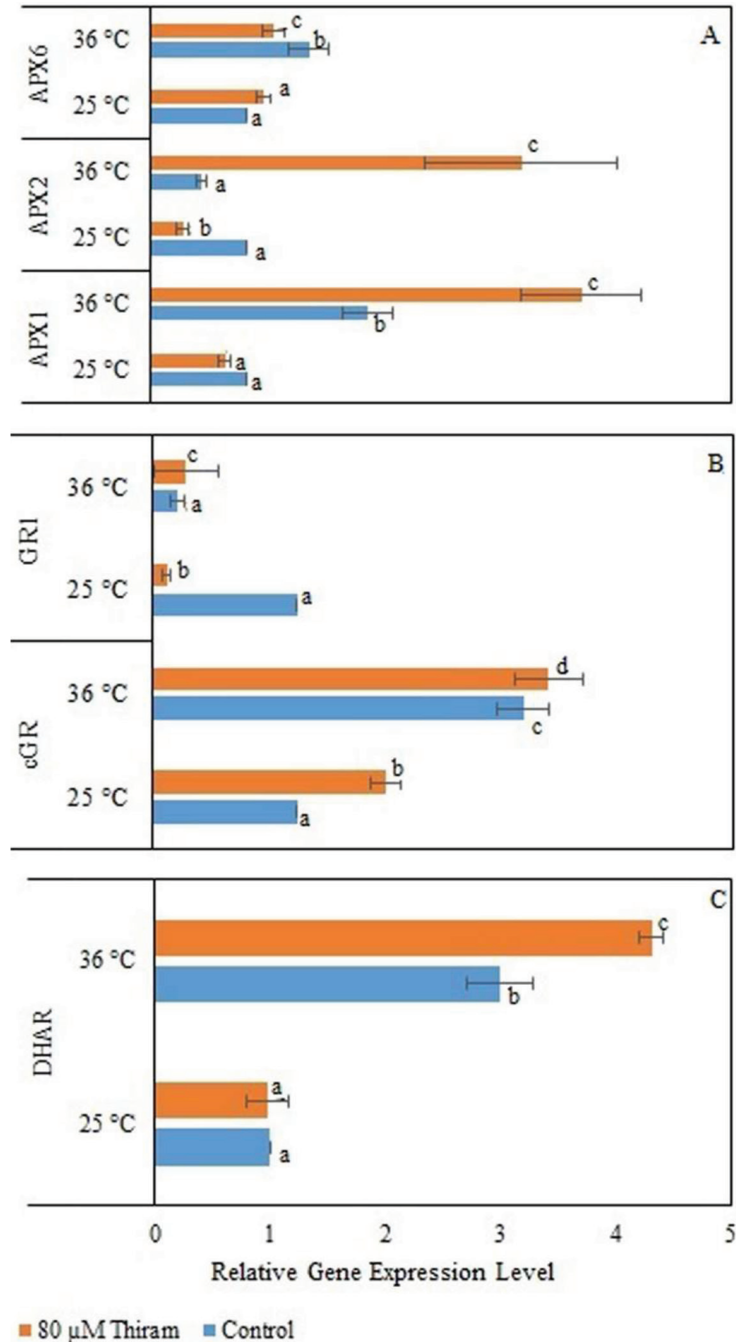

Figure 6. The effects of 80 µM thiram pretreatment on relative gene expression level of *LeApx1*, *LeApx2*, *LeApx6*, *LeDhar*, *LecGr,* and *LeGr1* genes in control (25 °C) and high (36 °C) temperatures. Tomato leaves were harvested in the absence or presence of 80 µM thiram pretreatment 7 days after the treatment. The different letters indicate the statistically significant differences ($P < 0.05$) according to one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test analysis. Bars represent standard deviations (SD).

factor for improving plant adaptation to environmental stresses. Recent evidence has shown that agrochemical fungicides have a role as a chemical pretreatment agent for plant stress management. For example, three different fungicides, paclobutrazol, propiconazole, and tetraconazole protect wheat seedling against heat stress treatment (Gilley and Fletcher, 1997). Another study was conducted that strobilurin-type fungicides enhanced tolerance to transplanting injury and chilling stress in rice seedlings (Takahashi et al., 2017). Considering the current understanding, the result of the present study revealed that fungicide pretreatment enhanced the tolerance in tomato

leaves exposed to high temperature stress. In this study, thiram fungicide was used as a pretreatment agent as a low dose (80 µM). Initially, thiram fungicide was applied to different concentrations at 40–240 µM. The analysis of H_2O_2 and MDA content showed that pretreatment of 80 µM thiram was the most effective concentration against HT stress in tomato leaves. In elevated concentration of thiram pretreatment caused an increase in H_2O_2 and MDA content. As well, in the previous study, it was indicated that 6.6 µM thiram treatment induced the toxicity in tomato leaves (Yüzbaşıoğlu and Dalyan, 2019). The result suggests that high-dose thiram application induced the toxicity but low-dose thiram showed a positive role under HT stress in tomato leaves.

The photosynthetic apparatus is more sensitive due to change in the structure and function of chloroplast under HT stress in plants. HT stress causes a decrease in photosynthetic capacity and chlorophyll accumulation in plants. The reduction of chlorophyll accumulation may result from increasing degradation enzyme activity as chlorophyllase or decreasing synthesis enzyme activity as 5-aminolevulinate dehydratase (ALAD). Besides, chlorophyll pigment can be negatively affected by the injurious effect of reactive oxygen species under HT stress (Mathur et al., 2014; Dikilitaş et al., 2019). In this study, high temperature stress caused a significant reduction in photosynthetic pigments by destroying chlorophyll a, chlorophyll b, and carotenoids. Besides, fungicide pretreatment enhanced photosynthetic pigment control and high temperature. The result suggested that fungicide pretreatment protected the photosynthetic pigment against high temperature stress in tomato leaves. Mohsin et al. (2019) showed that fungicide treatment significantly enhanced chlorophyll and carotenoid content under salt stress in cucumber seedlings.

It is well known that high temperature stress can enhance the accumulation of proline molecule, which is nontoxic, highly soluble, compatible osmolyte in plants and has a low molecular weight. Proline plays a major role in protecting plants by regulating the cellular osmotic balance in plant cells. In addition, it can stabilize the structure and function of membranes and proteins, also it can adjust the redox status and energy balance under stress conditions (Hayat et al., 2012). In this study, the high temperature stress caused an increase in proline content in tomato leaves. Moreover, fungicide pretreatment induced the proline accumulation under control temperature. Interestingly, proline content did not significantly change in fungicide-pretreated leaves 1 and 3 days after heat treatment. Further, fungicide pretreatment led to a decrease in proline accumulation 7 days after heat treatment. These decrements of proline accumulation may explain the elevated proline accumulation in nonstressed fungicide-pretreated plants. The induction of proline level may hinder the inhibition of the photosynthetic pigments and damage of membrane, and accumulation of the free radicals in under HT stress. These results indicated that fungicide pretreatment had a protective role by increasing proline content before the HT stress treatment in tomato leaves. Filippou et al. (2016) reported similar results in proline accumulation in fungicide-pretreated *Medicago truncatula* under drought stress.

The primary effect of HT stress disrupts cellular homeostasis because of the increase in membrane fluidity in plant cells. These increments in membrane fluidity can be caused by fragmentation of the lipid bilayer structure of the cell membrane. Commonly, HT stress has damaged the structure of plasmalemma, photosynthetic, and mitochondrial membranes (Asthir, 2015). Therefore, membrane lipid saturation is an important factor for indicating the effect of HT stress. Membrane damage is indicated by the presence of the form of polyunsaturated precursors that include small hydrocarbon fragments such as ketones, malondialdehyde (MDA) (Asthir, 2015). MDA is a highly reactive and low molecular three-carbon aldehydes that can be produced from a byproduct of polyunsaturated fatty acid peroxidation. Another adverse effect of HT is the excessive accumulation of reactive oxygen species (ROS) which causes oxidative stress in plant cells (Awasthi et al., 2015). To cope with the negative effect of ROS, plant cells can develop complex antioxidant enzyme systems which include CAT, POX, GST, APX, GR, MDHAR, and DHAR. Antioxidant enzymes are important defense systems of plants against oxidative damage which hindering ROS accumulation in many plant species (Dalyan et al., 2018). In the normal cycle of the plant, the cells have a balance between the level of ROS and antioxidant enzymes. However, these balances are disrupted under HT stress due to excessive production of ROS and insufficient antioxidant enzyme activities in plant cells (Awasthi et al., 2015). The present study has shown that high temperature stress triggers the oxidative stress and result in the accumulation of H_2O_2 and MDA in tomato leaves. The highest accumulation of H_2O_2 and MDA was observed 7 days after heat treatment. Also, H_2O_2 production is more abundant than MDA production under HT stress in tomato plants. The most accumulation of H_2O_2 may explain that ROS is produced as a byproduct of different metabolic pathways in various cellular compartments such as electron transport activities of chloroplasts, mitochondria, and plasma membranes (Sharma et al., 2012). Furthermore, HT stress induced a change in ROS scavenging enzyme systems as time-dependent in tomato leaves. Following high temperature exposure, CAT and POX activity significantly increased 7 days after treatment. In addition, APX, MDHAR, and GR

enzyme activities clearly triggered 7 days after treatment. However, GST and DHAR activities significantly increased at 1, 3, and 7 days after treatment. These results indicated that heat stress triggered oxidative stress that caused an increase in both H_2O_2 and MDA content and also, the increment of the antioxidant enzyme activities was not sufficient to overcome stressful conditions in tomato leaves. In our previous study, a similar effect of high temperature on oxidative stress and antioxidant enzyme activities in maize seedlings was observed (Yüzbaşıoğlu et al., 2017).

The expression level of antioxidant enzymes can provide detailed and reliable information about molecular adaptation to HT stress in plants. In this study, transcriptional level of *LeCat1*, *LeGst2*, *LeApx1*, *LeApx2*, *LeApx6*, *LeDhar*, *LecGr,* and *LeGr1* were determined in tomato leaves. Following high temperature exposure, the *Cat1* gene expression level further clearly enhanced 7 days after treatment in tomato. Catalase can localize in mitochondria, chloroplasts, and cytosol that are the sources of H_2O_2 in the plant cells. (Gill and Tuteja, 2010). Also, catalase has a high affinity for intracellular detoxication of H_2O_2 by being converted to H_2O and O_2 because it has the fastest turn-over rates and cannot use reducing molecule in the scavenging process (Soares et al., 2019). Thus, it is very important for scavenging H_2O_2 in plant cells. The CAT gene family consists of the three genes *Cat1*, *Cat2*, and *Cat3* in Arabidopsis and their expression level change as tissue and stress factors in plants (Soares et al., 2019). Scandalios et al. (2000) characterized the *Cat1*, *Cat2,* and *Cat3* gene expression in maize seedling under increasing temperature. Their result suggested that the *Cat1* gene played a protective role in responses to high temperature stress in maize. The other unique H_2O_2 scavenging enzyme system is ascorbate-glutathione cycle. The first enzyme of the ascorbate-glutathione cycle is APX that has various isoenzymes in a different part of cellular compartments, which has the ability for the capture of H_2O_2. The APX isoenzymes are localized in the cytosol, chloroplast, mitochondria, and peroxisom in the plant cell (Soares et al., 2019). In this study, three different *APX* genes including cytosolic (*Apx1* and *Apx2*) and thylakoid-bound *APX* gene (*Apx6*) were examined under heat stress in tomato. The result showed that *Apx1* and *Apx6* were up-regulated, whereas *Apx2* was down-regulated under HT stress in tomato. These findings revealed that cytosolic *Apx1* is more sensitive than *Apx2* and *Apx6* to heat-stimulated transcriptional regulation in tomato leaves. Koussevitzky et al. (2008) reported that cytosolic APX1 protein and transcriptional level increased in Arabidopsis plants under drought and heat stress. GR is mostly located in chloroplast but it also has been found in mitochondria and cytosol in Arabidopsis. Chloroplastic GR has commonly protected photosynthesis from oxidative stress (Trivedi et al., 2013). *Chloroplastic (cGR)* and *cytosolic (GR1)* GR genes were evaluated under HT stress in tomato. The real-time PCR result showed that *chloroplastic GR (cGR)* had a higher expression level than *cytosolic GR1* under HT stress. Shu et al. (2011) revealed that antisense transgenic tomato lines were obtained from chloroplast glutathione reductase gene that was cloned. The GR activity decreased in the transgenic tomato plants under chilling stress. Further, like DHAR enzyme activity, *DHAR* relative gene expression increased under HT stress in tomato leaves. However, the relative expression level of *Gst2* did not significantly change under HT stress.

Before the high temperature stress, the fungicide pretreatment regulated the ROS level and antioxidant status of the tomato cell under control temperature (25 °C). Fungicide pretreatment increased H_2O_2 content but it decreased MDA content first day after the treatment, and fungicide pretreatment did not significantly influence H_2O_2 and MDA content 3 and 7 days after treatment at control temperature. The rapid increase of H_2O_2 may stimulate priming signals in tomato leaves for inducing defense response. Hence, fungicide pretreatment enhanced the activities of CAT, POX, and GST enzyme under control temperature. The induction of POX and GST activity may explain due to being important members of the pesticide detoxification enzyme system that converts pesticides into less toxic molecules so, they have sensitive to fungicide application in tomato leaves. Moreover, spraying thiram fungicide as pretreatment agent did not clearly affect the ascorbate-glutathione cycle enzymes including APX, MDHAR, and DHAR, yet it only increased GR activity under control temperature on tomato leaves. These findings confirmed our previous study which showed that POX and GST activity increased under high concentration of thiram fungicide treatment (Yüzbaşıoğlu and Dalyan, 2019). In brief, low dose fungicide pretreatment did not cause oxidative stress but it significantly accelerated antioxidant enzyme activities including CAT, POX, and GST enzymes under control temperature in tomato leaves. There is less information about the effect of fungicide on the expression level of antioxidant enzyme genes. In the current study, fungicide pretreatment did not significantly alter the *LeApx1*, *LeApx2*, *LeApx6*, and *LeDhar* gene expressions; however, *LeCAT, LeGST2*, and *LecGR* gene expression were clearly induced under control temperature in fungicide pretreated-tomato leaves. These gene expression levels of antioxidant enzymes showed a correlation between antioxidant enzyme activities in fungicide pretreated leaves.

On the other hand, it is evident from the present study that fungicide pretreatment significantly accelerated tolerance against heat stress in tomato

leaves. The experimental finding revealed that fungicide pretreatment diminished the oxidative stress by reducing to the accumulation of H_2O_2 and MDA content when tomato leaves exposed to 36 °C. Furthermore, fungicide pretreatment led to increase in activities and gene expression of the antioxidant enzymes in tomato leaves under heat treatment compared to nonpretreated leaves. Especially, CAT, GST, POX and the ascorbate-glutathione cycle enzyme activities were remarkably enhanced in fungicide-pretreated leaves against high temperature stress. In addition, the expression profile of these enzymes showed similar results with the enzyme activity in fungicide-pretreated leaves under HT stress. Interestingly, fungicide pretreatment caused a highest expression level in *LeApx1* and *LeApx2* genes under HT stress. These results suggest that fungicide pretreatment may cause to mitigate the oxidative stress by enhancement of antioxidant enzyme systems under heat stress in tomato leaves. The protective effect of fungicide pretreatment may explain the stimulation of the de novo synthesis of antioxidant enzymes by increasing the protein level under HT stress. Many studies revealed that fungicides inhibited the senescence process by increasing cytokinins and polyamines and decreasing ethylene in many plant species (Wu and Tiedemann, 2002). The anti-senescent effect of fungicides may alleviate the antioxidant enzyme systems by decreasing the concentration of ROS levels during HT stress in tomato leaves. Wu and Tiedemann (2002) reported that foliar spray of two modern fungicides induced antioxidant enzyme activities for protection against ozone injury in spring barley leaves.

Consequently, fungicide pretreatment (1) increases basal resistance by enhancing proline accumulation and activities and gene expression of the antioxidant enzymes before the HT stress under control temperature, (2) reduces the lipid peroxidation and hydrogen peroxide level under HT stress, (3) improves pigment content under HT stress, (4) accelerates significantly the activities and gene expression of the antioxidant enzymes under HT stress.

5. Conclusion

Fungicide pretreatment has a positive role in regulating plant response to high temperature (HT) stress in tomato leaves. Thiram, a widely used fungicide has a critical role for protection against high temperature stress in tomato when it is used in low-dose. On this basis, fungicide pretreatment is applied for improving crop tolerance under abiotic stress conditions in agriculture. Also, fungicide has a low price and is abundant. Therefore, its future use is an advantage in agriculture under climatic changes such as high temperature. On the other hand, the studies about the fungicide pretreatment on the physiological and biochemical response of tomato under heat stress can contribute to developing new strategies for improving thermotolerance using genetic approaches.

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