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Amelioration of the adverse effects of thiram by 24-epibrassinolide in tomato (*Solanum lycopersicum* Mill.)

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Abstract: This study investigated thiram fungicide-induced-stress effects in tomato plants and the possible protective role of 24-epibrassinolide (24-EBL) in response to thiram (tetramethyl thiuram disulfide) toxicity. Tomato seedlings pretreated with 0, 10^{-11}, 10^{-9} and 10^{-7} M 24-EBL were treated with 6.6 mM thiram. Tomato leaves harvested 5 and 11 days after thiram treatment (DAT) were used for analysis. Thiram application caused oxidative stress by increased hydrogen peroxide and malondialdehyde levels, whereas the chlorophyll a, b and carotenoid amounts and total protein content decreased. In addition, the activities of antioxidant enzymes such as catalase, ascorbate peroxidase and glutathione reductase decreased in the thiram-treated tomato plants on DAT 5 and 11 while pesticide detoxification enzymes (peroxidase and glutathione-S-transferase) activities increased. The thiram-induced oxidative stress was alleviated after pretreatments with different concentrations of 24-EBL. The hydrogen peroxide and malondialdehyde levels decreased and the amounts of photosynthetic pigments and total protein content increased after 24-EBL pretreatments. In addition, the activities of antioxidant enzymes and pesticide detoxification enzymes further increased as the concentration of 24-EBL decreased in tomato under thiram stress, and the most effective concentration was determined as 10^{-11} M 24-EBL. The results suggested that 24-EBL could effectively alleviate thiram-induced phytotoxicity in tomato plants.

Keywords: fungicide, brassinosteroid, chlorophyll, oxidative stress, pesticide detoxification enzyme

INTRODUCTION

The use of pesticides has become a common agricultural practice for increasing the yield and quality of crops and meeting global food demands. Modern pesticides are complex compounds designed to influence target organisms. Although they help control crop pests, they have many undesirable side effects on non-target organisms. Excessive use of pesticides, especially in terms of high-dose applications, adversely affects plant growth, productivity and resistance to pests. Its redundant application on crops raises concerns about potential hazards to food safety and the quality of the harvested produce [1,2].

Tomato (*Solanum lycopersicum* Mill.), a member of the Solanaceae family, has an important place in world agriculture. Plant diseases cause significant product losses in the cultivation of tomatoes that are of high economic value. Due to the sensitivity of tomato plants to fungal diseases, various carbamate fungicides are widely used in agricultural applications [3]. Thiram, one of these fungicides, is one of the most widely and frequently used pesticides with broad-spectrum antibacterial properties for seeds, ornamental plants, vegetables, fruits and grass plants [4]. It is also used during the storage and transportation of post-harvest crops. At the same time, thiram is potentially dangerous to soil and aquatic life [4-6]. However, very limited information is available on the effects of thiram on the physiology of non-target plants [3].

Excessive use of pesticides causes oxidative stress by generating reactive oxygen species (ROS) in non-target organisms such as plants [7,8]. ROS cause oxidative damage to lipids, proteins and nucleic acids in the cell [9]. For instance, high amounts of hydrogen peroxide and malondialdehyde were detected in seedlings of *Brassica juncea* L. under the toxicity of imidacloprid [10]. Plants have developed an antioxidant defense system that is activated during oxidative stress to control

overproduced ROS. Catalase, ascorbate peroxidase and glutathione reductase are antioxidant enzymes that play a key role in combating pesticide toxicity in plants [11]. Catalase, especially in peroxisomes, directly catalyzes the conversion of hydrogen peroxide to water and molecular oxygen. Ascorbate peroxidase found in almost all parts of plant cells participates in the ascorbate-glutathione pathway and protects against oxidative stress through the detoxification of hydrogen peroxide, especially in photosynthetic tissues. Additionally, ascorbate peroxidase removes hydrogen peroxide by using ascorbate in the oxidation-reduction reaction in mitochondria and peroxisomes. Like ascorbate peroxidase, glutathione reductase, an NADPH-dependent enzyme, also plays an important role in protecting chloroplasts from oxidative damage by maintaining a high reduced/oxidized glutathione ratio [11].

ROS produced as a result of metabolic imbalance also mediate pesticide detoxification in plants. Plants can reduce pesticides to less toxic soluble metabolites through a three-phase enzyme-mediated degradation process via (i) enzymes such as peroxidases, cytochrome P450 monooxygenases and carboxylesterases involved in pesticide activation; (ii) glutathione-S-transferase and UDP-glycosyltransferase that cause the formation of more soluble and less toxic metabolites by providing the conjugation of activated pesticides with glucose and glutathione; (iii) the formation of metabolites formed, transferred and stored in vacuoles or the apoplast [12,13].

Brassinosteroids (BRs), a class of plant steroid hormones, contribute significantly to plant growth and development processes [14,15]. They also play a role in plant defense against fungal, bacterial and viral pathogens, and environmental stresses [16,17]. BRs are known to protect plants from abiotic stresses induced by pesticides [8,9,18]. 24-Epibrassinolide applications were observed to enhance the resistance to pesticides by increasing the antioxidant enzyme activities in plants such as rice and grapevine [19,20]. Also, it was reported [21] that 24-EBL plays an important role in the activation of pesticide-detoxifying enzymes such as peroxidase and glutathione-S-transferase. Sun et al. [22] determined that 24-EBL pretreatment under chlorpyrifos stress enhanced the transcription of genes encoding for antioxidant, detoxifying and defense proteins in tomato leaves. Sharma et al. [23] found that 24-EBL treatment decreased imidacloprid residues and increased the activities of antioxidant enzymes in *Brassica juncea* L. The effects of BRs on pesticide stress in plants have mostly been studied on herbicides and insecticides. However, studies in the context of fungicide toxicity are very limited, and there is no information about the exogenous application of BRs in thiram-induced stress in the plant.

In the present study, the toxic effects of thiram on tomato plants, and the ameliorative effects after exogenous pretreatment of 24-EBL, an active brassinosteroid, were investigated. Toxicity was evaluated in terms of photosynthetic pigment contents, oxidative stress indicators and total protein amounts, while detoxification was examined by determining the activation of antioxidative enzymes and pesticide metabolism enzymes.

MATERIALS AND METHODS

Plant material and growth conditions

Tomato *(Solanum lycopersicum* Miller var. SC2121) seeds were obtained from Balıkesir Küçükçiftlik Seed Corporation in Turkey. After surface sterilization with 1% sodium hypochlorite for 15 min, tomato seeds were kept in deionized water for 24 h. Seeds were sown in pots containing perlite and irrigated with 25% Hoagland solution [24] on alternate days. Tomato plants with six leaves 41 days after planting were sprayed with deionized water or solutions containing 24-EBL at different concentrations. Hormone pretreatments were repeated on the same plants on day 43. After 24 h, 24-EBL-treated and untreated plants were sprayed with thiram solution. On days 5 and 11 after thiram treatment (DAT), all leaves of the 49-day and 55-day-old plants were harvested. All plants were kept in a growth chamber (in a 16-8 h photoperiod) under a photosynthetic photon flux density of 150 μmol m^{-2} s^{-1}, day/night temperature of 20/25±2°C, and 60±5% relative humidity during growth and treatments.

Experimental design

The concentrations of 24-EBL and thiram solutions were determined according to preliminary studies. A stock solution of 10^{-2} M 24-EBL (Sigma Aldrich,

USA) was made by dissolving the required quantity of the hormone in ethanol (Sigma Aldrich). Solutions of 10^{-11}, 10^{-9} and 10^{-7} M concentrations were prepared by serial dilution with deionized water. For the 6.6 mM thiram solution, the required amount of pure thiram (Sigma Aldrich) was dissolved in ethanol and deionized water. Deionized water applied to control plants contained the same amount of ethanol. All solutions were sprayed on each leaf three times, and the nozzle of the sprayer was adjusted to release 0.3 mL in one pump. Each experiment was replicated five times, and each replicate consisted of nine plants. All leaves from nine plants per treatment were harvested, pooled and ground with liquid nitrogen, and a sample of 0.3 g was used for each analysis.

Determination of photosynthetic pigment contents

The fresh leaf sample was extracted in 100% acetone and then centrifuged at 3000 ×g at 4°C for 15 min. The absorbance of the obtained supernatant was measured at 661.6, 644.8 and 470 nm. The contents of chlorophyll a, b and carotenoids were calculated as µg/mL according to the method of Lichtenthaler and Buschmann [25].

Determination of hydrogen peroxide concentration

The fresh leaf sample was homogenized in 0.1% trichloroacetic acid and centrifuged at 12,000 ×g for 15 min. Potassium phosphate buffer (10 mM, pH 7.0) and potassium iodide (1 M) were added to the obtained supernatant and the absorbance of the mixture was determined spectrophotometrically at 390 nm. The hydrogen peroxide (H_2O_2) concentration was expressed in µmol on a standard curve [26].

Determination of lipid peroxidation

Lipid peroxidation was analyzed by determining the content of malondialdehyde (MDA). The fresh leaf sample was homogenized in 0.25% thiobarbituric acid and 10% trichloroacetic acid, then kept at 95°C for 30 min and chilled on ice. The absorbance of samples centrifuged at 5,000 ×g for 10 min was recorded spectrophotometrically at 532 nm and 600 nm. The concentration of MDA was expressed as µmol/g fresh weight (fw) [27].

Determination of enzyme activities

The fresh leaf sample was homogenized in potassium phosphate buffer (50 mM, pH 7.0) including 1% polyvinylpolypyrrolidone (PVPP) and 1 mM ethylenediaminetetraacetic acid (EDTA). The supernatant obtained after centrifugation at 13,000 ×g 4°C for 40 min was used to determine the total protein content and enzyme activity. Spectrophotometric measurements were made on an Epoch 2 Microplate Spectrophotometer (Winooski, USA). Enzyme activity was expressed as units (U)/mg protein [3].

Determination of the total protein content

Total protein content was measured by the Bradford method [33] and bovine serum albumin was used as a standard. The total protein content was given as mg/mL.

Catalase activity

Catalase (CAT, EC 1.11.1.6) activity was observed by the decomposition of H_2O_2 (extinction coefficient: 39.4 mM^{-1} cm^{-1}) at 240 nm for 2 min in a total volume including sodium phosphate buffer (50 mM, pH 7.0), H_2O_2 (0.3%), EDTA (0.1 mM) and plant extract [28].

Ascorbate peroxidase activity

Ascorbate peroxidase (APOX, EC 1.11.1.11) activity was estimated by observing the decrease in the absorbance at 290 nm for 2 min [29]. The reaction mixture contained sodium phosphate buffer (50 mM, pH 7.0), Na_2EDTA (0.1 mM), H_2O_2 (0.12 mM), ascorbate (0.5 mM) and plant extract. The concentration of oxidized ascorbate was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Glutathione reductase activity

Glutathione reductase (GR, EC 1.6.4.2) activity was assayed by the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) (extinction coefficient: 6.2 mM^{-1} cm^{-1}) at 340 nm for 2 min. The reaction mixture contained sodium phosphate buffer (25 mM, pH 7.8), NADPH Na_4 (0.12 mM), L-glutathione oxidized (GSSG) (0.5 mM) and plant extract [30].

Table 1. Time- and dose-dependent effects of 24-EBL pretreatments on photosynthetic pigments in tomato leaves under thiram stress. Tomato plants were sprayed with deionized water (control) or 10^{-11}, 10^{-9} and 10^{-7} M 24-EBL on days 41 and 43, followed by 6.6 mM thiram 24 h later. The fresh leaves were harvested 5 and 11 days after thiram treatment (5 DAT and 11 DAT, respectively) and used for photosynthetic pigment level determination.

		Chl a (µg/mL)	Chl b (µg/mL)	Car (µg/mL)	Total chl (µg/mL)	Total chl/car
5 DAT	**Control**	397.55±1.16	141.18±0.54	102.57±0.84	538.06±1.30	5.25±0.03
	Thiram	343.93±2.10^{a}	123.39±0.89^{a}	91.38±0.63^{a}	470.20±1.09^{a}	5.15±0.03^{a}
	10^{-11} M 24-EBL+Thiram	461.61±2.19^{ab}	162.59±0.95^{ab}	117.27±0.53^{ab}	619.88±2.32^{ab}	5.29±0.02^{b}
	10^{-9} M 24-EBL+Thiram	364.27±2.41^{ab}	129.97±0.92^{ab}	92.68±0.79^{ab}	492.68±0.96^{ab}	5.32±0.04^{ab}
	10^{-7} M 24-EBL+Thiram	369.33±2.0^{2a}b	131.00±0.89^{ab}	97.01±0.29^{ab}	500.23±1.81^{ab}	5.16±0.02
11 DAT	**Control**	315.49±2.48	112.76±1.55	86.32±0.70	434.16±2.58	5.03±0.04
	Thiram	279.05±2.84^{a}	102.01±0.97^{a}	75.91±0.55^{a}	376.48±2.09^{a}	4.96±0.01^{a}
	10^{-11} M 24-EBL+Thiram	287.41±1.47^{ab}	108.22±0.73^{ab}	80.38±0.35^{ab}	394.82±1.58^{ab}	4.91±0.03^{a}
	10^{-9} M 24-EBL+Thiram	319.44±1.57^{ab}	113.75±1.21^{b}	87.58±0.86^{b}	432.38±1.83^{b}	4.94±0.03^{a}
	10^{-7} M 24-EBL+Thiram	281.43±1.60^{a}	106.25±1.40^{ab}	77.37±0.53^{ab}	389.97±1.23^{ab}	5.04±0.04^{b}

DAT – day(s) after treatment, Chl – chlorophyll, Car – carotenoid. Five replicates, each with 9 plants, were prepared for each treatment. Data are presented as the means±standard deviations (SD). "a" and "b" letters indicate values that differ significantly from the control and thiram treatments, respectively, according to the Tukey test at $P<0.05$.

Peroxidase activity

Peroxidase (POX, EC 1.11.1.7) activity was measured as the decomposition of H_2O_2 (extinction coefficient: 2.47 mM^{-1} cm^{-1}) at 465 nm for 3 min in a total volume including 3,3'-diaminobenzidine (DAB) solution (50% gelatin and 0.15 M sodium phosphate-citrate buffer (pH 4.4)), H_2O_2 (0.6%) and plant extract [31].

Glutathione-S-transferase activity

Glutathione-S-transferase (GST, EC 2.5.1.18) activity was evaluated with the product of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation with reduced glutathione (GSH)-S-conjugate (extinction coefficient: 9.6 mM^{-1} cm^{-1}). The increase at 340 nm was measured for 5 min. The reaction mixture consisted of potassium phosphate buffer (50 mM, pH 6.5), CDNB (1 mM), GSH (5 mM), EDTA (1 mM) and plant extract [32].

Statistical analysis

All experiments consisted of five replicates. All data were obtained after using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test at $P<0.05$, with each treatment's mean and standard deviation calculated. All statistical analyzes were performed using GraphPad Prism Version 5.2 software (USA).

RESULTS

Photosynthetic pigment contents

The treatment with thiram adversely affected the amounts of photosynthetic pigments in tomato leaves. The chlorophyll a, b and carotenoid contents significantly decreased by 14%, 13% and 11%, respectively, on 5 DAT and by 12%, 10% and 12%, respectively, on 11 DAT compared to the control plants (Table 1). In addition, the thiram treatment caused a considerable decrease in the total chlorophyll content by 13% on both 5 and 11 DAT (Table 1). Besides, the total chlorophyll/carotenoid ratio under thiram stress was markedly reduced on 5 and 11 DAT relative to the control group (Table 1).

The pretreatments with 24-EBL under thiram toxicity increased the contents of photosynthetic pigments in tomato leaves. The amounts of chlorophyll a, b and carotenoids displayed a maximum increase of 34%, 32% and 28%, respectively, on 5 DAT with the 10^{-11} M 24-EBL pretreatment compared to the treatment with thiram (Table 1). The pretreatment with 10^{-9} M 24-EBL caused the highest increase on 11 DAT in chlorophyll a by 15%, chlorophyll b by 12% and carotenoids by 15% (Table 1). Moreover, all the concentrations of 24-EBL applied in the pretreatment under thiram stress considerably increased the total chlorophyll content. The pretreatment with 10^{-11} M 24-EBL showed the

Table 2. Time- and dose-dependent effects of 24-EBL pretreatments on hydrogen peroxide, malondialdehyde (MDA), and total protein contents in the tomato leaves under thiram stress. Tomato plants were sprayed with deionized water (control) or 10^{-11}, 10^{-9} and 10^{-7} M 24-EBL on days 41 and 43, followed by 6.6 mM thiram 24 h later. The fresh leaves were harvested 5 and 11 days after thiram treatment (5 DAT and 11 DAT, respectively) and used for hydrogen peroxide, malondialdehyde, and total protein levels determination.

		H_2O_2 content (µmol)	MDA content (µmol/g fr wt)	Total protein concentration (mg/mL)
5 DAT	**Control**	21.96±0.72	4.67±0.03	80.52±0.74
	Thiram	33.20±0.65[a]	5.39±0.04[a]	57.81±0.43[a]
	10^{-11} M 24-EBL+Thiram	27.84±0.50[ab]	4.46±0.03[ab]	72.95±0.72[ab]
	10^{-9} M 24-EBL+Thiram	30.31±0.75[ab]	5.25±0.0[3a]b	76.27±0.62[ab]
	10^{-7} M 24-EBL+Thiram	29.72±0.18[ab]	4.69±0.01[b]	106.64±0.85[ab]
11 DAT	**Control**	33.12±0.68	3.84±0.02	93.00±0.70
	Thiram	38.54±0.89[a]	6.51±0.05[a]	77.61±0.73[a]
	10^{-11} M 24-EBL+Thiram	34.54±0.26[b]	6.08±0.02[ab]	84.46±0.89[ab]
	10^{-9} M 24-EBL+Thiram	33.30±0.94[b]	5.06±0.03[ab]	107.50±0.9[8a]b
	10^{-7} M 24-EBL+Thiram	30.72±0.82[ab]	4.90±0.02[ab]	113.35±0.80[ab]

DAT – day(s) after treatment. Five replicates, each with 9 plants, were prepared for each treatment. The data are presented as the means±standard deviations (SD). "a" and "b" letters indicate values that differ significantly from the control and thiram treatments, respectively, according to the Tukey test at $P<0.05$.

highest increase on 5 DAT by 32% when compared to the thiram treatment (Table 1). With the 10^{-9} M 24-EBL pretreatment, the total chlorophyll content showed a maximum increase of 15% on 11 DAT (Table 1). In addition, the total chlorophyll/carotenoid ratio increased significantly at the concentrations of 10^{-11} M and 10^{-9} M 24-EBL on 5 DAT and the 10^{-7} M 24-EBL pretreatment on 11 DAT (Table 1).

Hydrogen peroxide concentration

Thiram toxicity led to a significant increase in H_2O_2 concentration in the tomato leaves. The H_2O_2 content in the thiram-treated plants increased by 51% and 16% on 5 and 11 DAT, respectively, compared to the control plants (Table 2). The pretreatments with 24-EBL under thiram stress considerably reduced the content of H_2O_2 in the tomato leaves. The amount of H_2O_2 showed a maximum decrease of 16% in the 10^{-11} M 24-EBL pretreatment on 5 DAT compared to the thiram treatment (Table 2). With the 10^{-9} M 24-EBL pretreatment, the H_2O_2 content decreased by 9% and 14% on 5 and 11 DAT, respectively (Table 2). The pretreatment of 10^{-7} M 24-EBL caused the greatest reduction in H_2O_2 of 20% on 11 DAT relative to the thiram treatment (Table 2).

Lipid peroxidation

The MDA content increased considerably with the thiram treatment in the tomato leaves. Thiram toxicity caused a significant increase in the concentration of MDA of 15% and 70% on 5 and 11 DAT, respectively, as compared to the control plants (Table 2). The pretreatments with 24-EBL of thiram-treated leaves significantly decreased the MDA content. The concentration of 10^{-11} M 24-EBL showed the greatest decrease on 5 DAT by 17% as compared to the thiram treatment (Table 2). MDA was decreased in the pretreatment with 10^{-9} M 24-EBL by 3% and 22% on 5 and 11 DAT, respectively (Table 2). The pretreatment with 10^{-7} M 24-EBL caused a maximum reduction of 25% in 11 DAT compared to the thiram-treated leaves (Table 2).

Total protein content

Thiram stress markedly reduced the total protein content in tomato leaves. The amount of total protein amount in the thiram-treated leaves decreased by 28% and 17% on 5 and 11 DAT, respectively, compared to the control plants (Table 2). The pretreatments with 24-EBL under thiram toxicity significantly enhanced the total protein content. The concentration of 10^{-7} M 24-EBL showed the highest increases by 85% and 46% on 5 and 11 DAT, respectively, compared to the thiram-treated plants (Table 2).

Antioxidant enzyme activities

The tomato leaves under thiram toxicity displayed a significant reduction of 11% and 21% in CAT activity on 5 and 11 DAT, respectively, compared to the control plants (Fig. 1A). On 5 DAT, the activity of CAT increased by 35% and 8% in the 10^{-11} M and 10^{-9} M 24-EBL pretreatment groups, respectively, but decreased by 26% in the 10^{-7} M 24-EBL pretreatment

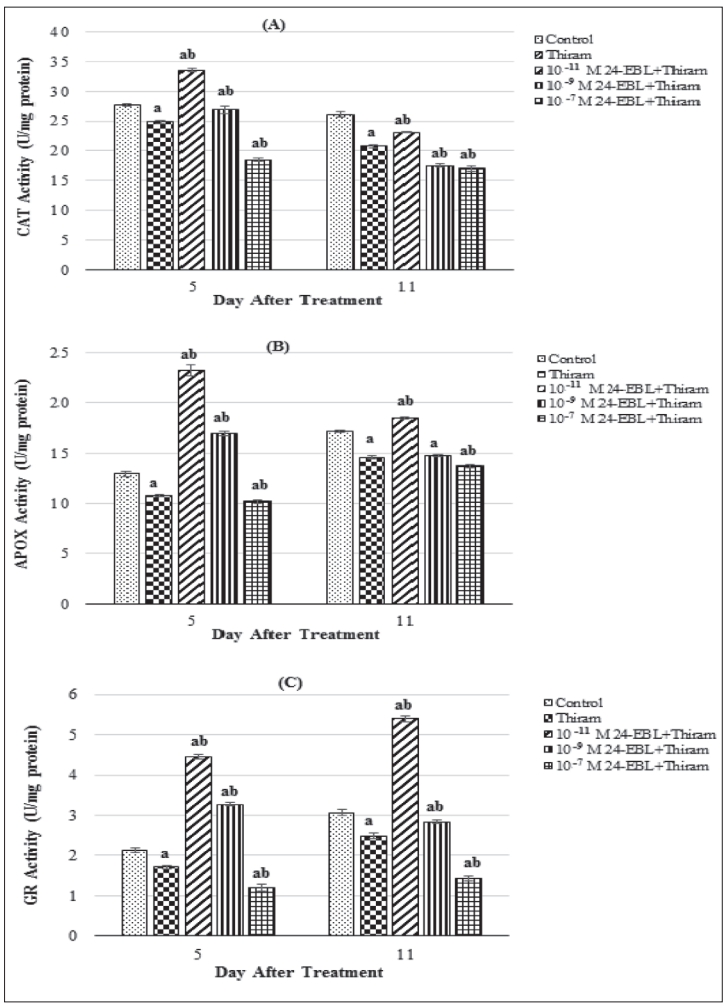

Fig. 1. Time- and dose-dependent effects of 24-EBL on (**A**) catalase, (**B**) ascorbate peroxidase, and (**C**) glutathione reductase activities in the tomato leaves under thiram stress. Tomato plants were sprayed with deionized water (control) or 10^{-11}, 10^{-9} and 10^{-7} M 24-EBL on days 41 and 43 days, followed by 6.6 mM thiram 24 h later. The fresh leaves were harvested 5 and 11 days after thiram treatment (5 DAT and 11 DAT, respectively) and used for antioxidant enzyme activity determination. Five replicates, each with 9 plants, were prepared for each treatment. Bars represent mean values±standard deviations (SD). "a" and "b" letters indicate values that differ significantly from the control and thiram treatments, respectively, according to the Tukey test at $P<0.05$.

group relative to the thiram-treated plants (Fig. 1A). In addition, the pretreatment with 10^{-11} M 24-EBL increased CAT activity by 11% on 11 DAT compared to the thiram treatment, while the pretreatment with 10^{-9} M and 10^{-7} M 24-EBL decreased it by 16% and 18%, respectively (Fig. 1A).

APOX activity in the thiram-treated tomato leaves exhibited significant decreases of 17% and 15% on 5 and 11 DAT, respectively, compared to the control groups (Fig. 1B). APOX activity increased in the 10^{-11} M and 10^{-9} M 24-EBL pretreatment groups by 115% and 58%, respectively, on 5 DAT relative to the thiram treatment (Fig. 1B). In addition, pretreatment with 10^{-11} M 24-EBL considerably enhanced the activity of APOX by 27% on 11 DAT compared to leaves treated with thiram, while the 10^{-7} M 24-EBL pretreatment reduced it by 6% (Fig. 1B).

Thiram toxicity caused a considerable reduction in GR activity by 20% and 19% on 5 and 11 DAT, respectively, when compared to the control plants (Fig. 1C). On 5 and 11 DAT, GR activity was markedly increased in the 10^{-11} M and 10^{-9} M 24-EBL pretreatment groups while it decreased in the 10^{-7} M 24-EBL pretreatment group relative to the thiram-treated plants. The highest increase of 161% and 117% on 5 and 11 DAT, respectively (Fig. 1C), was recorded in the 10^{-11} M 24-EBL group.

Pesticide detoxification enzyme activities

Thiram treatment significantly increased the activity of POX by 13% and 29% on 5 and 11 DAT, respectively when compared to the control group (Fig. 2A). On 5 DAT, POX activity increased significantly in pretreatment groups 10^{-11} M and 10^{-9} M 24-EBL by 53% and 12%, respectively, relative to the thiram treatment, while they decreased by 14% in the 10^{-7} M 24-EBL pretreatment group (Fig. 2A). In addition, the 10^{-11} M 24-EBL pretreatment considerably enhanced the activity of POX by 6% on 11 DAT compared with the thiram-treated group, but the pretreatment with 10^{-7} M 24-EBL reduced it by 30% (Fig. 2A).

GST activity under thiram toxicity was significantly increased in the tomato leaves relative to the control group by 25% and 9% on 5 and 11 DAT, respectively (Fig. 2B). The 10^{-11} M and 10^{-9} M 24-EBL pretreatment markedly increased the activity of GST on 5 DAT by 31% and 4%, respectively, as compared to the thiram treatment, while the pretreatments with 10^{-7} M 24-EBL reduced it by 22% (Fig. 2B). On 11 DAT, GST

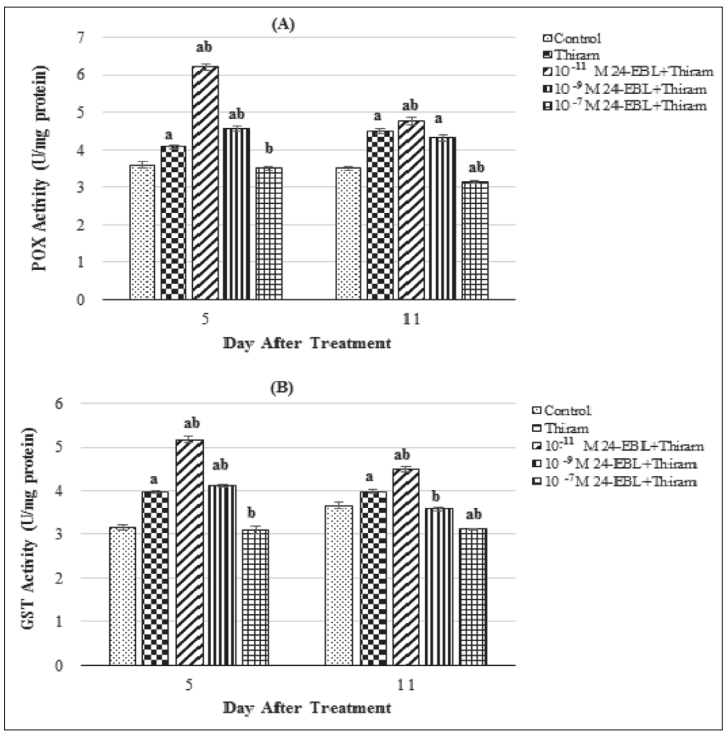

Fig. 2. Time- and dose-dependent effects of 24-EBL on (**A**) peroxidase and (**B**) glutathione-S-transferase activities in the tomato leaves under thiram stress. Tomato plants were sprayed with deionized water (control) or 10^{-11}, 10^{-9} and 10^{-7} M 24-EBL on days 41 and 43 days, followed by 6.6 mM thiram 24 h later. The fresh leaves were harvested 5 and 11 days after thiram treatment (5 DAT and 11 DAT, respectively) and used for antioxidant enzyme activity determination. Five replicates, each with 9 plants, were prepared for each treatment. Bars represent mean values±standard deviations (SD). "a" and "b" letters indicate values that differ significantly from the control and thiram treatments, respectively, according to the Tukey test at $P<0.05$.

activity remarkably increased in the 10^{-11} M 24-EBL pretreatment group by 13% when compared to the thiram-treated group but decreased in the 10^{-9} M and 10^{-7} M 24-EBL pretreatment groups by 10% and 22%, respectively (Fig. 2B).

DISCUSSION

Photosynthetic pigments (chlorophyll a, b and carotenoids) are very important for evaluating a plant's response to pesticide stress. Chlorophyll a, b and carotenoids bound to light-harvesting complex (LHC) proteins capture light energy and transfer it to the reaction center of the chloroplast thylakoid membrane [34]. Carotenoids also play a role in protecting chlorophyll pigments against oxidative stress by quenching free radicals. In the present study, a decrease in the amounts of chlorophyll a, b and carotenoids on 5 and 11 DAT indicated the destructive effect of thiram on photosynthetic pigments. These decreases could be the result of the impairment of LHC proteins, the breakdown of chloroplast structure and/or changes in chlorophyll fluorescence [3,21]. Sing and Sahota [35] detected a decreased chlorophyll content in chickpeas under fungicide toxicity. Also, a reduction in the total chlorophyll/carotenoid ratio showed a faster breakdown of chlorophyll than carotenoids after the thiram treatment in tomatoes. This decrease could be the result of free radical-induced oxidation of chlorophyll pigments or of the increase in the activity of chlorophyllase [36]. BR application to plants causes an increase in the amount of total chlorophyll depending on the concentration, time and plant species [37]. In the present study, the amounts of photosynthetic pigments and total chlorophyll on 5 and 11 DAT increased with the 24-EBL pretreatments under thiram stress. In addition, the increase in the total chlorophyll/carotenoid ratio in the groups pretreated with 24-EBL and exposed to thiram stress pointed to a decrease in the stress-related destruction of the photosynthetic apparatus. These effects of BRs on the photosynthetic pigments might be due to increased biosynthesis of these pigments, their reduced degradation, or changes in the levels of LHC proteins or early light-inducible proteins related to these pigments, especially in the chloroplast thylakoid membrane [9,37]. This result is compatible with the study of Sharma et al. [8] who detected an increase in photosynthetic pigment contents in *Brassica juncea* after 24-EBL application under imidacloprid stress. Moreover, BRs can regulate the expression of genes encoding key enzymes involved in the biosynthesis pathways of these pigments. Sharma et al. [9] reported that BRs improved chlorophyll biosynthesis by regulating *CHLASE* (chlorophyllase) expression under abiotic stress and increased the expression of the gene encoding phytoene synthase, the key enzyme of the carotenoid biosynthesis pathway.

Oxidative stress causes oxidative damage to macromolecules in the cell and is used as a marker of phytotoxicity [11]. In the present study, thiram induced

oxidative stress by enhancing H_2O_2 production in tomato leaves on 5 and 11 DAT. The increase in H_2O_2 might be related to plasma membrane NADPH oxidases involved in the production of H_2O_2 [8]. The conducted studies indicated that the expression of the respiratory burst oxidase homolog 1 (*RBOH1*) gene, which encodes the essential components of plant NADPH oxidase, was upregulated by pesticide stress [8,38]. Similarly, Zhou et al. [7] showed that excess H_2O_2 induced oxidative stress in chlorothalonil pesticide-treated tomatoes. BR-mediated oxidative stress responses might exhibit differences in a concentration-, time- and plant-species-dependent manner [39]. In the present study, the 24-EBL pretreatments alleviated thiram-induced oxidative stress by decreasing the concentrations of H_2O_2 on 5 and 11 DAT. BRs could ameliorate the concentrations of ROS by reducing NADPH oxidase activity under different stress conditions [40]. It was also reported that BRs led to the downregulation of respiratory burst oxidase (*RBO*) transcript levels under pesticide toxicity [7,41]. Likewise, Sharma et al. [9] showed that exogenous BR applications with insecticide stress generally reduced ROS levels.

Environmental stresses damage membrane lipids by inducing excessive production of ROS in plants. Lipid peroxidation can disrupt the bilayer structure of the cell membrane and further alter membrane fluidity and its permeability [20]. In the present study, the thiram treatment caused membrane lipid peroxidation by increasing the concentration of H_2O_2 on 5 and 11 DAT in tomatoes. Consistent with this result, an increase in lipid peroxidation was determined with the applications of imidacloprid in *Brassica juncea* [8] and nicosulfuron in *Zea mays* [42]. BR applications under different stress conditions caused a decrease in the amount of MDA depending on the concentration, time and plant species [43]. The present study showed that the decrease in the MDA level reflected lipid peroxidation by BR pretreatments under thiram stress on 5 and 11 DAT in tomatoes. BRs can alleviate lipid peroxidation by decreasing the concentration of ROS with its effects on the antioxidant enzyme activities under stress conditions [20]. Similarly, Liu et al. [44] and Bakshi et al. [38] revealed that BR application under different pesticide stress decreased lipid peroxidation by reducing the level of ROS.

Pesticide stress causes a decrease in the amount of total soluble protein [19]. The present study revealed a decrease in total protein under thiram stress on 5 and 11 DAT in tomatoes. This reduction could be the result of protein degradation because of increased protease activity or from autophagy that degrades oxidized proteins under stress [45]; this result is compatible with the reports of Sharma et al. [46]. BRs play a positive role in the regulation of protein metabolism. Under normal or stress conditions, the increase in the amount of protein could be the result of the regulation of the synthesis of polypeptides and proteins by BRs [47]. Herein, the decrease in protein content caused by thiram toxicity was restored with the 24-EBL pretreatments on 5 and 11 DAT. BRs can promote the synthesis of different proteins by affecting transcriptional and translational processes under pesticide stress [45]. Likewise, Sharma et al. [19] observed that 24-EBL applications under imidacloprid stress enhanced the amount of protein in rice.

Alterations in the activities of antioxidant enzymes in plants indicate abiotic stress. CAT, especially in peroxisomes, directly catalyzes the conversion of H_2O_2 to water and molecular oxygen. APOX, which is found in almost all parts of plant cells, participates in the ascorbate-glutathione pathway and protects against oxidative stress by detoxification of H_2O_2, especially in photosynthetic tissues. In addition, APOX removes H_2O_2 by using ascorbate in the oxidation-reduction reaction in mitochondria and peroxisomes. Like APOX, GR, an NADPH-dependent enzyme, also plays an important role in protecting chloroplasts from oxidative damage by maintaining a high reduced/oxidized glutathione ratio. These enzyme activities increase during initial exposure to pesticides but decrease considerably as pesticide toxicity increases [48]. In this study, the activities of CAT, APOX and GR under thiram stress decreased on 5 and 11 DAT when compared to the control plants. This result is consistent with other studies that reported changes in enzyme activities in response to imidacloprid stress [19,41]. Modulation of the antioxidant system plays a critical role in BR-mediated stress improvement. In the 24-EBL pretreatments under thiram stress, the activities of CAT, APOX and GR varied depending on the concentration and time. The 24-EBL pretreatments displayed the opposite effect to the thiram treatment. On 5 and 11 DAT, the increase was achieved with the 24-EBL pretreatments despite the reduction in enzyme activities because of thiram toxicity. These changes in enzyme activities could have been the result of 24-EBL-modulated protein synthesis or altered enzyme kinetics

[8,41]. In addition, increases were also observed in CAT, APOX and GR activities on 5 and 11 DAT as the concentration of 24-EBL used in the pretreatments decreased, with the most effective concentration determined to be 10^{-11} M 24-EBL. On the other hand, these enzyme activities that were observed in the 10^{-7} M 24-EBL pretreatment exhibited decreases on 5 and 11 DAT compared to the thiram treatment. Therefore, a higher 24-EBL concentration under thiram toxicity might possess an inhibitory effect on the activities of antioxidant enzymes. Wang et al. [20] reported that antioxidant enzyme activities were changed in 24-EBL-treated grape plants under chlorothalonil stress depending on hormone concentration.

As mentioned in the introduction, pesticide degradation in plants occurs in a three-step enzyme-mediated detoxification system. GST detoxifies xenobiotic chemicals to glutathione-S-conjugates by binding glutathione to hydrophobic substrates. POD and GST play an important role in pesticide detoxification through the oxidation of toxic chemicals [49]. This study revealed that the activities of POD and GST in the pesticide detoxification system increased in the thiram treatment on 5 and 11 DAT in tomato plants. Xia et al. [21] reported similar results in cucumber plants treated with chlorpyrifos. Regulation of the functioning of enzymes involved in pesticide detoxification by BR provides further degradation of pesticides [7,8]. In the current study, the activities of POD and GST significantly increased in tomato plants that were pretreated with 24-EBL under thiram stress in a concentration-dependent manner on 5 and 11 DAT. As the 24-EBL concentration decreased, detoxification of enzyme activities further increased, and the most effective concentration was 10^{-11} M 24-EBL. BRs might play a role in the regulation of plant resistance by improving the activities of POD and GST in response to pesticide-induced stress in plants [20]. Since the activity of POD changes under different environmental stresses, the epibrassinolide-mediated induction of the detoxification enzymes and thiram metabolism may be part of the stress response. Furthermore, the conversion of pesticides into intermediates with reduced phytotoxicity by the glutathionylation reaction catalyzed by GSTs is very important in this process. GST can also assist the detoxification process by transporting glutathione-pesticide conjugates to the vacuole or apoplast, thus participating in their storage [50]. In addition, the regulation of the transcript levels of genes encoding enzymes such as POD and GST by BR might result in the reduction of pesticide residues. Sharma et al. [9] detected an increase in the expression of GST1 and POD after castasterone application under imidacloprid stress; the authors proposed that regulated gene expression was the result of BR cell signaling initiated from brassinosteroid-sensitive 1 and BRI1 kinase inhibitor 1 receptors [51]. This signaling probably further regulates biochemical processes that lead to increased plant resistance to pesticide stress [10,18].

CONCLUSIONS

This study is the first to show the time- and dose-dependent stress-protective properties of 24-EBL in tomato plants against thiram-induced oxidative stress. 24-EBL pretreatments under thiram toxicity possessed ameliorative effects on oxidative stress by increasing photosynthetic pigment and protein contents, decreasing H_2O_2 and MDA levels and changing the activities of antioxidant enzymes and pesticide detoxification enzymes. As the concentration of 24-EBL pretreatments decreased, higher increases in CAT, APOX and GR activities, as well as POX and GST activities, were observed, with the most effective concentration being 10^{-11} M 24-EBL.

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