

RESEARCH PAPERS

## Comparison of Physiological Responses in Some *Pinus* Species Attacked by Pine Processionary Moth

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**Abstract**—*Pinus* species, used in traditional medicine and are an important industrial plant, suffer severe ecological and economic losses with the invasion of *Thaumetopoea wilkinsoni* (the pine processionary moth). Due to the damage caused by *T. wilkinsoni*, a biotic stress factor, the changes occur in the ecological and physiological parameters of *Pinus* species, which also try to cope with abiotic stress. The changes in fresh/dry weight, percent water content, chlorophyll *a* and *b* content, the total protein content, malondialdehyde (MDA) amounts, and superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) activities in leaf samples of the pine processionary moth-infested and non-infested *Pinus sylvestris* L. and *P. nigra* Arn. species were compared in the study. The leaf samples of *P. nigra* and *P. sylvestris* were collected from the Ondokuz Mayıs University Kurupelit Campus in Samsun, Turkey, in 2020. Individuals of the moth-infested and non-infested *P. sylvestris* and *P. nigra* formed two distinct experimental groups. It was found that the fresh/dry weight, percent water content, chl *a* and *b* content of the moth-infested individuals of both species were significantly decreased compared with non-infested individuals; the total protein contents, lipid peroxidation and SOD, CAT, APX activities were found to be significantly increased. It was also determined that the moth-infested individuals of both species had the most sesquiterpenes and the least monoterpenes. Our results clearly show that *P. nigra* and *P. sylvestris* defend themselves when attacked by the pine processionary moth, both by changing the amount of secondary metabolites and by reacting as antioxidative defense responses.

**Keywords:** *Pinus sylvestris*, *Pinus nigra*, antioxidant enzymes, ecological parameters, pine processionary moth, secondary metabolites

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### INTRODUCTION

The genus *Pinus* L. is composed of evergreen, resinous trees or shrubs with 130 species. The buds, young needles, and female cones of the *Pinus* species contain the essential oil and resin. They are used in traditional medicine because of these properties [1]. The needle leaves are used in green paint, and the durable bodies are used in the furniture industry [2].

Many taxa that can create biotic stress prefer the food in coniferous species [3]. Of these taxa, insects are the most important organisms that conifers must contend with to survive. *Thaumetopoea wilkinsoni* Tams, 1926 (Lepidoptera, Notodontidae) is an important insect species that threatens pine species. Besides its economic and ecological damage, the insect poses a threat to human health due to its toxic hairs causing allergic reactions [4–6].

Insect activity causes biotic stress in plants; the secondary metabolites synthesized in the plants provide stress tolerance and environmental adaptation [3]. Plant secondary metabolites involved in environmental adaptation and stress tolerance are generally phenolics, flavonoids, alkaloids, and terpenoids. Terpenoids are compounds with essential properties regarding chemical ecology [3, 7]. Some terpenoids are precursors of phytohormones and plant growth regulators, photosynthetic pigments, electron carriers, and essential components of membrane structures, while others play a unique role in the plant-environment relationship. Terpenoids play an important role in abiotic stress-induced plant defense and many above-ground and below-ground biotic interactions [8]. The relationships of the secondary metabolites with organisms and their effects on ecosystem functioning are greater than expected [9].

Oxidative stress occurs as a secondary effect because of damage to plants exposed to insect infestation. The disruption of membrane integrity decreased photosynthesis, disturbances in water transport, inhibition of protein synthesis, and disruption of carbohy-

**Abbreviations:** APX—ascorbate peroxidase; CAT—catalase; H<sub>2</sub>O<sub>2</sub>—hydrogen peroxide; MDA—malondialdehyde; OH<sup>•</sup>—hydroxyl radicals; O<sub>2</sub><sup>•-</sup>—superoxide radicals; ROS—reactive oxygen species; SOD—superoxide dismutase; TBA—thiobarbituric acid; TCA—trichloroacetic acid.

drate metabolism can be counted among the effects of oxidative stress. It is known that some physiological and biochemical parameters play a role in the formation of resistance to oxidative stress in plants [10, 11]. Changes in lipid peroxidation are one of the most important indicators of stress in plants [12]. The amount of malondialdehyde (MDA) is used to determine membrane lipid peroxidation in plants. It is well known that there is a correlation between insect damage and MDA amount in the damaged plants [13, 14]. Also, the stress in plants causes the accumulation of reactive oxygen species (ROS) such as hydroxyl radicals ( $\text{OH}^-$ ), superoxide radicals ( $\text{O}_2^-$ ), and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) [14–16]. Excessive ROS accumulation causes protein oxidation, peroxidation of membrane lipids, DNA and RNA damage, and even the death of the organism [17]. Plants have developed antioxidative defense systems to combat these oxidative stressors. They increase the activity of various enzymatic and non-enzymatic antioxidants to maintain cellular homeostasis and reduce cell damage [18]. Some enzymatic components include superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) [12, 19–21].

The population density of *T. wilkinsoni* in pine species has increased significantly due to global warming that has affected the whole world recently. This further increases the extent of damage to pine species. Considering the richness and importance of pine forests worldwide and in Turkey, the damage caused by *T. wilkinsoni* and the subsequent effects of oxidative stress caused by biotic stress require a detailed investigation. Based on this, the aim of the study, i) to investigate the responses of oxidative stress in the antioxidant defense system that occurs secondary to trees infested by *T. wilkinsoni* and exposed to biotic stress, ii) to compare the secondary metabolite contents of *Pinus* samples infested by *T. wilkinsoni* and exposed to biotic stress with those of non-infested *Pinus*, iii) to determine whether the difference in secondary metabolite content is effective in insect infestation of trees.

## MATERIALS AND METHODS

**Collection of samples.** Two different *Pinus* species, *Pinus sylvestris* L. and *P. nigra* Arn., were used in our study. The leaf samples of plant species were collected from the Ondokuz Mayıs University Kurupelit Campus in Samsun, Turkey (N 41°22'26.5116", E 36°13'17.6340"), in 2020. For both species, individuals of the moth-infested and non-infested *P. sylvestris* and *P. nigra* formed two distinct experimental groups. Needle samples were collected in April 2020, at the last larval stage of *T. wilkinsoni*. The trees from which the needle samples were collected are 7 m long. 3 trees were determined for each experimental group. Needle samples were collected from each tree from 4 directions as north, south, east, and west. To eliminate the variability of the parameters to be examined, the needles of

2020 at a height of about 5 m were collected from the infested and non-infested trees. Each experiment was carried out in triplicate.

**Measurements of fresh and dry weight.** The fresh weight (FW; g) of the leaf samples collected was determined. A certain amount of needle leaves was taken from each pine tree (10 pieces) in order to be of a certain standard. The FW of these 10 needles was taken. Then, these 10 needles were kept in an oven at 70°C for 72 h, and their dry weights (DW) were weighed and recorded. Following the process, the percent water content of the samples was calculated according to the formula given below:

$$\text{Water content (\%)} = [(FW - DW)/FW] \times 100.$$

**Photosynthetic pigments.** The chlorophyll contents of the leaf samples were determined spectrophotometrically using the Arnon method [22]. A fresh leaf sample of 0.05 g was taken and homogenized in 15 mL of 80% acetone. The homogenate was filtered through the filter paper, and absorbance values at 645 and 663 nm wavelengths were measured by spectrophotometer AgileSpec (Delta Electronics Inc., China). Chl *a* and *b* content in leaf samples was determined as mg/mL using the following formulas [22]:

$$\text{chl } a = (12.7A_{663} - 2.69A_{645})(V/1000 \times W),$$

$$\text{chl } b = (22.9A_{645} - 4.68A_{663})(V/1000 \times W),$$

where *V* is a volume of 80% acetone, *W* is wet weight (g) of the extracted leaf sample.

**Measurements of the enzyme activities and the amounts of MDA.** After homogenizing 0.5 g of plant leaf sample with 10 mL of 0.1% trichloroacetic acid (TCA), the homogenate was centrifuged at 35000 g for 5 min, then 1 mL of the supernatant was taken and mixed with 0.5% thiobarbituric acid (TBA) dissolved in 4 mL of 20% TCA. After the mixture was kept at 95°C for 30 min, it was cooled in an ice bath and centrifuged at 16000 g for 10 min, and then the 1 mL of clear part was taken, and its absorbance was measured in a spectrophotometer Genesys 10S UV-VIS (Thermo Fisher Scientific, USA) at 532 and 600 nm wavelengths. The amounts of MDA were calculated using these absorbance values according to Kabay and Şensoy [23].

SOD is an important enzyme that converts superoxide anion ( $\text{O}_2^-$ ) to hydrogen peroxide and molecular oxygen. The SOD activity was determined using a SOD determination kit (19160 SOD Determination Kit, Sigma-Aldrich, USA). The SOD activity IC50 (50% inhibition activity) was determined by the colorimetric method. The isolated protein samples were prepared in three replicates into a 96-plate to determine the SOD activity. The 96-plate was then incubated at 37°C for 20 min, and the absorbance values were determined using a micro-plate reader (Thermo Fisher Scientific, USA) at 450 nm.

The CAT activity was determined using spectrophotometric monitoring of the change in absorbance due to the decomposition of hydrogen peroxide into water and oxygen by the CAT enzyme, 1 mL of a CAT reaction buffer (50 mM  $K_2HPO_4$ , 50 mM  $KH_2PO_4$  and 30 mM  $H_2O_2$ ) was added to a 20  $\mu$ L protein extract. The kinetic measurements of the samples at absorbance 240 nm were recorded at 15-s intervals for 3 min. The changes in absorbance were recorded. The total CAT activity was calculated according to Tepe and Aydemir [24].

To determine the APX activity, 1 mL of an APX reaction buffer (50 mM  $K_2HPO_4$ , 50 mM  $KH_2PO_4$ , 250  $\mu$ M ascorbic acid and 5 mM  $H_2O_2$ ) was added to a 20  $\mu$ L protein extract. The samples were kinetically measured at 290 nm wavelengths for 3 min at 15-s intervals, and the decrease in absorbance was recorded. The total APX activity was calculated as U/( $\mu$ g protein) according to Cervilla et al., [25].

**Measurement of total protein content.** The total protein content in the leaf samples was determined according to the Bradford method [26]. To determine the total protein contents, 20  $\mu$ L of enzyme solution was put on 780  $\mu$ L  $dH_2O$ . 200  $\mu$ L of 5 $\times$  Bradford solutions was added to this mixture. The mixture was kept in the dark and at room temperature for 5 min before the absorbance values were measured in triplicate in the spectrophotometer at 595 nm. The standard chart was created using 0.1 mg/mL BSA (Sigma) stock. Taking 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10  $\mu$ g of protein from the BSA stock yielded the absorbance values. The absorbance values of the standards were used to create a graph, and the concentrations of the samples with unknown concentrations were calculated using the formula obtained from this graph ( $y = 0.042x + 0.033$ ).

**Analysis of the secondary metabolite content.** *Pinus* samples for secondary metabolite analysis were collected on the same day and at the same time of the day and analyzed simultaneously. The secondary metabolite composition of the needle samples was determined by a GC/MS (Shimadzu GCMS-QP2010 Ultra, injector port: 250°C, detector: 280°C, split rate: 25, Helium gas flow: 1.44 mL/min, injected volume: 0.1 mL, 1% solution (diluted in hexane), detector voltage: 70 eV), and a Rxi-5ms capillary column (30 m  $\times$  0.25 mm ID, film thickness of 0.25 mm) was used. The Rxi-5ms capillary column was connected to the mass spectrometer. A sample (1 g) was placed in a 40 mL vial and heated for 30 min at 50°C. The fiber (Supelco 57328-U) was then inserted into the vial and heated for 30 min at 50°C again before being read into the GC-MS device. Essential oil analyzes were made qualitatively (Table 1). Qualitative analysis of the samples was performed using NIST 2020 and Wiley 12th edition libraries integrated into the GC-MS instrument.

**Data analysis.** The experimental groups of *P. sylvestris* and *P. nigra* samples infested by *T. wilkinsoni* were

compared with the non-infested experimental groups in our study. Each study with experimental groups was conducted in 3 repetitions. One-Way ANOVA (Graph-Pad program) analysis of variance was used for statistical analysis firstly, and then Duncan multiple comparison tests (SPSS 21.0) were used.

## RESULTS

### *Biometric Parameters*

The fresh and dry weights of the leaves of the moth-infested and non-infested *P. sylvestris* and *P. nigra* were compared. In both species, it was determined that the FW and DW of the non-infested individuals were significantly higher than those of the moth-infested individuals ( $P < 0.05$ ) (Fig. 1). The FW of the non-infested *P. sylvestris* samples was  $0.787 \pm 0.067$  g, while the FW of the moth-infested samples was  $0.500 \pm 0.026$  g. The DW of the non-infested samples was  $0.420 \pm 0.053$  g, whereas the moth-infested samples had a DW of  $0.220 \pm 0.044$  g. The FW of the non-infested *P. nigra* samples was  $0.909 \pm 0.017$  g, while the FW of the moth-infested samples was  $0.755 \pm 0.011$  g. While the DW of the non-infested samples was  $0.476 \pm 0.019$  g, the moth-infested samples had a DW of  $0.417 \pm 0.016$  g.

When the water contents (%) of leaf samples of moth-infested and non-infested *P. sylvestris* and *P. nigra* species were compared, it was determined that the water contents of the infested samples of both species were less than the non-infested ones (Fig. 2).

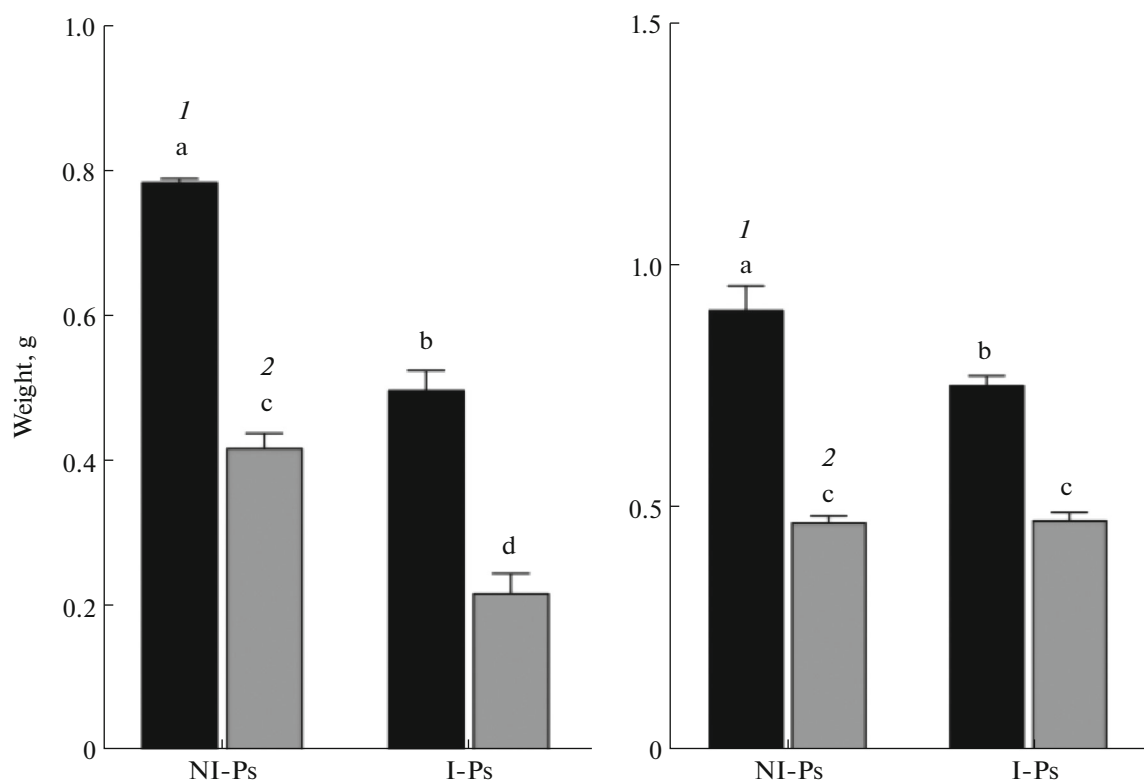
### *The Secondary Metabolite Content*

There were four main essential oil groups in the *P. sylvestris* and *P. nigra* species used in our study: monoterpenes, monoterpenoids, sesquiterpenes, and sesquiterpenoids (Fig. 3). The essential oil contents of the moth-infested and non-infested *Pinus* species are shown in Table 1. Accordingly, the non-infested *P. sylvestris* species had the most sesquiterpenes (53.96%) (Fig. 3). Among the sesquiterpenes,  $\alpha$ -Pinene was the most abundant (3.2%), while 3- $\delta$ -Carenene was the least one (Table 1). Monoterpenes were the least common essential oil group of this type (6.61%) (Fig. 3). Aromadendrene was the most abundant monoterpene (25.27 percent), while the least one was  $\beta$ -Elemene (0.87%) (Table 1). Sesquiterpenoids were the most abundant (52.79%) essential oil group in the *P. sylvestris*, in which the insects were actively fed, whereas monoterpenes were the least abundant (5.5%) (Fig. 3). Among the sesquiterpenoids,  $\alpha$ -Himachalene had the highest rate (50.36%), whereas caryophyllene oxide had the lowest (0.97%) (Table 1). The moth-infested *P. nigra* had the highest concentration of sesquiterpenes with 42.25% and the lowest concentration of monoterpenes with 4.31%. The sesquiterpene with the highest rate was  $\gamma$ -Cadinene with 15.01%, and the lowest rate was  $\beta$ -Bourbonene with 0.54%. Among the

**Table 1.** Volatile organic compounds identified using GC-MS analysis in the infested and non-infested *Pinus sylvestris* and *Pinus nigra* species

No.	Compounds	Area (%)			
		NI-Ps	I-Ps	NI-Pn	I-Pn
1	$\alpha$ -Pinene	3.2 $\pm$ 0.001	1.82 $\pm$ 0.003	1.42 $\pm$ 0.001	34.86 $\pm$ 3.0
2	Sabinene	1.1 $\pm$ 0.0002	0.79 $\pm$ 0.0001	–	–
3	$\beta$ -Pinene	0.94 $\pm$ 0.004	0.73 $\pm$ 0.0001	2.33 $\pm$ 0.005	5.55 $\pm$ 0.03
4	3- $\delta$ -Carene	0.6 $\pm$ 0.0007	0.28 $\pm$ 0.00003	–	–
5	P-Cymene	–	0.67 $\pm$ 0.0002	–	–
6	Limonene	–	0.51 $\pm$ 0.0002	–	1.15 $\pm$ 0.002
7	Tripinolene	0.77 $\pm$ 0.0003	0.7 $\pm$ 0.0003	–	–
8	Pulegone	–	–	–	0.37 $\pm$ 0.0001
9	Ethyl chrysanthemumate	–	–	0.56 $\pm$ 0.0001	0.97 $\pm$ 0.0005
10	Terpinen-4-ol	–	2.25 $\pm$ 0.1	0.7 $\pm$ 0.0005	–
11	$\alpha$ -Terpinyl acetate	–	3.55 $\pm$ 0.3	6.67 $\pm$ 0.8	–
12	$\alpha$ -Terpineol	–	–	0.72 $\pm$ 0.0003	–
16	Nerol	–	–	–	0.54 $\pm$ 0.0002
17	Valencene	1.3 $\pm$ 0.0005	0.54 $\pm$ 0.0003	–	–
18	$\alpha$ -Humulene	6.53 $\pm$ 0.7	9.74 $\pm$ 0.3	8 $\pm$ 1.6	2.25 $\pm$ 0.003
19	$\gamma$ -Cadinene	3.69 $\pm$ 0.2	4.05 $\pm$ 0.8	15.01 $\pm$ 2.1	8.97 $\pm$ 0.1
20	$\alpha$ -Muurolene	5.48 $\pm$ 0.4	4.5 $\pm$ 0.2	3.88 $\pm$ 0.7	2.92 $\pm$ 0.002
21	$\delta$ -Cadinene	4.39 $\pm$ 0.3	4.66 $\pm$ 0.5	11.9 $\pm$ 1.0	4.98 $\pm$ 0.02
22	$\beta$ -Elemene	0.87 $\pm$ 0.0005	–	1.04 $\pm$ 0.004	–
23	$\alpha$ -Cubebene	6.43 $\pm$ 0.02	–	0.71 $\pm$ 0.0002	1.95 $\pm$ 0.08
24	Germacrene B	–	–	1.17 $\pm$ 0.02	–
25	$\beta$ -Bourbonene	–	–	0.54 $\pm$ 0.0007	–
26	Aromadendrene	25.27 $\pm$ 1.3	–	–	12.3 $\pm$ 1.6
28	$\alpha$ -Himachalene	–	50.36 $\pm$ 2.8	30.17 $\pm$ 2.1	–
29	Cyclosativene	–	1.46 $\pm$ 0.001	4.19 $\pm$ 0.3	2.02 $\pm$ 0.0007
30	Viridiflorol	0.71 $\pm$ 0.0001	–	–	–
31	Caryophyllene oxide	10.41 $\pm$ 0.8	0.97 $\pm$ 0.0004	0.73 $\pm$ 0.0002	7.06 $\pm$ 0.2
32	Spathulenol	–	–	–	0.45 $\pm$ 0.0005
33	Phenethyl alcohol	2.38 $\pm$ 0.1	–	–	0.69 $\pm$ 0.006
34	2.5 dimethyl Furan	–	–	–	0.87 $\pm$ 0.002
35	Citronellyl butyrate	0.7 $\pm$ 0.0005	–	–	–
36	Civetone	0.97 $\pm$ 0.0004	–	–	–
37	10 $\beta$ H-Cadina-1(6).4-diene	0.61 $\pm$ 0.0008	0.53 $\pm$ 0.0005	2.35 $\pm$ 0.02	0.59 $\pm$ 0.0001
38	Phenethyl 2-methylbutyrate	1.22 $\pm$ 0.06	1.74 $\pm$ 0.001	1.3 $\pm$ 0.0006	–
39	Phenethyl isovalerate	10.7 $\pm$ 0.4	7.95 $\pm$ 0.9	3.56 $\pm$ 0.5	0.53 $\pm$ 0.0001
40	Acetoin	2.54 $\pm$ 0.1	–	0.67 $\pm$ 0.0003	2.46 $\pm$ 0.2
41	Capronaldehyde	0.8 $\pm$ 0.0002	–	–	1.9 $\pm$ 0.003
43	Pelargonaldehyde	–	–	–	0.68 $\pm$ 0.0003
44	$\alpha$ -Copaene	1.21 $\pm$ 0.03	2.18 $\pm$ 0.2	2.38 $\pm$ 0.4	0.61 $\pm$ 0.0004
46	Diethyl phthalate	2.19 $\pm$ 0.1	–	–	1.58 $\pm$ 0.002
47	2.6-Nonadienal. (E.E)	0.67 $\pm$ 0.0002	–	–	–
	Total	100	99.98	100	100

Means ( $n = 3$ )  $\pm$  SD are shown. NI-Ps—non-infested *P. sylvestris*, I-Ps—infested *P. sylvestris*, NI-Pn—non-infested *P. nigra*, I-Pn—infested *P. nigra*.



**Fig. 1.** Fresh (1) and dry (2) weights of leaf samples of moth-infested and non-infested *Pinus* species. NI-Ps—non-infested *P. sylvestris*, I-Ps—infested *P. sylvestris*, NI-Pn—non-infested *P. nigra*, I-Pn—infested *P. nigra*. Means ( $n = 3$ )  $\pm$  SD are shown. Different letters above the bars indicate statistically significant differences at 0.05 levels in Duncan's multiple range tests.

monoterpenes,  $\beta$ -Pinene had the highest rate of 2.33%, while ethyl chrysanthemumate had the lowest rate of 0.56% (Table 1). Monoterpenes were the most abundant essential oil group (42.91%) in the moth-infested *P. nigra* species. The most abundant monoterpene was  $\alpha$ -Pinene with 34.86%, and the least one was pulegone with 0.37%. Monoterpenoids, which contained only nerol (0.54%), were the least common essential oil group in *P. nigra* (Table 1).

#### Photosynthetic Pigments

When the experimental groups of the moth-infested and non-infested *P. sylvestris* and *P. nigra* species were compared, it was found that the contents of chl *a* and *b* were significantly lower in the moth-infested experimental groups ( $P < 0.05$ ) (Fig. 4). The content of chl *a* and *b* in the leaf samples of the non-infested *P. sylvestris* were  $1.272 \pm 0.027$  mg FW and  $0.937 \pm 0.069$  mg FW, respectively. The content of chl *a* and *b* of the samples in the moth-infested experimental group were determined to be  $0.965 \pm 0.021$  mg FW and  $0.660 \pm 0.013$  mg FW, respectively. While the contents of chl *a* and *b* of the leaf samples of the non-infested *P. nigra* were  $2.076 \pm 0.093$  mg FW and  $2.524 \pm 0.065$  mg FW, respectively; the contents in the moth-infested experimental group were determined to be

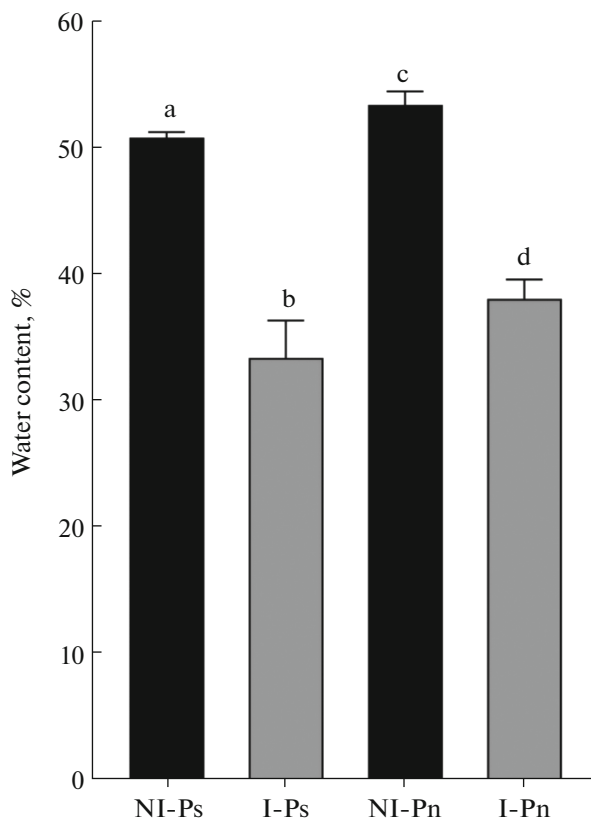
$1.087 \pm 0.021$  mg FW and  $0.587 \pm 0.014$  mg FW, respectively (Fig. 4).

#### MDA Abundance

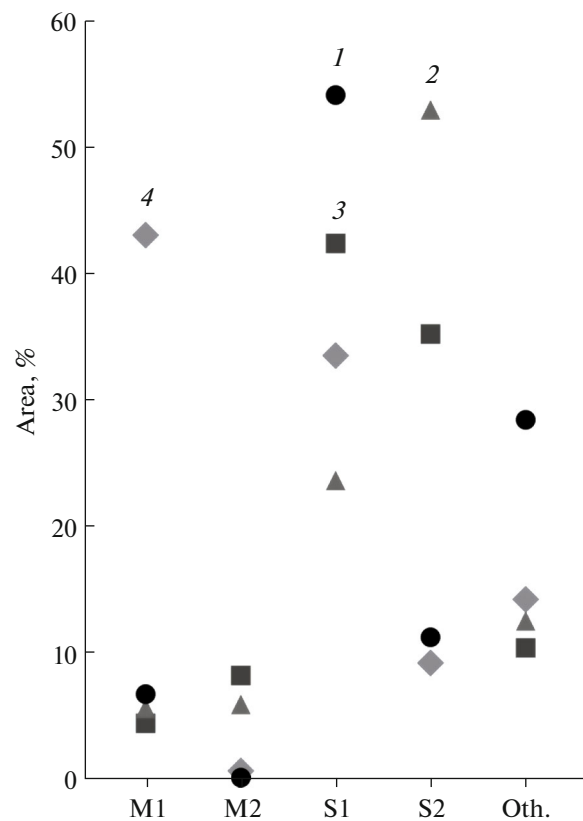
When the amounts of MDA indicating lipid peroxidation, one of the key markers of stress in plants, were compared, it was determined that the amounts of MDA in both the non-infested *P. sylvestris* and *P. nigra* were significantly lower than those of the moth-infested experimental groups ( $P < 0.05$ ) (Fig. 5a). The amount of MDA in the non-infested *P. sylvestris* was  $1.022 \pm 0.014$  nmol/mL, while it was  $2.324 \pm 0.011$  nmol/mL in the moth-infested experimental group. While the amount of MDA in the non-infested *P. nigra* was  $1.053 \pm 0.037$  nmol/mL, the amount in the moth-infested experimental group was  $1.686 \pm 0.010$  nmol/mL.

#### The Total Protein Contents

The total protein content of the non-infested *P. sylvestris* showed a statistically significant increase compared with that of the moth-infested ( $P < 0.05$ ) (Fig. 5b). The total protein content of the non-infested individuals was  $659.726 \pm 20.179$  mg/mL, while the total protein content of the moth-infested experimental group was  $469.315 \pm 56.961$  mg/mL.



**Fig. 2.** Water content of leaf samples of moth-infested and non-infested *Pinus* species. NI-Ps—non-infested *P. sylvestris*, I-Ps—infested *P. sylvestris*, NI-Pn—non-infested *P. nigra*, I-Pn—infested *P. nigra*. Means ( $n = 3$ )  $\pm$  SD are shown. Different letters above the bars indicate statistically significant differences at 0.05 levels in Duncan's multiple range tests.



**Fig. 3.** Secondary metabolite compound groups of moth infested and non-infested *Pinus* species NI-Ps—non-infested *P. sylvestris* (1), I-Ps—infested *P. sylvestris* (2), NI-Pn—non-infested *P. nigra* (3), I-Pn—infested *P. nigra* (4); M1—Monoterpenes, M2—Monoterpenoids, S3—Sesquiterpenes, S4—Sesquiterpenoids, Oth—others; means ( $n = 3$ )  $\pm$  SD are shown. Different letters above the bars indicate statistically significant differences at 0.05 levels in Duncan's multiple range tests.

When the total protein contents in the moth-infested and non-infested *P. nigra* were compared, the protein content of the non-infested experimental group was higher than that of the moth-infested experimental group ( $718.630 \pm 71.628$ ,  $638.265 \pm 70.815$  mg/mL, respectively), although there was no statistically significant difference ( $P < 0.05$ ).

#### Antioxidant Enzyme Activities

When the activities of SOD, CAT, and APX of the experimental groups of *P. sylvestris* and *P. nigra* were compared, it was determined that all three enzyme activities showed a statistically significant increase in the experimental groups of the moth-infested individuals compared with the non-infested groups ( $P < 0.05$ ) (Fig. 6). While SOD activity was  $33.921 \pm 6.473$  U/(mg protein) in the non-infested individuals and the activity was  $61.391 \pm 4.840$  U/(mg protein) in the moth-infested individuals of *P. sylvestris*; it was determined to be  $28.597 \pm 2.774$  U/(mg protein) in the non-infested individuals and  $41.499 \pm 5.261$  U/(mg protein)

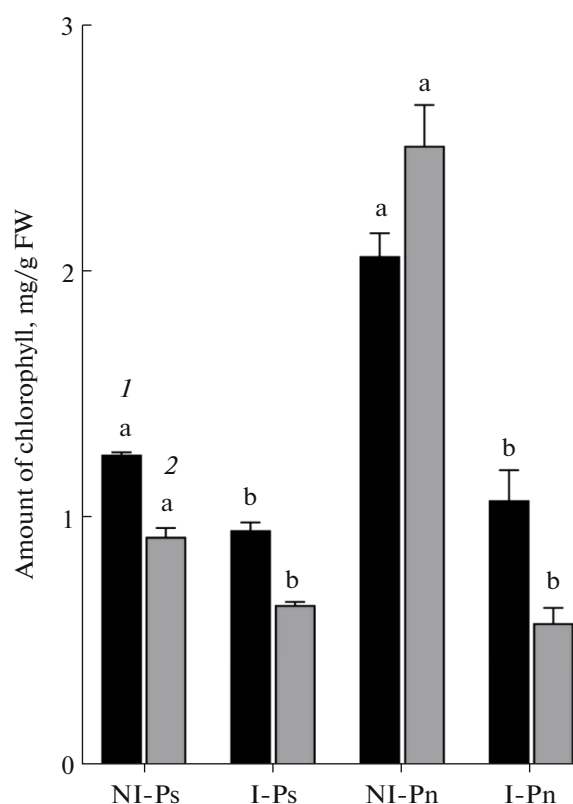
in the moth-infested *P. nigra* individuals (Fig. 6a). CAT activity was  $0.0070 \pm 0.0009$  U/(mg protein) in the non-infested individuals and  $0.0109 \pm 0.0007$  U/(mg protein) in the moth-infested *P. sylvestris* individuals; additionally, the enzyme activity was  $0.0064 \pm 0.0007$  U/(mg protein) in the non-infested individuals and  $0.0097 \pm 0.0005$  U/(mg protein) in the moth-infested *P. nigra* individuals (Fig. 6b). The APX activity was  $0.011 \pm 0.0009$  U/(mg protein) in the non-infested individuals and  $0.017 \pm 0.005$  U/(mg protein) in the moth-infested *P. sylvestris* individuals; however, it was determined that the APX activity was  $0.006 \pm 0.00012$  U/(mg protein) in the non-infested individuals and  $0.0097 \pm 0.00008$  U/(mg protein) in the moth-infested individuals of *P. nigra* (Fig. 6c).

#### DISCUSSION

The pine processionary moth causes various damages to the tree species on which it feeds, and these

damages are particularly pronounced and effective on trees in afforestation and natural regeneration areas. Epidemics are especially common in young pines; they cause sapling deformities and stunted growth in seedlings [27]. The pine processionary moth, which causes biotic stress, has a negative impact on plant ecological and physiological characteristics in addition to morphological deformations [28, 29]. Moreover, the secondary effect of the destruction caused by the pine processionary moth is the occurrence of abiotic stress [30, 31]. A significant decrease was detected in the fresh-dry weight and percent water contents of the leaves in the moth-infested individuals in our study ( $P < 0.05$ ). The decrease in the water contents, which is one of the most significant effects of insect damage, causes the plants to dry out by interfering with all metabolic events in the plant. Recent studies have focused on the changes caused by the lack of water in the plant in photosynthetic metabolism and secondary metabolite profile [32]. Although there have been studies in the literature regarding the fresh-dry weight and water contents of the leaves of various trees, there has been no study that correlates insect infestation with these ecological characteristics [33].

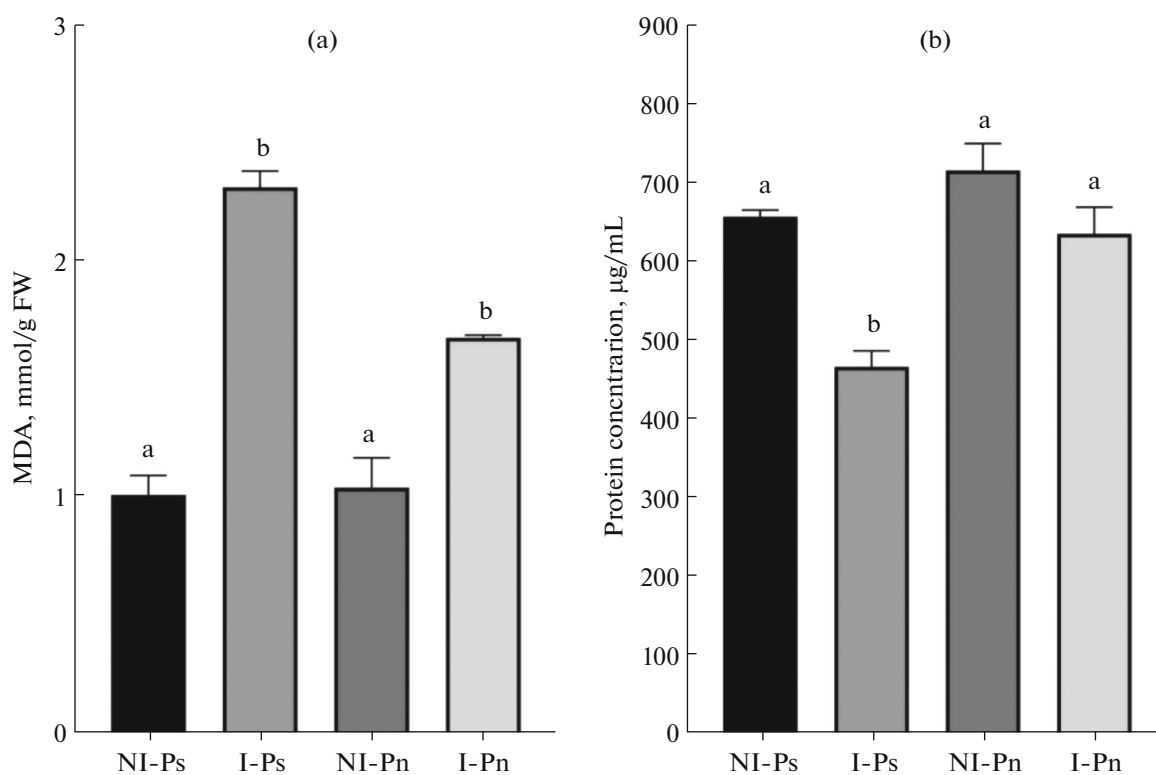
Plant secondary metabolites are a diverse group of chemical compounds produced by most plant species in response to stress. The secondary metabolites, unlike primary metabolites, are metabolites that are not required for the growth, development, and reproduction of an organism [9]. However, they play a central role in the defense of plants against insects and pathogens. There are numerous studies on the secondary metabolite contents of *Pinus* species [1, 8, 34]. In our study, when the GC-MS analysis results of infested and non-infested individuals are compared, it is seen that there is a remarkable increase in monoterpenoids and sesquiterpenoids in *P. sylvestris* and monoterpenes in *P. nigra*. These results suggest that *P. sylvestris* and *P. nigra* try to cope with herbivorous attacks by increasing the synthesis of these essential oils. The secondary metabolite contents of the moth-infested and non-infested *P. sylvestris* and *P. nigra* species were determined in our study. From monoterpenes, monoterpenoids, sesquiterpenes, and sesquiterpenoids, 47 different secondary metabolites were determined. According to our findings, there was a remarkable increase in monoterpenoids and sesquiterpenoids in the moth-infested *P. sylvestris* compared to the non-infested individuals and in monoterpenoids in the moth-infested *P. nigra* individuals. The results suggest that *P. sylvestris* species try to cope with herbivore attack using monoterpenoids and sesquiterpenoids, whereas *P. nigra* uses monoterpenes. The secondary metabolite contents of the non-infested *P. sylvestris* and *P. nigra* species were similar. The most common essential oil compounds in both species were in the sesquiterpene group, and the least common were in the monoterpene group. The most abundant and the least abundant essential oil compounds in the



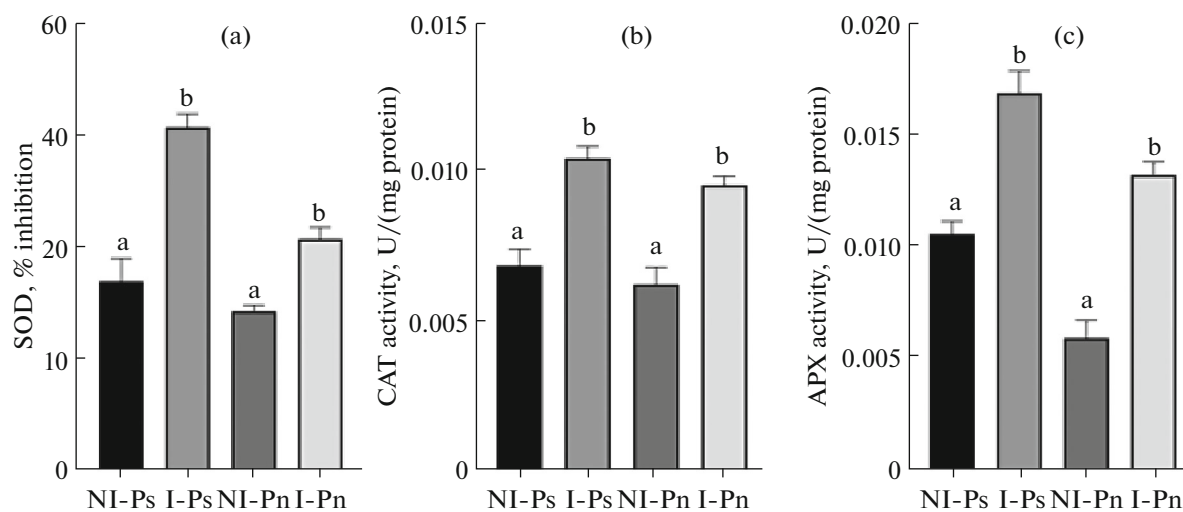
**Fig. 4.** Chlorophyll *a* (1) and *b* (2) levels of moth-infested and non-infested *Pinus* species. NI-Ps—non-infested *P. sylvestris*, I-Ps—infested *P. sylvestris*, NI-Pn—non-infested *P. nigra*, I-Pn—infested *P. nigra*. Means ( $n = 3$ )  $\pm$  SD are shown. Different letters above the bars indicate statistically significant differences at 0.05 levels in Duncan's multiple range tests.

moth-infested *P. sylvestris* and *P. nigra* were in different groups. Similar essential oil compounds were found in the literature in parallel with our findings, but the rate of their presence in the plant was determined to be [1, 8, 35].

*Pinus* species are severely damaged by insect infestation as well as by factors such as drought and anthropogenic activities. *T. wilkinsoni*, which prefers *Pinus* species for active nutrition, damages the leaves of the plant. The damage to the leaves negatively affects the content of chl *a* and *b*, which play a role in photosynthesis. The high levels of photosynthetic pigment indicate that the plant's light absorption capacity and organic matter synthesis are high. The insect infestation decreases photosynthetic pigments [36]. Photosynthetic pigments, stems, and shoots of *P. yunnanensis* infested by *T. yunnanensis* and *T. minor* were compared with non-infested *P. yunnanensis* individuals [14]. As a result of the study, they reported that the contents of chlorophyll *a* and chlorophyll *b* decreased in the shoots and stems of the infested individuals of both *Tomiscus* species compared with the non-infested individuals. Besides, in a study, in which the contents of



**Fig. 5.** MDA amount (a) and protein concentration (b) of leaf samples of moth-infested and non-infested *Pinus* species. NI-Ps—non-infested *P. sylvestris*, I-Ps—infested *P. sylvestris*, NI-Pn—non-infested *P. nigra*, I-Pn—infested *P. nigra*. Means ( $n = 3$ )  $\pm$  SD are shown. Different letters above the bars indicate statistically significant differences at 0.05 levels in Duncan's multiple range tests.



**Fig. 6.** The enzyme activities of leaves of moth-infested and non-infested *Pinus* species. (a) The SOD activities, (b) the CAT activities, and (c) the APX activities of leaves of moth-infested and non-infested *Pinus* species. NI-Ps—non-infested *P. sylvestris*, I-Ps—infested *P. sylvestris*, NI-Pn—non-infested *P. nigra*, I-Pn—infested *P. nigra*. Means ( $n = 3$ )  $\pm$  SD are shown. Different letters above the bars indicate statistically significant differences at 0.05 levels in Duncan's multiple range tests.

chlorophyll a and chlorophyll b in leaves of two different varieties of *P. pineaster* were investigated, the content of chlorophyll in the Maghrebiana cultivar was found to be higher than that in the Renoui cultivar [37]. The results obtained in our study supported the studies in the literature. It was determined that the contents of chl *a* and *b* decreased significantly in the moth-infested experimental groups of *P. nigra* compared with the non-infested experimental groups ( $P < 0.05$ ).

MDA is a marker of membrane lipid peroxidation and one of the most significant indicators of membrane damage. Studies on lipid peroxidation are extremely limited in the literature. In a study conducted with shoots and stem of *P. yunnanensis* infested by two different *Tomicus* species, it was found that both species caused serious damage to shoots and stem; therefore, the amounts of MDA in both shoots and phloem were significantly increased compared with uninfested individuals [14]. We found similar findings in our study. The amounts of MDA in *P. sylvestris* and *P. nigra* leaves exposed to the pine processionary moth infestation were found to be significantly higher than those of the non-infested individuals. The results indicate that the pine processionary moth, a biotic stress factor, causes abiotic stress in the plant due to the damage it causes.

The insect infestation reduces protein content in plants [38]. The total protein contents were found to be lower in the experimental groups of both *P. sylvestris* and *P. nigra* species compared with those of the non-infested individuals in the current study. While the decrease in *P. sylvestris* was statistically significant, the decrease in *P. nigra* was determined to be insignificant. The study conducted by [14] supported our findings. The total protein contents in both leaf and stem phloems were found to be lower in *P. yunnanensis* infested by *Tomicus yunnanensis* and *T. minor*. It has been stated that this situation significantly impairs the health of the plant, depending on the time of exposure and the degree of injury [14]. In another study by Skwarek et al. [39], it was reported that the total protein contents were lower in the leaf and root samples taken from *P. sylvestris* and *Larix decidua* species infested by *Melolontha melolontha* at the third and sixth weeks, and the samples were damaged compared with the control group.

Abiotic stress in plants causes an excessive accumulation of toxic components in cells. The development of stress tolerance is based on the activation of antioxidant enzyme systems that can neutralize the reactive oxygen species that are continuously produced in response to stress [40]. Antioxidative systems, such as SOD, CAT and APX activity, increase to eliminate ROS and prevent plant cells [12]. There are very few studies in the literature on antioxidant defense systems in trees exposed to insect infestation. [14] investigated the activities of SOD, CAT and POD in the leaves and stems of *P. yunnanensis* infested by *T. yun-*

*nanensis* and *T. minor*. As a result of their studies, it was reported that the increase in all three enzyme activities was significant compared with the non-infested individuals, and the activities of SOD and CAT in leaves were sensitive to insect infestation. In another study, it was stated that the leaves of *P. sylvestris* and *L. decidua* seedlings infested by *M. melolontha* had higher activities of SOD and POD than non-infested seedlings and that these enzymes played a direct role in the plant's response to *M. melolontha* damage [39]. In our study, in parallel with the literature, it was determined that the activities of SOD, CAT, and APX of both moth-infested *Pinus* species were significantly increased compared with the non-infested experimental groups. The increase in all three enzymes was found to be statistically significant.

Because of changing ecological conditions, there is a growing interest in biotic-abiotic stress interactions in plants. The changes in ecological and physiological characteristics that occurred in *P. sylvestris* and *P. nigra* depending on the plant-insect relationship were determined in this study. It was found that the fresh-dry weight, percent water contents, and the contents of chl *a* and *b* of the moth-infested individuals of both species were significantly decreased compared with the non-infested individuals; additionally, the total protein contents, MDA amounts, and the activities of SOD, CAT, and APX were determined to be significantly increased. The remarkable increase in monoterpenoids and sesquiterpenoids in infested *P. sylvestris* and a remarkable increase in monoterpenes in *P. nigra* reveal that both species can develop defenses against the herbivorous insect by using secondary metabolites. The increase in different secondary metabolites even in these two species against herbivore attack suggests that plants can use their secondary metabolite defense power in different ways.

#### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

#### COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving animals or human participants as objects of research.

#### AUTHOR CONTRIBUTIONS

SL and OY contributed to the study design; SL, FDB, EFT and OY performed the data analyzes. All authors helped the original draft of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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