Anatolian Journal of Botany
9(2): 141-149 (2025)
doi:10.30616/ajb.1701359

Research article

Gall wasps change the biochemical composition of *Eucalyptus* leaves

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Received : 17.05.2025
Accepted : 08.08.2025
Online : 26.08.2025

Gal arıları *Eucalyptus* yapraklarının biyokimyasal kompozisyonunu değiştirir

Abstract: Determining the effects of gall wasps on leaf biochemistry may contribute to the understanding of damage mechanisms. The study was carried out in eucalyptus plantations in the coastal regions of the Northeastern Mediterranean (Tarsus/Mersin/Turkey). Biochemical analyses were performed on *Eucalyptus camaldulensis* leaves infested and uninfested with two different gall wasps *(Leptocybe invasa* and *Ophelimus maskelli*) and the data were compared. The flavonoid content of the leaves reduced 16.5% in leaves infested with *L. invasa* and 33.7% with *O. maskelli*. Total antioxidant capacity decreased with *O. maskelli* infestation but did not change with *L. invasa* infestation. Copper ion reduction capacity increased significantly with both pest infestations. Ascorbic acid increased by 87% in *L. invasa* infestation and 120% in *O. maskelli* infestation compared to control. Superoxide generation rates of noninfested and infested leaves from the infested trees were found to be higher than those of the control samples. Differences in the biochemical composition of infested and non-infested leaves of infested plants were determined. The invasion of gall wasps triggers oxidative stress by increasing the rate of superoxide production in eucalyptus leaves.

Key words: Antioxidant, insect pest, *Leptocybe invasa, Ophelimus maskelli,* photosynthetic pigments

Özet: Gal arılarının yaprak biyokimyası üzerindeki etkilerinin belirlenmesi, zarar mekanizmalarının anlaşılmasına katkı sağlayabilir. Çalışma Kuzey Doğu Akdeniz (Tarsus/Mersin/Turkiye) sahil bölgelerindeki okaliptus plantasyonlarında gerçekleştirildi. İki farklı gal arısı (*Leptocybe invasa* ve *Ophelimus maskelli)*, ile istila edilmiş ve edilmemiş *Eucalyptus camaldulensis* yapraklarında biyokimyasal analizler yapıldı ve veriler karşılaştırıldı. Yaprakların flavonoid içeriğinde *L. invasa* istilasında %16,5, *O. maskelli* istilasında %33,7 azalma belirlendi. Toplam antioksidan kapasite, *O. maskelli* istilasıyla azaldı ancak *L. invasa* istilasıyla değişmedi. Bakır iyonu indirgeme kapasitesi, her iki pest istilasında da önemli ölçüde arttı. Askorbik asit, *L. invasa* istilasında kontrole göre %87 ve *O. maskelli* istilasında %120 arttı. İstila edilmiş ağaçlardan istila edilmemiş ve istila edilmiş yaprakların süperoksit üretim oranlarının kontrol örneklerinden daha yüksek olduğu bulundu. İstila edilmiş bitkilerin istila edilmiş ve edilmemiş yapraklarının biyokimyasal kompozisyonunda farklılıklar belirlendi. Gal arılarının istilası, okaliptüs yapraklarında süperoksit üretim oranını artırarak oksidatif stresi tetiklemektedir.

Anahtar Kelimeler: Antioksidan, böcek pest, *Leptocybe invasa*, *Ophelimus maskelli*, fotosentetik pigment, süperoksit dismutaz

Citation: Aytar F, Keleş Y (2025). Gall wasps change the biochemical composition of *Eucalyptus* leaves. Anatolian Journal of Botany 9(2): 141-149.

1. Introduction

The genus *Eucalyptus* L'Hér. includes about 800 tree species common in tropical, subtropical, and temperate regions. Since it contains medicinal and aromatic compounds, in addition to those grown in the natural environment, it is also cultivated by humans (Sahin Basak and Candan, 2010). *Eucalyptus* leaf extracts are of economic importance as they are used in medicine and cosmetics. Fast-growing eucalyptus trees also have an important place in the forestry industry. Eucalyptus species are grown for wood and paper production as well as for biofuel production. (Hinchee et al., 2011).

Eucalyptus species originating from Australia can easily adapt to temperate climatic regions and can be grown with high productivity. However, these trees are adversely affected by pests and pathogens that are due to their natural characteristics or that come from the environment in which they are grown. (Wingfield et al., 2013). Herbivorous insects that cause damage to eucalyptus species are polyphage pests that either spread from Australia to other regions or migrated from native Myrtaceae species to eucalyptus species with similar anatomic and metabolic features (Paine et al., 2011).

Insect pests *Leptocybe invasa* Fisher & La Salle (Blue gum chalcid, *Hymenoptera: Eulophidae*) (Billings, 2011) and *Ophelimus maskelli* (Ashmead) (*Hymenoptera: Eulophidae*) (Floris et al., 2018) can cause loss of productivity by causing damage to eucalyptus leaf tissues. The single Australian insect that migrated to Asia and caused damage to eucalyptus trees is *L. invasa*, which emerged after 2002 (Zhang et al., 2021). *L. invasa* produces galls that inflate the stem, petiole, and midrib (Mendel et al., 2004). The female wasp stabs and lays her eggs on the upper part of the leaves. The larvae develop in the gall, pupate, and the adults burrow out and are released. By forming galls twice a year, at the beginning and end of summer, it causes injury, weakening, and stunting, especially of young trees. In severe infestation, wasp attacks can completely stop growth (Billings, 2011). *Ophelimus maskelli* (Badmin, 2008), one of the common species of the European wasp fauna, has been recorded as a pest of *E. camaldulensis* Dehnh in many countries in the Mediterranean basin. In contrast to *L. invasa*, galls caused by *O. maskelli* only occur on the upper surface of the eucalyptus leaf lamina. It is typically observed as round button-like projections (Protasov et al., 2007). Under favorable environmental conditions, the surface of infested leaves can become completely covered with galls (Branco et al., 2016). *Ophelimus maskelli* populations in the Mediterranean basin reach their highest point in early spring (Floris et al., 2018; Adel-Sellami et al., 2025).

In addition to the characteristics of the invading insects and environmental factors, the established and induced defense systems of the host plant may also be decisive in the emergence of virulence (Naidoo et al., 2014). The lines of defense that protect plants from pests and pathogens are mechanical barriers such as bark, wall and leaf cuticles, protective secretions, and toxic secondary metabolites, respectively (Glazebrook, 2005). When pests and pathogens overcome all these lines of defense and cause damage to cells, resistance responses are stimulated by activating PR genes through hormonal regulation and the production of signaling compounds such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Mithöfer and Boland, 2008). Since ROS and RNS accumulation caused by pest invasion may cause structural and metabolic damage, they should be eliminated by antioxidant defense compounds (ADC) and antioxidant enzyme activities (AEA). Antioxidant defense requires the use of compounds such as ascorbate, glutathione, phenolics, carotenoids, tocopherols, and activities of enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate perozidase (APX), and glutathione reductase (GR) (Hakiman and Maziah, 2009).

Threats from insect pests can be reduced by planting tolerant eucalyptus genotypes (hybrids) or by using biological control methods (Dittrich-Schröder et al., 2012). The resistance of forest trees to pests can be gained by genetic, biochemical, and physiological modifications such as the formation of genetic variations, the development of immune response, plasticity, and interaction with environmental conditions (Naidoo et al., 2014; Oates et al., 2015; Mhoswa et al., 2020). Studies are showing that eucalyptus leaves have a high antioxidant capacity (Elansary et al., 2017). A comparison of the biochemical compositions of uninfested and infested leaves may be useful to elucidate the roles of ADC and AEA in protecting eucalyptus leaves against attack by insect pests. This study was planned to determine the biochemical changes in the infested and uninfested leaves of *E. camaldulensis* infested with two different gall wasps.

2. Materials and Method

2.1. Material

Eucalyptus camaldulensis Dehnh. leaf samples were taken from the trees grown from cuttings obtained from the "Eucalyptus Clone Garden" of the Eastern Mediterranean Forestry Research Institute Karabucak / Mersin / Turkiye. Karabucak region is located on the coast of the Mediterranean. The average elevation of the region is 50 m (Geographical Coordinate: 36°52'42.25"N, 34°52'55.43" W). Mediterranean climate prevails in the region. The annual average temperature is 19˚C, the relative humidity is 67% and the annual precipitation is 503 mm. Eucalyptus leaves were collected on September 10, 2020, from trees infested by gall wasps *Leptocybe invasa* Fisher & La Salle and *Ophelimus maskelli* (Ashmead). Infested (IL) and noninfested (NL) leaf samples were collected from the same infested trees (IT). Control samples were taken from noninfested trees (NT). In the collection, leaves were sampled from 3 different individuals of each species in 3 repetitions. The leaves were randomly selected from the leaves that had completed their development on the last shoot. The leaves, which were brought to the laboratory for surface cleaning, were dried in a lyophilizer and ground in the mill, and kept at 4˚C in plastic containers until analysis.

2.2. Methods

2.2.1. Soluble Carbohydrates

100 mg leaf sample was homogenized in 10 mL 80% ethanol with homogenizer for 1 min, centrifuged at 10,000 g for 10 min and the supernatant was diluted 1/100 fold. Then 2 mL of anthron reagent was added to 1 mL of the extract and incubated at 100°C for 5 min for glucose, at 40°C 30 min for fructose. The absorbance measurement of the cooled mixture was carried out at 620 nm. The amount of glucose and fructose was calculated from the curve formed with the glucose and fructose standards (Halhoul and Kleinberg, 1972).

2.2.2. Free Proline

100 mg leaf material was extracted for 24 h at room temperature in 10 mL of 3% 5-sulfosalicylic acid solution. The extracts were centrifuged at 5000 g for 5 min. 2 mL of each of the ninhydrin reagent, glacial acetic acid, and the extracts were mixed in a test tube and incubated at 100°C for 60 min, then cooled and vortexed by adding 4 mL of cold toluene. The absorbance of the toluene phase at 520 nm was measured and the amount of proline was determined from the curve formed with the proline standard (Bates et al., 1973).

2.2.3. Soluble Phenolics

200 mg leaf material in 20 mL of methanol and 1 mL of 1% $NaHSO_3$ were mixed with vortex for 2 min. The methanol and extract were incubated in a water bath set at 75°C for 3 min. After filtration through Whatman no. 1 filter paper, methanol was eliminated from the filtrate by evaporation in a vacuum. Total soluble phenolics in the remaining water phase were determined spectrophotometrically with the Folin-Ciocalteu reagent (prepared by 1/1 dilution with distilled water), against the chlorogenic acid standard (Ferraris et al., 1987).

2.2.4. Total Flavonoids

Total flavonoid content was extracted by the aluminum chloride method. Accordingly, 2 mL of aqueous extract (10 mg/mL) or standard solution of quercetin (25-200 µg mL^{-1}) was added to 2 mL of 2% $AlCl_3$ solution and 2 mL of 120 mM potassium acetate. Samples were incubated for one hour at room temperature. Absorbance was measured at a wavelength of 425 nm by using a UV-Vis spectrophotometer. The total flavonoid content obtained is calculated as quercetin equivalent (Pekal and Pyrzynska, 2014).

2.2.5. Total Antioxidant Capacity

Phosphomolybdenum complex formation (PCF) method: 200 mg leaf sample was taken and extracted in 5 mL of 96% methanol and the extract was centrifuged at 5000 g for 5 min. A reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate was prepared. The final volume was adjusted to 3 mL by taking 150 µL of the sample solution and 2.85 mL of the reagent solution. The samples were then incubated at 90°C for 90 min and cooled to room temperature to determine their absorbance at a wavelength of 765 nm. The ascorbic acid solution was used as a standard and the results were calculated as ascorbic acid equivalents (Prieto et al., 1999).

Cupric ion reducing capacity (CUPRAC) method: 100 mg leaf tissue was homogenized in 10 mL of cold ethanol. The homogenized mixture was centrifuged at 5000 g for 10 min. 0.1 mL of the extract was added to the reaction mixture (containing 1 mL of 10 mM $CuCl_2$, 1 mL of ammonium acetate buffer pH 7, and 1 mL of 7.5 mM solution of neocuprion). The final volume was made up to 4.1 mL with water and incubated at 50°C for 20 min. After 30 min at room temperature, absorbance was measured at 450 nm. Gallic acid dissolved in 96% ethanol was used as a standard and the results were given as gallic acid equivalents (Apak et al., 2004).

2.2.6. Total Glutathione

100 mg leaf tissue was homogenized in 3 mL of 6% meta-phosphoric acid. 0.1 mL was taken from the samples centrifuged at 5000 g for 5 min and diluted 1/30 with sodium citrate buffer containing 2 mM EDTA (pH 5.0). 0.1 mL of the diluted sample contains 2 mL of solution A (contains sodium phosphate buffer (66 mM, pH 7.0 + 2 mM EDTA + 0.3 mM 5,5' dithiobis-(2-nitrobenzoic acid) + 0.4 mL L^{-1} BSA) and 0.9 mL of solution B (contains sodium phosphate buffer 66 mM, pH 7.0 + 2 mM EDTA + 50 mM imidazole + 0.2 mL L^{-1} BSA and 1.5 units of glutathione reductase). The reaction was started with 50 µL of NADPH (8.5 mM). The increase in absorbance due to the reduction of glutathione was measured at a wavelength of 412 nm (Gosset et al., 1994).

2.2.7. Ascorbic Acid

100 mg leaf tissue was homogenized by adding 2 mL 10% (w/v) trichloroacetic acid (TCA), cooled to 4°C and centrifuged at 5000 g for 5 min. 200 µL supernatant was mixed with 500 µL phosphate buffer (pH 7.0, 150 mM + 5 mM EDTA) and 100 µL dithiothreitol (10 mM) and incubated at room temperature for 10 min. 50 µL of the mixture was added to 2.95 mL of chlorophenol-indophenol solution (containing 13 mg L^{-1} DCPIP + 3 g L^{-1} sodium acetate) and the decrease in absorbance at 520 nm was measured with a spectrophotometer (Chen et al., 1991).

2.2.8. Superoxide Dismutase (SOD, EC 1.15.1.1.)

0.5 g leaf material was homogenized in 6 mL 0.1 M potassium phosphate extraction buffer (pH 7.0, containing 100 mg insoluble PVP and 0.1 mM EDTA) with Ultra Turrax. The homogenate was centrifuged for 5 min at 6000 g and 4°C. The supernatant was filtered through a Whatman GF/A glass fiber disc with a vacuum filtration system (Schöner and Krause 1990). SOD activity was determined according to Beyer and Fridowich (1987). The reaction mixture (3 mL) contained potassium phosphate buffer (pH 8.0, 0.025% Triton X-100 and 0.1 mM EDTA), enzyme extract, 12 mM L-methionine 75 µM nitro blue tetrazolium chloride (NBT) and 2 µM riboflavin. The reaction mixture was kept under fluorescent light for 10 min at 25°C. One SOD unit was described as the amount of enzyme where the NBT reduction ratio was 50%. The NBT reduction ratio was measured with a spectrophotometer at 550 nm wavelength.

2.2.9. Chlorophyll Contents

Chlorophyll was extracted with 8 mL of 80% acetone (buffered to pH 7.8 with phosphate buffer) from dried-ground leaf material. The chlorophyll a, chlorophyll b, and total chlorophyll concentrations were measured with a spectrophotometer (447 and 664 nm wavelength). The chlorophyll contents were calculated according to the equations of Porra et al. (1989). (As µg mL^{-1} Chl-a $12.25*A^{664}-2.55*A^{647}$, Chl-b $20.31*A^{647}-4.91*A^{664}$, Total Chl $17.76*A^{647}+7.34*A^{664}$)

2.2.10. Carotenoids

200 mg leaf material was homogenized in ethanol and centrifuged at 5000 g for 5 min, then the supernatant was concentrated by evaporation at 40°C in a rotary evaporator. The residue adhering to the glass surface was dissolved in 2 mL of chloroform. The obtained extract was applied with a micropipette in 100 µL on silica gel coated on a carrier layer with a thickness of 0.5 mm. Then these layers; It was placed in a chromatography tank containing hexane/diethyl ether/acetone as a solvent at a ratio of 60/35/20 by volume. The tank was kept in a dark environment so that the stains on the layers would not deteriorate. Carotene and xanthophyll stains that became evident after the running process were scraped from silica gel with a spatula, and 5 mL of acetone was added to it and centrifuged at 6000 g for 5 min. The absorbance values of the clarified supernatants were measured in a spectrophotometer adjusted to 450 nm wavelength. As standard, β-carotene and xanthophyll (lutein, Sigma) were used (Moore, 1974).

2.2.11. Hidrogene Peroxide

H_2O_2 content was analyzed in 0.2 g leaf tissue according yo Tian et al. (2015) as described. Leaf tissue was homogenized in 10 mL of 0.1% TCA at 4°C in a mortar and centrifuged at 10,000 g for 5 min. 0.5 mL of supernatant, 1 mL of phosphate buffer (100 mM, pH 7.0), and 1 mL of potassium iodide (1 M) were mixed and incubated at 25°C for 60 min in the dark. A separate control was prepared for each sample to determine the absorbance due to the color of the extracts. The absorbance was measured at 390 nm and H_2O_2 serial solution was used as a standard and results are given as a percentage of control.

2.2.12. Superoxide Generation Rate

The rate of superoxide formation was determined by hydroxylamine oxidation in 0.2 g leaf tissue. (Tian et al. 2015). Leaf tissue was homogenized in a mortar in 2 mL of potassium phosphate buffer (50 mM, pH 7.8, containing 1% PVP and 0.1 mM EDTA) at 4°C and centrifuged at 10,000 x g for 5 min. 0.5 mL of supernatant, 0.5 mL of phosphate buffer, and 1 mL of hydroxylamine chloride (1 mM) were mixed and incubated at 25°C for 60 min. 1 mL of sulfanilic acid (17 mM) and 1 mL of α-naphthylamine (7 mM) was added to the mixture and incubated at 25°C for 20 min and absorbance was measured at 530 nm. Results are given as a percentage of control.

2.2.13. Statistics

All analyses and measurements were performed in at least three replicates. Whether there was a difference between the groups was determined with the Kruskal-Wallis (KW) test and between which groups the difference was determined with the Least Significant Difference (LSD) test. The results of KW and LSD tests are shown in tables and graphs.

3. Results

To determine the effects of insect pests on carbohydrate metabolism in eucalyptus leaves, glucose, and fructose contents were analyzed by the antron method. In addition to the free glucose and fructose contents, this method also determines those that depend on the sucrose structure. Noninfested and infested leaf samples from trees infested with *L. invasa* and *O. maskelli* were compared with leaf samples from non-infested trees as controls. Glucose and fructose contents in leaves of infested trees by both gall wasps were higher than in control samples. The effect of *L. invasa* and *O. maskelli* infestation on glucose contents was quite similar (Table 1). Noninfested leaves of the infested trees had glucose values approximately 10% higher than the infested leaves. The fructose content of all samples was measured as 2/3 of the glucose content. Fructose contents were also low in control samples and high in noninfested leaves of infested trees (Table 1).

Free proline is the most characteristic indicator of direct or indirect osmotic stress. It is expected to increase in case of lack of water caused by insect damage on the leaves. The free proline content of leaves infested with *L. invasa* was found to be significantly higher than control samples. This increase was not observed in the noninfested leaves of the infested trees. On the other hand, the proline content in the damaged leaves of the infested trees by *O. maskelli* was found close to the control samples, while it was found to be significantly higher in the healthy leaves (Table 1).

Insect pests did significantly affect the total soluble phenolic content of eucalyptus leaves. The soluble phenolic content of leaves from infested trees by *L. invasa* was found to be significantly higher. It was higher in healthy leaves of infested trees than in damaged leaves. A change in the ratio of soluble phenolic compounds in the healthy leaves of trees infested by *O.maskelli* could not be determined compared to the control samples. It was observed that the phenolic content of the infested leaves of the infested trees with *O. maskelli* was 28.3% lower than the infested leaf (Table 1).

Analysis of the total flavonoid content of eucalyptus leaves revealed that the flavonoid content decreased with insect infestations. It was determined that *O. maskelli* was more effective than *L. invasa* in the decrease observed in flavonoid contents. The reduction in flavonoid content of infested trees was observed in the entire tree, although more pronounced in the infested leaves. The reduction rate of 16.5% in leaves infested with *L. invasa* was 33.7% with *O. maskelli* (Table 1).

Infestation of eucalyptus leaves by insect pests can cause disruptions in metabolic processes. If the damage in metabolism is due to the formation of free radicals, stimulation of the antioxidant defense system can be expected. In this study, antioxidant defense capacity was determined by two different methods. According to the PCF method findings, the total antioxidant capacity of trees infested with *L. invasa* showed values close to the control samples. Whereas, antioxidant capacity was found to be significantly lower in infested trees with *O. maskelli* (Table 2). The CUPRAC method findings showed that both gall wasps increased the total antioxidant capacity in eucalyptus leaves. Moreover, this increase was observed in all leaves, not just infested leaves.

Glutathione is a water-soluble antioxidant compound that has an important role in protecting the aqueous phases of the cell against oxidation. Extremely high glutathione values (6.72 mg g^{-1}) were determined in control samples taken from eucalyptus trees that were not infested by insect pests. Glutathione levels were significantly reduced in the leaves of trees infested by both *L. invasa* and *O. maskelli*. The lowest values were determined in the noninfested leaves of the infested plants (Table 2).

It was determined that the concentrations of ascorbic acid, one of the water-soluble antioxidant compounds of plants, increased significantly in response to insect pests. The increase in ascorbic acid concentrations is particularly higher in directly-infested leaves. It was determined that the amount of ascorbic acid increased by 87% in *L. invasa* infestation and 120% in *O. maskelli* infestation compared to control (Table 2).

While the highest total SOD activity was found in control samples, lower SOD activity was detected in non-infested leaves of infested trees by insect pests. It was observed that the total SOD activity was higher in the infested leaves of the infested trees than in the noninfested leaves (Table 2). The increase observed in damaged leaves was calculated as 29% for *L. invasa* and 20% for *O. maskelli*.

The total chlorophyll content in leaf samples taken from non-infested trees was determined as 4.53 mg g^{-1}. Chlorophyll content was found to be higher in both

Table 1. Metabolites in eucalyptus leaves infested and noninfested by gall wasps, *L. invasa,* and *O. maskelli* (NT noninfested tree, ITNL infested tree noninfested leaves, ITIL infested tree infested leaves), KW Kruskal Wallis test results are shown with stars (** indicates significant difference at the 0.01 level between leaf samples from infested and uninfested trees), and LSD test results are shown with letters, different letters indicate a significant difference between the means.

Pests		Glucose mg g^{-1}	Fructose mg g^{-1}	Proline µmol g^{-1}	Phenolics mg g^{-1}	Flavonoids mg g^{-1}
Control	NT	83.2±17.6 b	55.5±09.4 b	9.4±1.0 ab	22,0±2.1 b	5.2±0.7 a
L. invasa	ITNL	98.7±08.2 a	67.4±13.1 a	7.9±0.8 b	33.5±5.3 a	4.4±1.1 ab
	ITIL	87.8±12.0 ab	60.6±10.9 ab	13.6±1.4 ab	29.4±4.0 ab	4.3±0.9 ab
O. maskelli	ITNL	95.9±07.7 ab	65.5±12.9 ab	15.6±1.1 a	22.0±5.0 b	3.5±0.8 b
	ITIL	88.3±07.8 ab	60.1±11.3 ab	10.4±0.8 ab	28.0±3.4 ab	3.4±0.4 b
KW test		**	**	**	**	**

Table 2. Antioxidant capacity, antioxidant compounds and SOD activities in eucalyptus leaves infested and noninfested by gall wasps, *L. invasa,* and *O. maskelli* (NT noninfested tree, ITNL infested tree noninfested leaves), ITIL infested tree infested leaves), KW Kruskal Wallis test results are shown with stars (** indicates significant difference at the 0.01 level between leaf samples from infested and uninfested trees), and LSD test results are shown with letters, different letters indicate a significant difference between the means.

Pests		PCF mg AAE g^{-1}	CUPRAC mg GAE g^{-1}	Glutathione mg g^{-1}	Ascorbate mg g^{-1}	SOD U g^{-1}
Control	NT	111±23.5 ab	13.2±1.7 b	6.72±1.4 a	8.0±1.5 b	223±30 b
L. invasa	ITNL	111±12.6 ab	27.1±3.1 a	1.23±0.3 b	9.0±1.6 ab	311±57 ab
	ITIL	114±17.5 a	19.5±2.4 ab	5.44±1.3 ab	14.9±1.7 ab	401±74 a
O. maskelli	ITNL	92±18.6 ab	21.6±2.4 ab	1.99±0.2 ab	14.2±1.8 ab	290±87 ab
	ITIL	85±17.8 b	22.1±2.7 ab	4.18±0.6 ab	18.5±3.2 a	348±52 ab
KW test		**	**	**	**	**

noninfested (5.54) and infested (5.07) leaves of trees infested with *L. invasa*. While the non-infested leaves (total 6.20, Chl a/b 1.5) of the infested trees by *O. maskelli* had very high chlorophyll values, the damaged leaves (total 3.22, Chl a/b 2.1) had significant chlorophyll loss.

The total carotenoid content in control samples was determined as 5.73 mg g^{-1}. Although the infestation of *L. invasa* caused a slight decrease in the carotenoid content of *E. cameldulensis* leaves, the carotenoid values of noninfested and infested leaves were found to be close to each other. *O. maskelli* infestation caused an increase in total carotenoid content in noninfested leaves (total 7.29, Xan/Cds 5.0) but significantly decreased it in infested leaves (total 2.98, Xan/Cds 3.2) (Fig. 1).

The hydrogen peroxide content in leaves from non-infested trees was higher than in all samples from infested trees. *L. invasa* infestation caused lower H_2O_2 values than *O. maskelli* infestation. The values found in terms of superoxide formation rate were lower in the control samples, in contrast to the values found for the H_2O_2 content. Superoxide generation rates of non-infested and infested leaves from the infested trees were found to be higher than those of the control samples (Fig. 2).

4. Discussions

Eucalyptus trees are widespread in temperate regions with their rapid growth and high adaptability. Its cultivation is also widespread due to its use as an industrial raw material or energy source. Even a single insect can cause widespread damage due to the use of eucalyptus breeding clones (Wingfield et al., 2013). Eucalyptus species can be damaged by a large number of pathogens and pests, despite their success in adapting to their new habitats (de Souza Tavares et al. 2023). Two of these insect pests, *L. invasa* and *O. maskelli*, invade the leaves of *E. camaldulensis* trees and cause yield loss (Wingfield et al., 2008). Studies investigating the susceptibility and resistance properties of *E. cameldulensis* trees to insect pests at the genomic level Naidoo et al. (2014) compiled by the resistance of plants to harmful insect pests depends on the effectiveness of the plant defense system. Anatomical barriers play a role in the first stage of the plant defense system, physiological changes in the second stage, and biochemical regulations in the third stage (Franceschi et al., 2005; Eyles et al., 2010). The antioxidant defense system, which constitutes an important part of biochemical regulation, is the focus of this research. Studies on the biochemical responses of plant cells are scarce, as research on the interaction between insect pests and plants mostly focuses on pests.

Insect pests can affect the metabolism of plant cells by causing physiological and biochemical changes (Bari and Jones, 2009). The accumulation of sugars in plants infested by insect pests may have resulted from the inhibition of the transport of photosynthetic products from the leaves to the storage organs or their reduced use (Table 1). It has been reported that reducing sugar levels in eucalyptus leaves infested with *L. invasa* are highly variable (22.3-112.7 mg/g), but not related to tolerance (Vastrad and Ramanagouda, 2014). Proline accumulation is the result of primary or secondary water stresses. Damage caused by *L. invasa*, which produces gall in leaf veins, can cause leaves to become water-stressed. It was determined that the

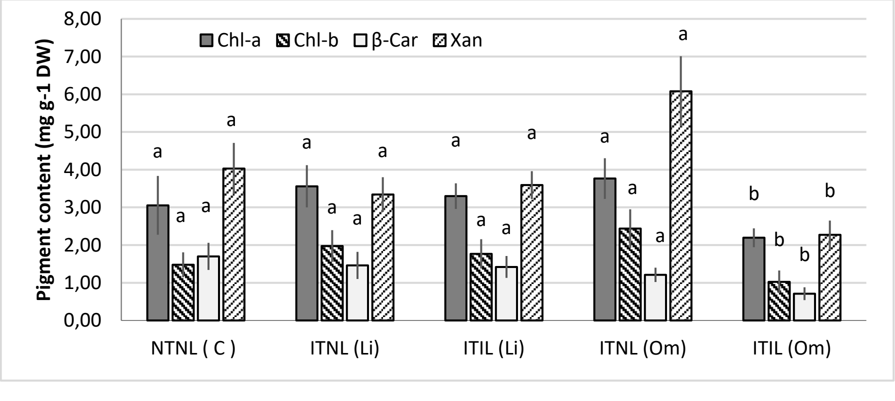

Figure 1. Photosynthetic pigments in eucalyptus leaves infested and noninfested by gall wasps, *L. invasa* (Li) and *O. maskelli* (Om). NT noninfested tree, ITNL infested tree noninfested leaves, ITIL infested tree infested leaves. KW test: Chl a (**), Chl b (**), β-Car (**), Xan (**). ** indicates significant difference at the 0.01 level between leaf samples from infested and uninfested trees), and LSD test results are shown with letters, different letters indicate a significant difference between the means.

damage caused by *O. maskelli* on the leaf surface was less effective on water transmission.

Pathogen and pest invasions cause oxidative damage by accelerating the formation of reactive oxygen species (ROS) in plants. The increase in ROS acts as secondary messengers that stimulate the antioxidant defense system that protects plants against oxidative damage (Singh et al., 2016). Some of the important elements of the antioxidant defense system, both enzymatic and non-enzymatic, were discussed in this study. Ascorbate, one of the water-soluble antioxidant compounds, increased significantly in infested plants, while glutathione levels decreased compared to control samples. Ascorbate and glutathione levels of damaged leaves of trees infested with *L.invasa* and *O.maskelli* were higher than healthy leaves. This finding can be interpreted as ascorbate synthesis is stimulated in damaged leaves and the stimulus is transmitted to healthy leaves as well. It can be thought that glutathione synthesis is not involved in the defense mechanism of eucalyptus trees against pest invasion.

One of the defense elements of plants against oxidative stress is phenolic compounds with their strong antioxidant effects. According to Vastrad and Ramanagouda (2014), the total phenol content is 115.0 mg g^{-1} in the *E. cameldulensis* (C-2045) clone infested with *L. invasa*, while it is 141 mg g^{-1} in the non-infested clone (C-526). Of the 48 eucalyptus genotypes of the 3 species screened, 13 were classified as highly susceptible, 2 as susceptible, 22 as tolerant, 2 as resistant, and 9 as immune. Invasion of *L. invasa* in *E. cameldulensis* trees increased phenolic synthesis in the whole plant, but more in healthy leaves. *O maskelli* infestation induced phenolic synthesis only in damaged leaves (Table 1). Such differences can be attributed to the level of damage and stimulation of the synthesis of signal carrier molecules. In addition to studies showing that compounds such as jasmonic acid and salicylic acid are synthesized and initiate defense responses in pathogen and pest invasions, there are also studies showing the roles of secondary message carrier compounds (Orozco-Cárdenas et al., 2001). The increase determined in phenolic compounds was not observed in flavonoids, a subgroup of phenolic compounds (Table 1).

There are many tests used to determine the antioxidant defense capacity of plants. These tests may give different results as they better measure the effects of some of the compounds that contribute to antioxidant defense (Pisoschi et al., 2016). Of the tests used in this study, PCF better represents the antioxidant capacity resulting from phenolic compounds, while CUPRAC rather measures the reducing capacity. The PCF test showed that the invasion of *L. invasa* did not affect the total antioxidant capacity, but the invasion of *O. maskelli* weakened the antioxidant capacity. The CUPRAC test showed that both insect infestations increased the total reducing capacity compared to the control samples (Table 2). The difference between these two test results shows that while pest attacks stimulate the synthesis of some antioxidant compounds in plant cells, they do not affect or suppress others.

One of the defense responses induced by ROS damage is the synthesis of pathogen-related (PR) proteins. While PR-15 proteins from this protein family are required for oxidase activity in the production of H_2O_2, which is toxic to pathogens and pests (Ali et al., 2018; van Loon, 2009), PR-9 proteins are peroxidase enzymes and protect plant cells from H_2O_2 damage. (Passardi et al., 2004). Studies show that essential oils produced and stored in eucalyptus tissues protect against insect pests (Chen et al., 2002). The results of this study showed decreased SOD activity in the leaves of eucalyptus trees infested with both *L. invasa* and *O. maskelli*. The SOD activity of the damaged leaves of the infested plants was higher than the healthy leaves (Table 2). Decreased H_2O_2 levels of leaves and increased rate of superoxide production in infested plants may be consequences of decreased SOD activity (Fig. 2).

Some effects of pest infestations on the pigmentation of eucalyptus leaves were observed. *L. invasa* infestation did not have a significant effect on pigmentation, whereas *O. maskelli* infestation significantly reduced the pigment content of infested leaves, while higher pigment content was detected in healthy leaves of infested trees. Since the invasion of *L. invasa* was observed in the leaf veins and stem, no adverse effects were observed in the photosynthesizing mesophyll cells. Whereas, *O. maskelli* invasion directly affects photosynthetic mesophyll cells and reduces pigment synthesis. To reduce the effects of pigment loss in leaves directly affected by the invasion, trees synthesize more pigment in their unaffected leaves. Pigment analysis results show that the major pigment of eucalyptus leaves is xanthophylls. Zeaxanthin, one of the xanthophyll molecules, is more effective than β-carotene in protecting from the harmful effects of high light and heat (Mortensen et al., 2001). This shows that the daily xanthophyll cycle (X-cycle) plays an important role in protecting eucalyptus trees from photoinhibition under high

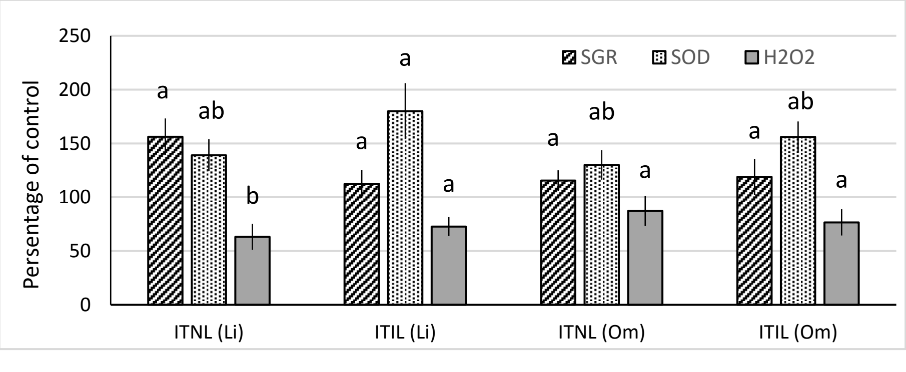

Figure 2. Superoxide generation rates (SGR), superoxide dismutase (SOD), and hydrogen peroxide (H_2O_2) as a persentage of control in Eucalyptus leaves infested and noninfested by gall wasps, *L. invasa* (Li) and *O. maskelli* (Om). NT noninfested tree, ITNL infested tree noninfested leaf, ITIL infested tree infested leaf. KW test: SGR (**), SOD (**), H_2O_2 (**).

temperature and light conditions. Oates et al. (2015) identified significant differences in mono and sesquiterpene profiles both between genotypes and between control and invaded material. Ultimately, they proposed a model based on transcriptomic and chemical data for the interaction between *E. cameldulensis* and *L. invasa*. According to this model, the release of terpenoids into the atmosphere may be intended to attract predator insects. The observed increase in terpenoid synthesis in response to *L. invasa* infestations should also be evaluated in terms of its contribution to carotenoid synthesis.

The effects of these two insects on leaf biochemistry were different from each other. This difference may be because *L. invasa* affects the veins and *O. maskelli* affects the leaf blades. Different localities on the leaf may affect water and solute transport and accumulation. Significant differences were found in the biochemistry of the infested leaf and the non-infested leaf from infested trees for some parameters. For example, proline was high in *L. invasa* and ITIL, while *O. maskelli* was high in ITNL. In terms of phenolic content, the situation was the opposite.

The infestation of *L. invasa* and *O. maskelli*, which are two of the gall bees that caused damage to the leaves of *E. cameldulensis*, caused significant changes in leaf biochemistry. *L. invasa* produces gall on the petiole and veins, and *O.maskelli* on the leaf blade. Different gall formation sites caused different effects on leaf biochemistry. *L. invasa* caused osmotic stress and promoted the accumulation of proline, while the invasion of *O. maskelli* led to the loss of pigment. The biochemical composition of the damaged and intact leaves of the infested plants also differs. The invasion of gall bees triggers oxidative stress by increasing the rate of superoxide production in eucalyptus leaves. The protective roles of ADC and AEA may be critical in reducing the damage of insect pests.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

FA identification and collection of plant samples, checking and editing of the article. YK: biochemical analyses, statistical evaluations and writing of the article.

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