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Role of Salicylic Acid in Resistance to Everzol Navy ED in *Lemna minor* L. (Duckweed)

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Abstract

The dyes used in the production of the textile industry are toxic substances that are resistant to biological treatment. Most of them have highly complex polymer structures. Salicylic acid (SA) is an important hormone produced by plants that provides tolerance to many biotic and abiotic stress factors. Duckweed (*L. minor* L.) are free floating plants. These plants have been used in laboratory toxicological studies by many researchers for reasons such as their high adaptability to aquatic environments, their small biomass and their high ability to accumulate contaminants. In this study was to evaluate some biochemical changes in duckweed (*L. minor* L.) of 75 ppm, 150 ppm and 300 ppm Everzol Navy ED (ENED) reactive dyestuff and 0.5 mM SA and ENED application. Total chlorophyll (TChl) and carotenoid (Car) contents were found higher at 0.5 mM SA and 300 ppm dye applied groups on day 7. Peroxidase (POD), ascorbate peroxidase (APX) and Glutathione S-transferase (GST) activities and total glutathione (GSH) content were increased at 0.5 mM SA with ENED application groups. (Superoxide dismutase) SOD and Catalase (CAT) activity increased on days 1, 4 and 7 in both treatment groups. GR activity decreased in dye applied groups and 0.5 mM SA with dye applied groups on day 7. MDA content decreased on day 7 with 300 ppm dye application and 0.5 mM SA and 300 ppm dye application.

Keywords: *Lemna minor*, lipid peroxidation, pigmentation, salicylic acid

Lemna minor L. (Su mercimeği)'de Everzol Lacivert ED'ye Dirençte Salisilik Asitin Rolü

Öz

Tekstil endüstrisinin üretiminde kullanılan boyalar biyolojik uygulamalara dayanıklı toksik maddelerdir. Çoğunun oldukça karmaşık polimer yapıları vardır. Salisilik asit (SA), bitkiler tarafından üretilen, birçok biyotik ve abiyotik stres faktörüne tolerans sağlayan önemli bir hormondur. Su mercimekleri serbest yüzen bitkilerdir. Bu bitkiler, sucul ortamlara yüksek adaptasyonları, küçük biyokütleleri ve kirletici biriktirme kabiliyetleri gibi nedenlerle birçok araştırmacı tarafından laboratuarda toksikolojik çalışmalarda kullanılmıştır. Bu çalışmada 75 ppm, 150 ppm ve 300 ppm Everzol Lacivert ED (ELED) reaktif boyarmadde ile 0.5 mM SA ve ELED uygulamasının su mercimeğindeki (*L. minor* L.) bazı biyokimyasal değişiklikleri değerlendirilmiştir. Toplam klorofil (TKl) ve karotenoid (Kar) içerikleri 7. günde 0.5 mM SA ve 300 ppm boya uygulanan gruplarda daha yüksek bulunmuştur. Peroksidaz (POD), askorbat peroksidaz (APX) ve Glutatyon S transferaz (GST) aktiviteleri ve toplam glutatyon (GSH) içeriği 0.5 mM SA ve ELED uygulanan gruplarda artmıştır. Süperoksit dismutaz (SOD) ve katalaz (CAT) aktivitesi her iki uygulama grubunda da 1., 4 ve 7. günlerde artış göstermiştir. GR aktivitesi hem boya uygulanan gruplarda hem de 0.5 mM SA ve boya uygulanan gruplarda 7. günde azalmıştır. MDA içeriği 7. günde 300 ppm boya uygulanan gruplarda ve 0.5 mM SA ve 300 ppm boya uygulanan gruplarda azalmıştır.

Anahtar kelimeler: *Lemna minor*, lipid peroksidasyonu, pigmentasyon, salisilik asit

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INTRODUCTION

Any unsuitable condition or substance that affects or impedes the plant's metabolism, growth and development is considered stress and is closely related to plant tolerance. Most dyes are carcinogenic and mutagenic in nature. Plants are potential candidates for improvement textile dye effluents from contaminated areas.

Protecting the environment and natural resources against pollution is extremely important in terms of preventing environmental pollution. Phytoremediation is a new technology shown as an alternative to physical remediation methods that destroy the treatment of pollutants in soil (Ashraf et al., 2019), sediment, surface and groundwater. The most important advantages of phytoremediation is that it is an effective, easy and inexpensive method. One of the plants used for phytoremediation is *L. minor*. It is a perennial, monoic plant that lives floating in water. It spreads in lakes, pools, swamps and canals. It is an important nutritional source for aquatic creatures as it contains high amounts of protein (Iatrou et al., 2018; Al-Snai, 2019).

SA, which is considered a plant hormone, is a group of phenolic substances (Demirci et al., 2021). SA has a regulatory role in the flowering of thermogenic plants (Aziz and Kapoor, 2018). Also exogenous SA applications in plants, inhibit ethylene biosynthesis, delay senescence and stimulate the synthesis of pathogen-related protein (Di et al., 2017; Akbulut, 2020; Bozbuga, 2020; Feng et al., 2020; Wang et al., 2020).

Reactive oxygen species (ROS) act as signaling molecules to regulate development and initiate response to environmental stressors. Plants have developed mechanisms that protect themselves against the damage caused by ROS. These mechanisms consist of clearance of ROS by non-enzymatic antioxidants and enzymatic antioxidant (Karaaslan et al., 2018; Sarker and Oba, 2020). By reducing the chlorophyll content of plants under stress, the photosynthesis content is reduced. Carotenoids play accessory light-absorbing role in photosynthesis. They are also potent scavengers for protecting pigments (Dorina et al., 2020) and unsaturated lipid fatty acids against oxidative harm (Strzałka et al., 2003).

POD utilities have been documented in plants, such as H_2O_2 degradation, toxic compound elimination, insect herbivore protection and many other stress-related responses (Bansal and Kanwar, 2013). APX and GR play a key role in the ascorbate-glutathione cycle by reducing H_2O_2 to water. SOD is a functional antioxidant enzyme in plants against biotic and abiotic stress (Stephenie et al., 2020). CAT is an enzyme in protein structure. It breaks down H_2O_2 into O_2 and H_2O (Sharma and Mathur, 2020; Yu et al., 2020). Glutathione is a small molecule of intracellular thiol that is known as a potent antioxidant that is not enzymatic (Hasanuzzaman et al., 2017). It is stated that GST enzymes are associated with xenobiotic detoxification, oxidative damage and stress in plants (Gong et al., 2005).

Hydroxyl radicals attack polyunsaturated fatty acids, causing a hydrogen atom to be removed from the methylene group and thus start lipid peroxidation. MDA is the end product of lipid peroxidation (Teixeira et al., 2020).

In this study, some biochemical parameters such as pigment, antioxidant enzyme activites and lipid peroxidation of ENED reactive dyestuff on *L. minor* L., an aquatic plant that plays an important role in phytoremidation studies were investigated. In addition, the effect of SA application, which is an important plant hormone, on *L. minor* exposed to dye was tried to be determined.

MATERIAL AND METHODS

Experimental Design

Healthy mature plans of *L. minor* were obtained from Erciyes seed company, located at Kayseri, Turkey. Reactive dyestuff Everzol navy ED (ENED) was selected for a study. Before dye treatment, *L. minor* plants were acclimated in Hoagland medium (Hoagland and Arnon, 1938) for seven days in the greenhouse. After one week of cultivation, healthy fronds (30-40 g) of plants were separated and placed in 250 mL glass beakers in 1/30-dilute Hoagland culture solution containing one of the following treatments: (1) control: Hoagland medium: (2) 75 ppm ENED; (3) 150 ppm ENED; (4) 300 ppm ENED; (5) 0.5 mM SA; (6) 0.5 mM SA + 75 ppm ENED; (7) 0.5 mM SA + 150 ppm ENED and (8) 0.5 mM SA + 300 ppm ENED. All experiments were repeated in triplicate. The fronds were harvested on days 1, 4 and 7.

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Pigment analysis

TChl and Car concentrations were measured at 470 nm, 645 nm and 662 nm according to the method described by De-Kok and Graham (1980). The absorbance was calculated according to Lichtenthaler and Wellburn (1983).

Enzyme Extraction and Protein Content

Enzyme extractions were assayed according to Huang et al. (2013). 0.5 g of *L. minor* was homogenized in 5 ml, 0.1 M potassium phosphate buffer (pH 7.8). Homogenates were centrifuged at 4°C and 15000 g for 15 min. A Bradford protein assay was used to determine the protein concentration, using bovine serum albumin as standard (Bradford, 1976).

POD Activity

POD activity was assigned according to Peters et al. (1989) and Mac Adam et al. (1992). POD activity was calculated at 436 nm. Guaicol's extinction coefficient is 26.6 mM^{-1} cm^{-1}.

APX Activity

APX activity was determined according to Nakano and Asada (1981). The decrease in the optical density to ascorbic acid was recorded at 290 nm. APX activity was determined with an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

GST Activity

GST activity was estimated by the methods of Habig et al. (1974). Enzyme activity was determined at 344 nm. The extinction coefficient of CDNB is 9.6 mM^{-1} cm^{-1}.

GR Activity

GR activity was measured according to Carlberg and Mannervik (1985). GR activity was detected at 340 nm. The extinction coefficient NADPH is 6.2 mM^{-1} cm^{-1}.

GSH Content

GSH content was determined according to Akerboom and Sies (1981). GSH content was stated at 412 nm.

SOD Activity

SOD activity was established by the method of McCord Fridovich (1969). SOD activity was determined at 550 nm.

CAT Activity

CAT activity was measured by the method of Luck (1963). Enzyme activity was detected at 240 nm. The molar extinction coefficient for H_2O_2 is 0.0396 cm^2 μmol^{-1}.

Lipid Peroxidation Assay

MDA content was assayed by the thiobarbituric acid reactive substances method according to Heath and Packer (1968). Absorbance was calculated at 532 nm and 600 nm.

Statistical Analysis

Values were expressed as a mean of ±SE. For all experiments, the total data were statistically analyzed in version 21.0 of the SPSS. One-way variance analysis (ANOVA) and Duncan multiple-range tests were used to test differences (Duncan, 1955). The confidence coefficient was set to $p < 0.05$.

RESULTS

Changes in TChl and Car Contents

In the 150 ppm ENED groups, the highest TChl was found at 15.02 µg g^{-1} on day 4. The highest TChl content was stated in the 0.5 M SA and 300 ppm ENED as 16.09 µg g^{-1} on day 7. TChl content decreased, except for the groups treated with 75, 150 and 300 ppm ENED on day 7 and groups treated with 150 ppm on day 4 compared to control. TChl content was increased compared to the 0.5 mM SA control on day 7 in the 0.5 mM SA and 150 ppm groups, and on day 4 and on day 7 in the SA and 300 ppm dye applied groups. The highest Car content was determined as 3.12 µg g^{-1} on day 7 in the 75 ppm ENED group. Car content was increased in the 75 ppm dye applied groups and SA and 300 ppm dye applied groups on day 7 compared to the control. These changes were found to be statistically significant (Figure 1).

Changes in Antioxidant Activity

POD activity was found higher in the 75 ppm, 150 ppm and 300 ppm dye applied groups on days 1, 4 and 7. POD activity was increased due to increasing day and dye concentration. Also after 0.5 mM SA and dye application, POD activity was determined higher

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according to 0.5 mM SA control. The highest POD activity was determined as 14.57 U mg^{-1} protein and 16.13 U mg^{-1} protein in 0.5 mM SA and 300 ppm ENED on days 4 and 7, respectively. These changes were determined to be statistically significant (Figure 2).

APX activity was determined higher in the 75 ppm, 150 ppm and 300 ppm dye applied groups on days 1, 4 and 7. APX activity was increased due to increasing day and dye concentration. The highest APX activity was found as 15.34 U mg^{-1} protein in 0.5 mM SA and 300 ppm dye applied groups. It was determined that 0.5 mM SA with dye application was more effective than dye application alone. These changes were found to be statistically significant (Figure 3).

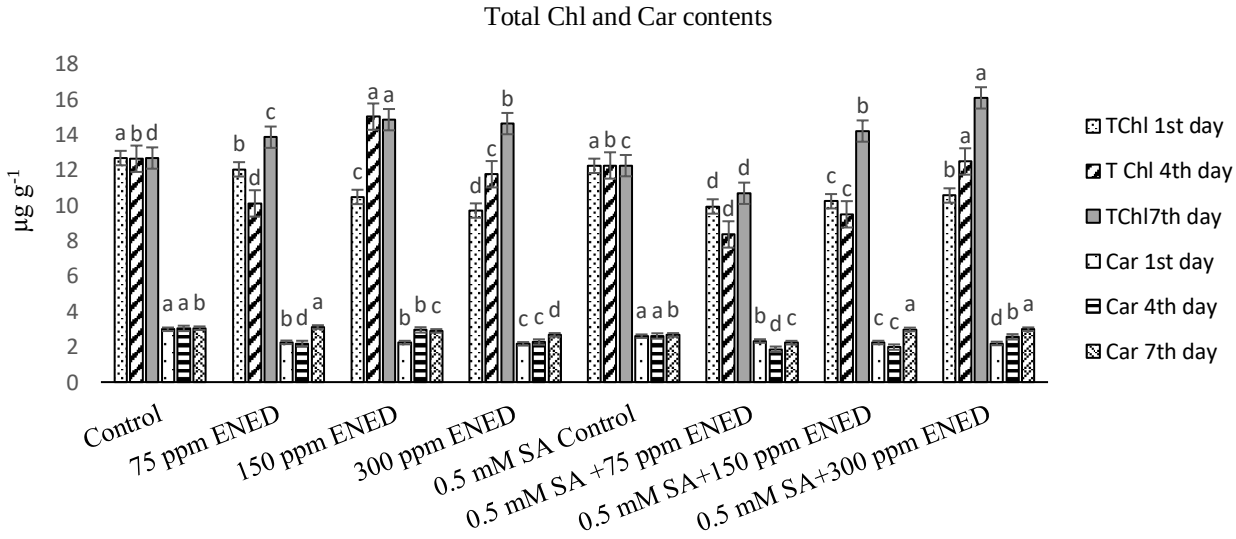

Figure 1. Changes in Total Chl and Car content in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

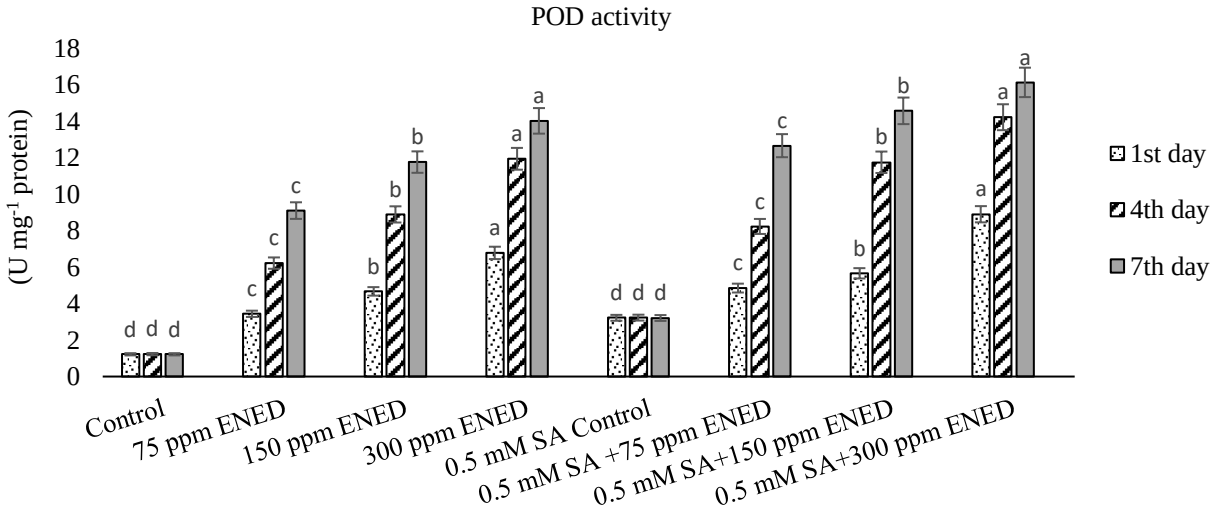

Figure 2. Changes in POD activity in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

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The highest GST activity was detected as 12.33 U mg^{-1} protein in 300 ppm dye applied groups on day 7. GST activity was found higher in 0.5 mM SA with dye applied groups compared to dye application alone. The highest GST activity was found as 14.26 U mg^{-1} protein in the groups treated with 0.5 mM SA + 300 ppm dye applied groups on day 7. These changes were determined to be statistically significant (Figure 4).

GR activity was increased in 75 ppm, 150 ppm and 300 ppm dye applied groups on day 1 and decreased in 150 ppm and 300 ppm dye applied groups compared to the control on days 4 and 7. GR activity was determined higher in 0.5 mM SA and dye applied groups compared to dye applied groups. GR activity decreased in the SA and dye applied groups on days 4 and 7. These changes were found to be statistically significant (Figure 5).

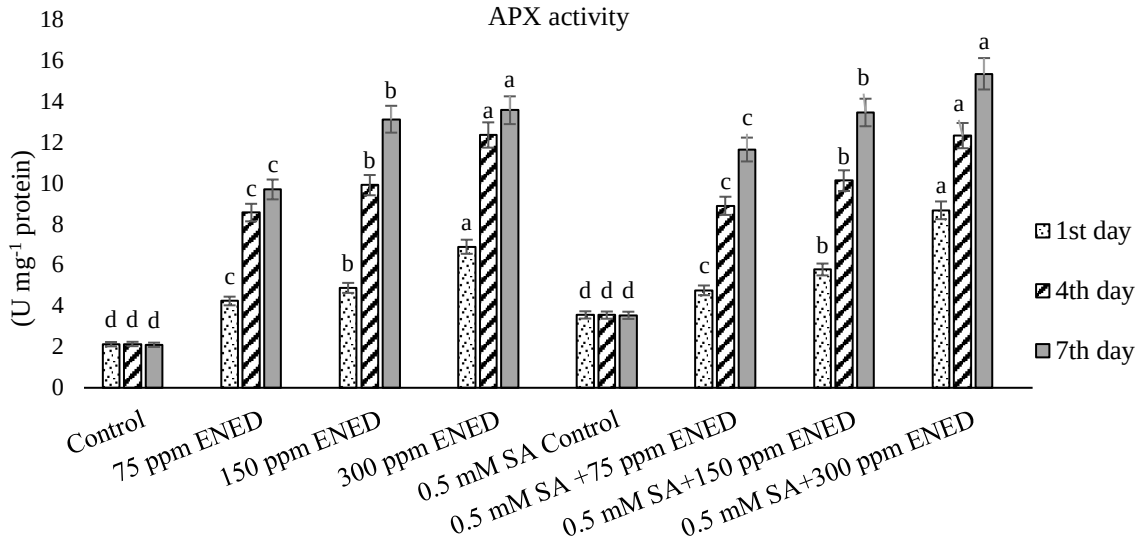

Figure 3. Changes in APX activity in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

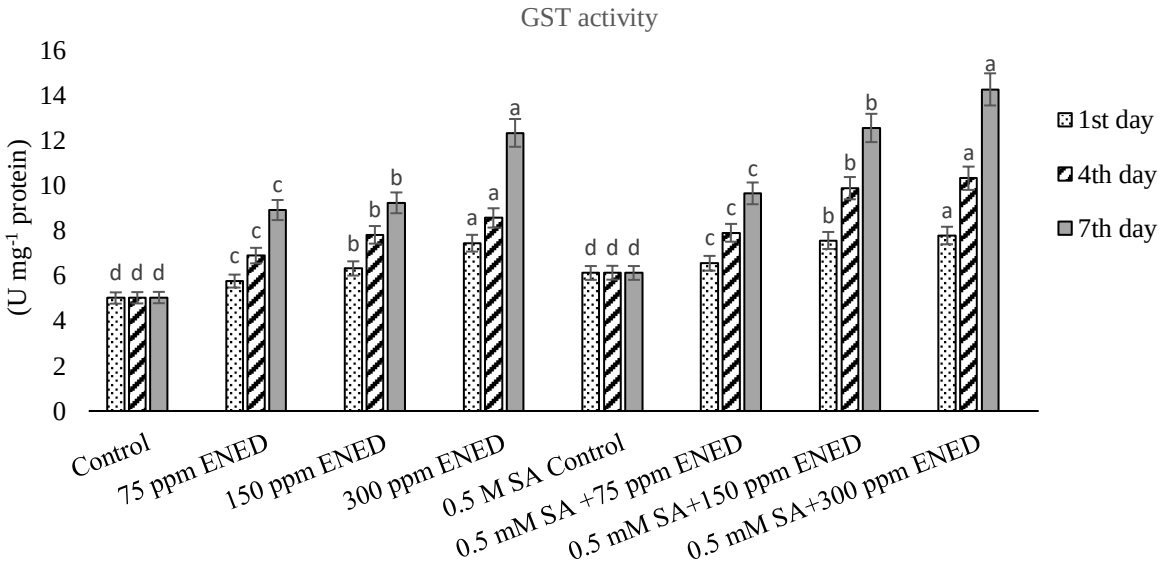

Figure 4. Changes in GST activity in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

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GSH content was higher in 75 ppm, 150 ppm and 300 ppm dye applied groups compared to control. There was an increase in the GSH content in the 0.5 mM SA and dye applied groups depending on both concentrations and days. The highest GSH content was determined as 1.9 U mg^{-1} protein in 300 ppm dye applied groups, while it was 2.38 U mg^{-1} protein in groups treated with 0.5 mM SA and 300 ppm dye applied groups. These changes were determined to be statistically significant (Figure 6).

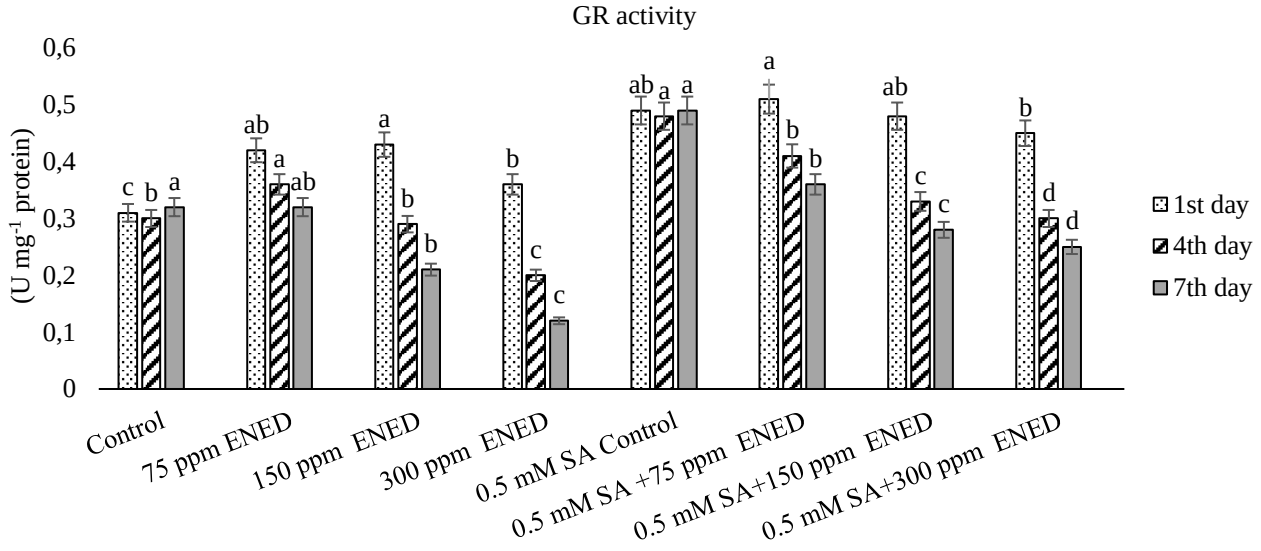

Figure 5. Changes in GR activity in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

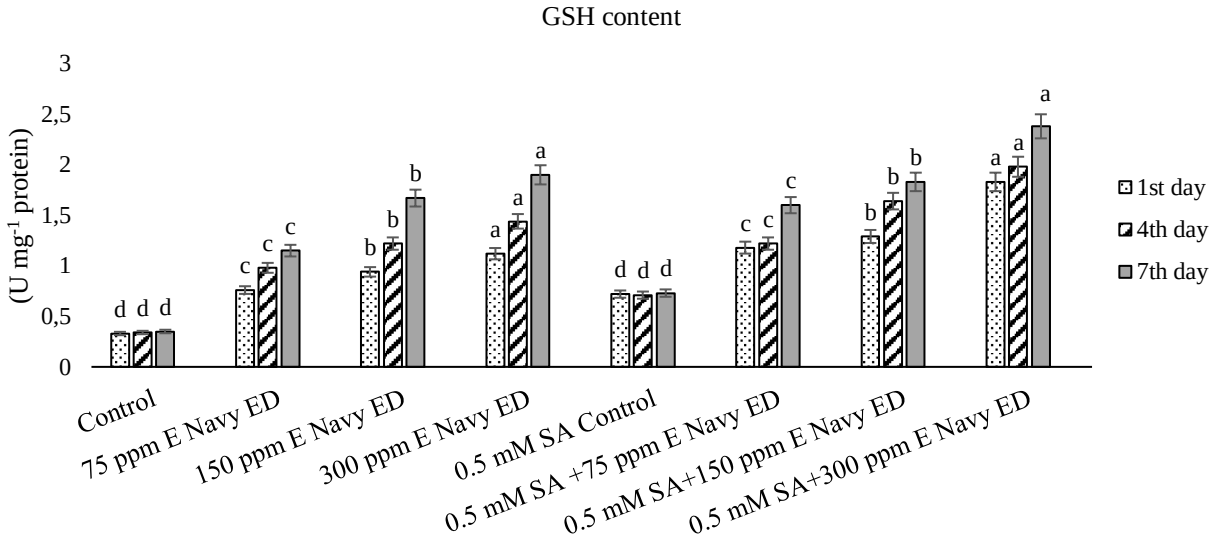

Figure 6. Changes in GSH content in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

SOD activity increased in the dye applied groups on days 1, 4 and 7. SOD activity was determined higher in the groups treated with 300 ppm dye as 1.8 U mg^{-1} protein. SOD activity increased on days 4 and 7 in the 0.5 mM SA and 75 ppm dye groups and in the 0.5 mM SA and 150 ppm dye groups, while it increased on day 4 and decreased on day 7 in the 0.5 mM SA and 300 ppm dye groups (Figure 7). In the groups treated with 0.5 mM SA and dye and dye alone, CAT activity increased on day 4. CAT activity decreased

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on the 7th day in both treatment groups. The highest CAT activity was calculated as 1.89 U mg^{-1} protein on day 4 in the 0.5 mM SA and 300 ppm dye groups, while the lowest CAT activity was found in the 0.5 mM SA control group (Figure 7).

Changes in lipid peroxidation

MDA content was increased on days 4 and 7 in the groups treated with 75 ppm and 150 ppm, while it was found to increase on day 4 and decrease on day 7 in the groups with 300 ppm dye applied groups. MDA content was increased in the 75 ppm and 150 ppm SA and dye applied groups on days 4 and 7. In SA and 300 ppm dye applied groups an increase was determined on day 4 and a decrease was found on day 7. It was determined that SA and dye application was more effective than dye application alone. These changes were found to be statistically significant (Figure 8).

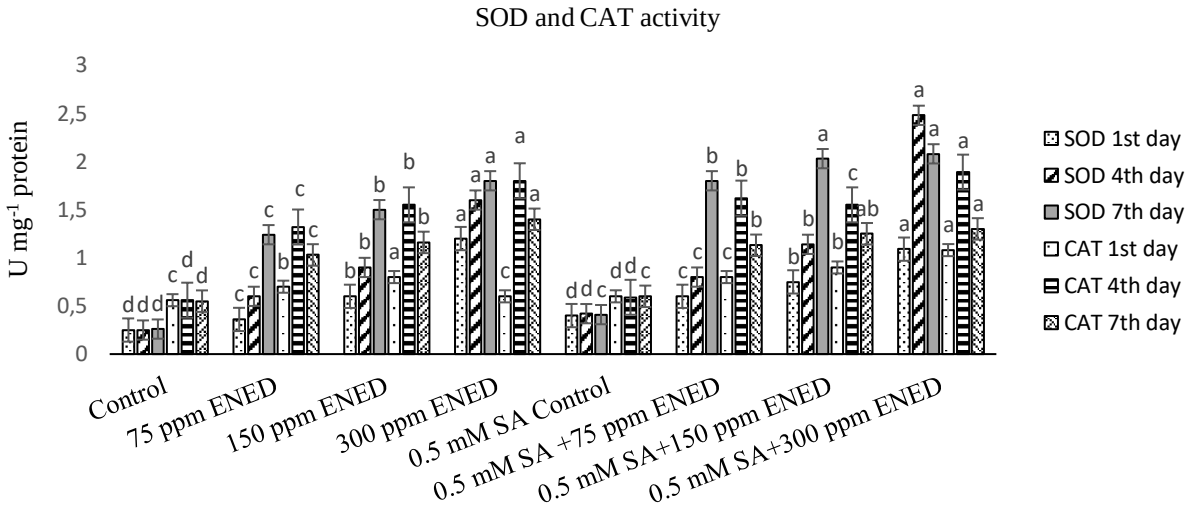

Figure 7. Changes in SOD and CAT acitivites in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

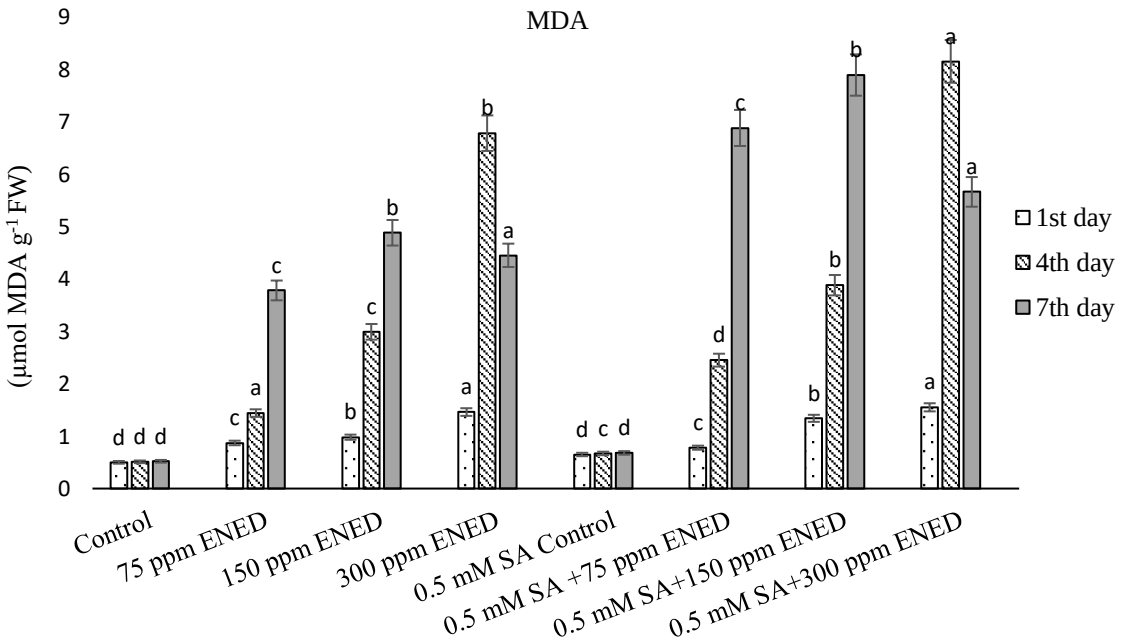

Figure 8. Changes in MDA content in *L. minor* exposed to different concentrations of ENED and 0.5 mM SA+ ENED

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DISCUSSION

Synthetic dyes are widely used in the textile, food, paper and leather industries. The synthetic origin and complex structure of these dyes make them strong and difficult to degrade. *L. minor* L. has been identified as a suitable plant material for ecotoxicological investigations in recent years (Sackey et al., 2020). SA mediates plant responses to many biotic and abiotic stresses (Hernández-Ruiz and Arnao, 2018; Cohen and Leach, 2019). In this study, the protective role of 0.5 mM SA against oxidative stress caused by ENED, an anionic dye in *L. minor* was determined.

The photosynthetic pigments are directly connected to the growth. Car are important antioxidants that play a role in oxidative stress tolerance (Havaux, 2014; Chavoushi et al., 2020; Li et al., 2020). Souza et al. (2019) showed that the chlorophyll content decreased at high iron oxide nanoparticle concentrations, which disrupted the light absorption mechanism in *L. minor*. In this study, total Chl content was determined higher in the 75 ppm, 150 ppm and 300 ppm dye groups compared to the control groups on day 7. Total Chl content in 0.5 mM SA and 150 ppm dye applied groups and 0.5 mM SA and 300 ppm dye applied groups were found higher than 0.5 mM SA control group on day 7. Car content was higher in the 75 ppm dye applied groups, 0.5 mM SA and 75 ppm dye applied groups and 0.5 mM SA and 300 ppm dye applied groups compared to control groups (Figure 1). Chlorophyll breakdown can be caused, in particular, by increased chlorophyllase enzyme activity. POD and APX play an important role in the antioxidant system of plants (Buttar et al., 2020). Li et al. (2020) determined that Ag^+ treatment caused an evident reduction in the activities of SOD, POD and CAT in *L. minor*. Also photosynthetic pigment contents of *L. minor* decreased. In this study, it was determined that 0.5 mM SA and the ENED application increased the POD and APX activities more than the dye-only groups (Figure 2, 3).

GSH plays a direct role in neutralizing free radicals by chemically reacting with singlet oxygen with superoxide and hydroxyl radicals (Cicerali, 2004). The reduction of oxidized glutathione (GSSG) to GSH is catalyzed by the GR enzyme. GST plays a key role in detoxification mechanism (Lo et al., 2007). Teisseire and Vernet (2000) showed that GSH and GSSG contents in *L. minor* increased after diuron and folpet-exposure. Razinger et al. (2007) reported that treatment of *L. minor* with $CuSO_4$ resulted in an immediate decrease in the glutathione pool. GR, guaiacol peroxidase and CAT activities increased after 24 hours of exposure with $CuSO_4$. In this study, it was determined that GSt activity and GSH content increased on days 1, 4 and 7. GR activity decreased in both treatment groups on day 7. SA application was found to be more effective than dye application alone (Figure 4-6).

SOD is an important antioxidant enzyme in reducing and eliminating the content of ROS. CAT, which are mainly localized in peroxisomes, are enzymes containing tetrameric heme that convert H_2O_2 to O_2 and H_2O. Sun et al. (2019) reported that SOD and CAT failed to reflect the effects of nano-ZnO on Cd toxicity on *L. minor*. Alkimin et al. (2019) reported that SA application was capable of increasing CAT and provoking a variation in APX and GSTs, according to time and concentration on *L. minor*. In this stdy, while SOD activity increased on days 1, 4 and 7 in both treatment groups, CAT activity decreased after day 4. It was determined that SA application was more effective in both applications (Figure 7).

MDA appears as an important indicator in revealing lipid damage. Parlak and Yilmaz (2012) found that pigments and soluble proteins decreased exposure to high Zn concentrations. Also the content of MDA increased with increasing Zn concentration in *L. gibba*, *L. minor* and *Spirodela polyrrhiza*. In this study, it was observed that 0.5 mM SA application generally increased the MDA content in *L. minor* (Figure 8).

CONCLUSIONS

L. minor is one of the strongest candidate species for use in technologies to be installed in natural environments. In conclusion ENED dyestuff caused oxidative stress in *L. minor* L. Pigmentation was generally reduced. POD, APX, GST, GSH, SOD and CAT activities increased with dye and 0.5 mM SA and dye application on day 7. GSH content decreased in both dye application groups on day 7. MDA content increased. MDA content increased in both the dye applied groups and the 0.5 mM SA and dye applied groups. The knowledge obtained from this research indicates that exogenous SA application had a healing effect against the dye-induced stress in *L. minor*.

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CONFLICT OF INTEREST STATEMENT
The author declares that there is no conflict of interest in this study.

RESEARCH AND PUBLICATION ETHICS STATEMENT
The author declares that the research and publication ethics are complied with in the study.

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