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Alleviation of Everzol Red LFB Toxicity in Duckweed (*Lemna minor* L.) by Exogenous Salicylic Acid

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ABSTRACT
Salicylic acid (SA) has an important function in the formation of induced systemic resistance. The aim of this study was to determine effects of exogenous application of 0.5 mM SA on the stress response in duckweed (Lemna minor L.) exposed to the reactive dyestuff Everzol Red LFB (ER LFB). Phytotoxic responses induced by exposure to both ER LFB (75 ppm, 150 ppm and 300 ppm) and 0.5 mM SA+ ER LFB (75 ppm, 150 ppm and 300 ppm) applications were comparatively examined at 1st, 4th, and 7th days. The lowest chlorophyll a (Chl-a), chlorophyll b (Chl-b), and total chlorophyll (total Chl) contents were found in the 300 ppm ER LFB groups. The carotenoid (Car) content was decreased compared to control groups. The highest total glutathione (GSH), glutathione S-transferase (GST) and peroxidase (POD) activities were found after 0.5 mM SA + 300 ppm ER LFB groups at 7th day. Glutathione reductase (GR) activity was reduced at 7th day. The level of lipid peroxidation, measured as malondialdehyde (MDA) content was increased generally both ER LFB and SA+ER LFB groups compared to control groups. Results suggest that SA plays a positive role in L. minor against ER LFB.

Research Article

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Su Mercimeğinde (*Lemna minor* L.) Everzol Red LFB Toksisitesinin Eksojen Salisilik Asit ile Azaltılması

ÖZET
Salisilik asit (SA) sistemik direncin oluşmasında önemli bir role sahiptir. Bu çalışmanın amacı eksojen uygulanan 0.5 mM SA'nın reaktif boya maddesi Everzol Red LFB (ER LFB)'ye maruz kalan su mercimeğindeki (Lemna minor L.) stress tepkisi üzerindeki etkilerini belirlemektir. Hem ER LFB (75 ppm, 150 ppm ve 300 ppm) hem de 0.5 mM SA + ER LFB (75 ppm, 150 ppm ve 300 ppm) uygulamalarına maruz kalmanın neden olduğu fitotoksik tepkiler 1., 4. ve 7. günlerde karşılaştırmalı olarak incelenmiştir. En düşük klorofil a (Kl-a), klorofil b (Kl-b) ve toplam klorofil (toplam Kl) içeriği 300 ppm ER LFB grubunda bulunmuştur. Karotenoid (Kar) içeriği kontrol gruplarına göre azalmıştır. En yüksek toplam glutatyon (GSH), glutatyon S-transferaz (GST) ve peroksidaz (POD) aktiviteleri 7. günde 0.5 mM SA + 300 ppm ER LFB gruplarında bulunmuştur. Glutatyon redüktaz (GR) aktivitesi 7. günde azalmıştır. Malondialdehid (MDA) içeriği olarak ölçülen lipit peroksidasyon seviyesi, kontrol gruplarına kıyasla genellikle hem ER LFB hem de SA + ER LFB gruplarında artmıştır. Sonuçlar SA'nın L. minor'de ER LFB'ye karşı pozitif bir rol oynadığını göstermektedir.

Araştırma Makalesi

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INTRODUCTION

Aromatic compounds produced via chemical synthesis, termed as synthetic dyes (Kant, 2012). These dyes are widely used in textile, food, cosmetic, plastic and pharmaceutical industries (Kagalkar et al., 2010; Lin et al., 2011). Application of dye-containing wastes has

been a challenging problem for environmental technologies. Some dyes decompose, and the resulting compounds may also have a toxic effect on the aquatic environment (Carmen and Daniela, 2012). In recent years, the use of plants for removal of polluted environments has attracted much attention (Vafaei et al., 2013; Tagun and Boxall, 2018). Duckweed (*Lemna minor* L.) is a free-floating water plant from the *Lemnaceae* family (Cheng et al., 2002; Ozengin and Elmaci, 2007; Elmaci et al., 2009). Because of its small size, simple structure, morphology, rapid growth rate, short life span and sensitivity to environmental pollutants, it is widely used in ecotoxicological studies (Balen et al., 2011; Lu et al., 2016).

Reactive oxygen species (ROS) function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components, such as lipids, proteins, and DNA (Valko et al., 2006; Birben et al., 2012). Plants have developed various defensive mechanisms that allow for ROS removal (Jóźwiak and Politycka, 2019). The photosynthetic pigment in plants is considered to be one of the factors that is responsive to stress (Ozengin and Elmaci, 2007). Content of MDA is considered an indicator of lipid peroxidation and membranes damage (Sharma et al., 2012). GSH is a multifunctional tripeptide found in plants and animals (Noctor et al., 2002). GST plays an important role in the phytoremediation process (Kömives and Gullner, 2000). GR plays an important role in the oxidative stress response by maintaining the reduced state of the intracellular GSH pool (Miteva et al., 2010).

Plant growth regulators play important roles in the regulation of plant developmental processes and signalling networks (Khan et al., 2012; Asgher et al.2015). SA is an effective plant growth regulator. It is considered to be an important signal molecule involved in the induction of systemic resistance (Hayat et al., 2010; Kumar, 2014).

The purpose of this study was to determine pigment content, lipid peroxidation and some antioxidant enzymes, resulting from implementation of dyestuff ER LFB in *L. minor*. We also aim to evaluate if the toxic effects showed on *L. minor* by ER LFB can be regulated by pre-treatment with 0.5 mM SA.

MATERIALS and METHOD

Plant material, acclimatisation and dye treatment

Fresh samples of *L. minor* were obtained from Erciyes Seed Company, Kayseri, Turkey. ER LFB (R4504502), a commonly used commercial reactive dyestuff was chosen. Before dyestuff treatment, plants were cultivated in 1/30-dilute Hoagland culture solution (Hoagland and Arnon, 1938) under growth chamber (temperature: 23 ± 2°C; light/dark cycle: 16/8 h; light intensity: 10,000 lux) for acclimatization. The growth medium was changed every two days. After one week of cultivation, similar fronds (30-40 g) of *L. minor* 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 ER LFB; (3) 150 ppm ER LFB; (4) 300 ppm ER LFB; (5) 0.5 mM SA; (6) 0.5 mM SA + 75 ppm ER LFB; (7) 0.5 mM SA + 150 ppm ER LFB and (8) 0.5 mM SA + 300 ppm ER LFB. Three replicates were used for each treatment. The fronds were harvested after 1st, 4th and 7th days of treatment.

Photosynthetic pigments analysis

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

Enzyme extraction and protein content

Enzyme extractions were determined according to Huang et al. (2013). Approximately 0.5 g of *L. minor* was homogenized in 5 mL cold potassium phosphate buffer (0.1 M, pH 7.8). Homogenates were centrifuged at 4°C and 15000 g for 15 min. Protein content was measured by the method of Bradford (1976).

Enzyme activities

POD analysis was assayed according to Peters et al. (1989) and Mac Adam et al. (1992). GST activity was measured as described by Habig et al. (1974). GR activity was determined according to Carlberg and Mannervik (1985). GSH activity was assayed according to Akerboom and Sies (1981).

Determination of MDA content

MDA content was determined according to Heath and Packer (1968). Absorbance of the supernatants was measured at 532 and 600 nm. MDA content was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Statistical Analysis

Statistical software package SPSS, version 21.0 were used for the analysis. The comparison of the averages was used for the Duncan's Multiple Range test at the probability 5% (Duncan, 1955).

RESULTS

Effect of ER LFB and 0.5 mM SA+ER LFB on Pigmentation

In *L. minor*, the Chl-a content was decreased in both ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) groups compared with that in control groups at both 1st and 4th days. In 0.5 mM SA+ 75 ppm ER LFB group at 7th day, Chl-a content

was found to be higher than SA control group. The highest Chl-a content was determined as 20.09 µg/g in the control group and the lowest Chl-a content was found in the 0.5 mM SA+300 ppm ER LFB groups at day 1st (Fig. 1).

When Chl-b content was examined in *L. minor* to which ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) were applied, the highest Chl-b content was determined as 7.67 µg/g in SA control group at 4th day. The lowest Chl-b content was found to be 1.16 µg/g in the 300 ppm ER LFB groups at day 1st (Fig. 2).

The total Chl content was decreased in both ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) groups compared to control groups at 1st, 4th, and 7th days. The lowest total Chl content was found in the 300 ppm ER LFB groups at day 1st (Fig. 3).

When Car activity was examined in *L. minor* to which ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) were applied the highest Car content was found to be 7.19 µg/g in the control groups at day 7th, and the lowest Car content was found to be 2.62 µg/g in 0.5 mM SA+ 150 ppm ER LFB groups (Fig. 4).

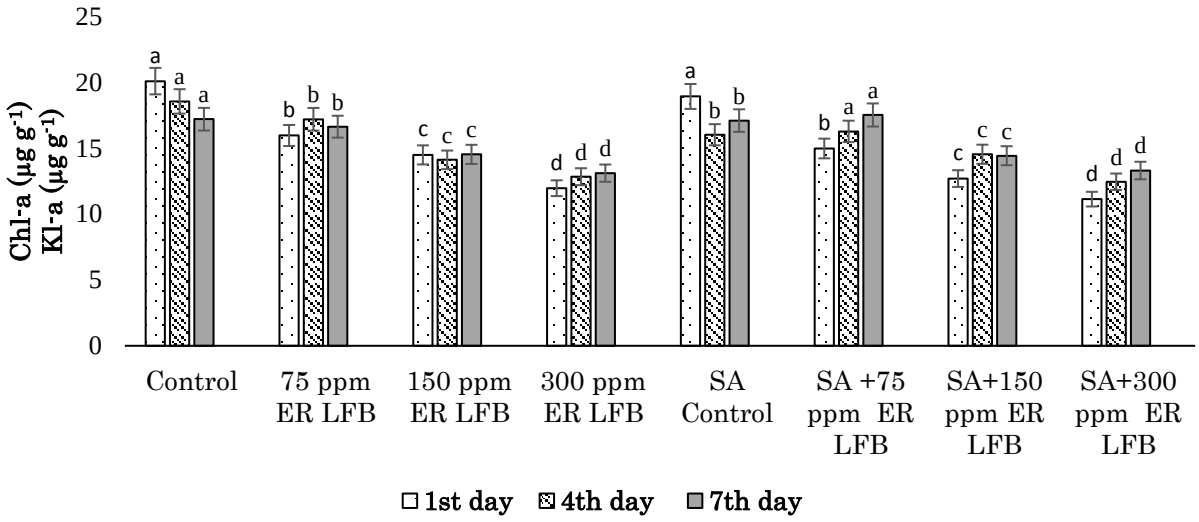

Figure 1 Changes in Chl-a in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ ERLFB
Şekil 1. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki Kl-a değişimi

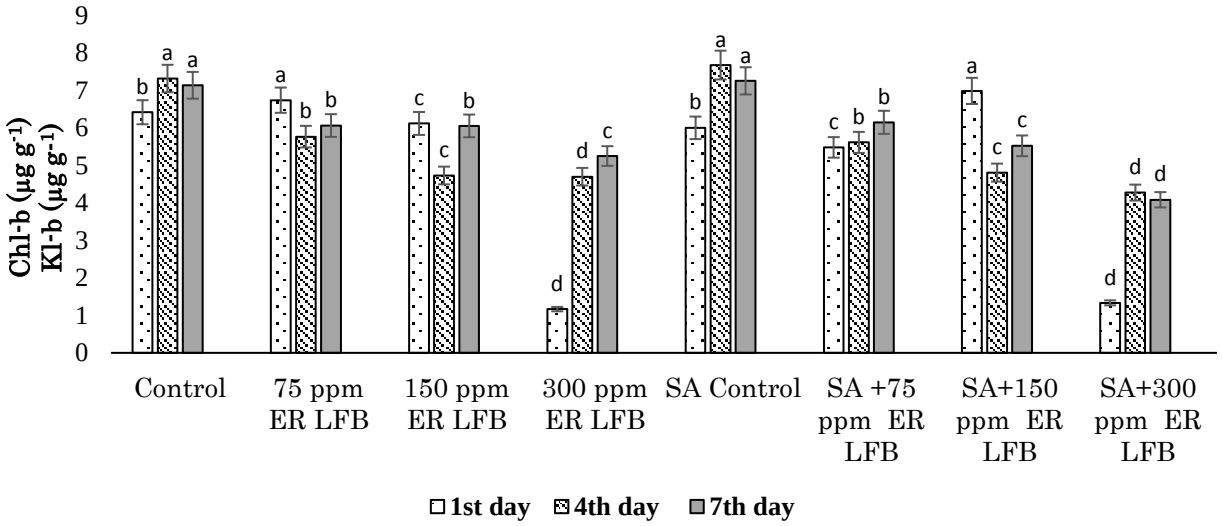

Figure 2. Changes in Chl-b in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ ERLFB.
Şekil 2. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki Kl-b değişimi

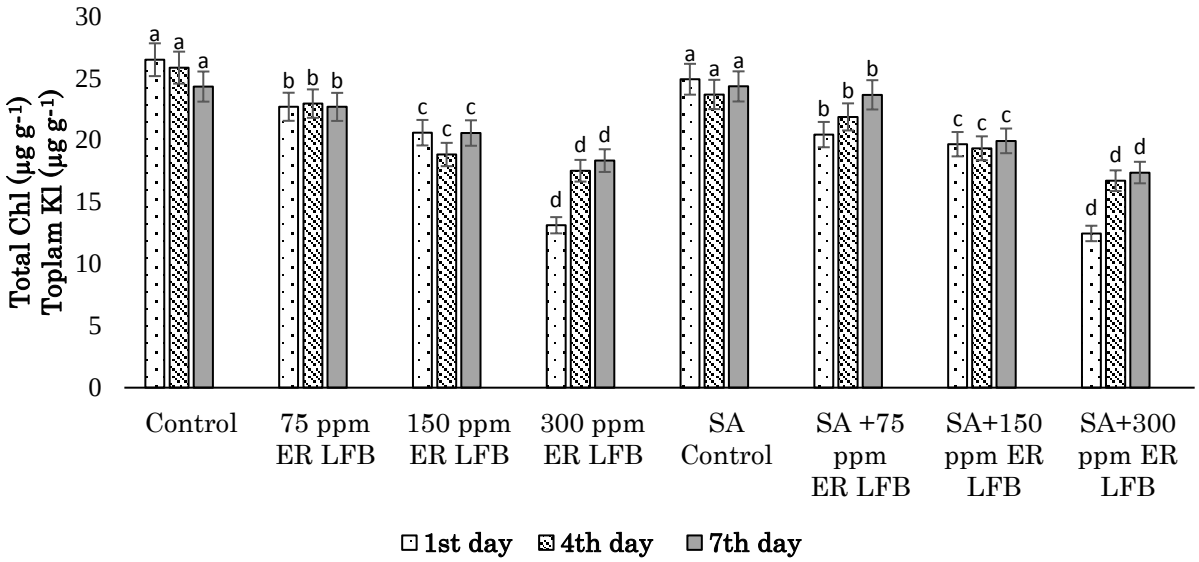

Figure 3. Changes in Total Chl in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ ER LFB.

Şekil 3. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki toplam Kl değişimi

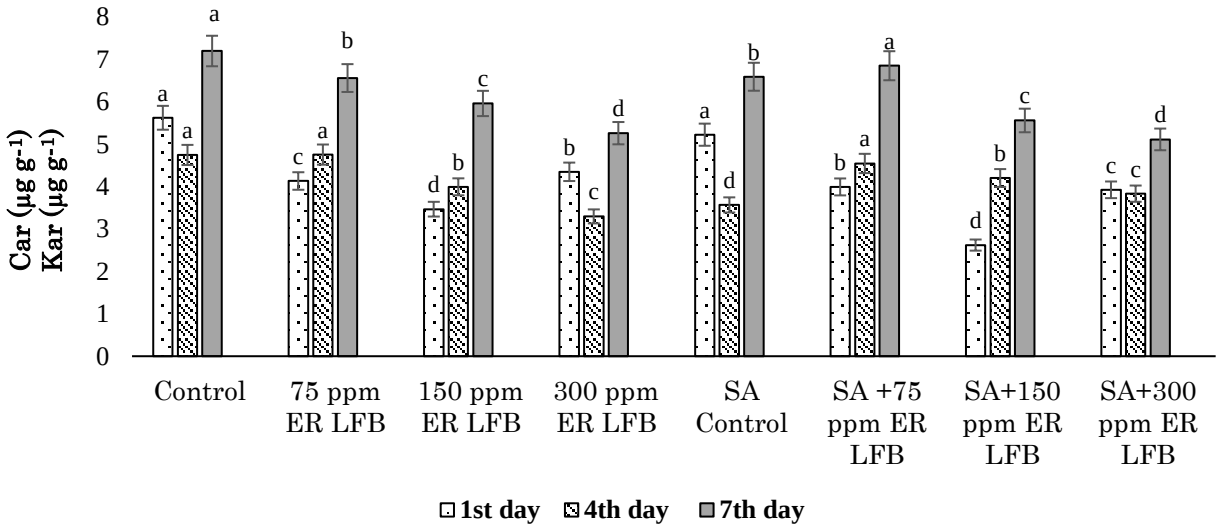

Figure 4. Changes in Car in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ ERLFB.

Şekil 4. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki Kar değişimi

Effect of ER LFB and 0.5 mM SA+ER LFB on MDA

When MDA content was evaluated in *L. minor* to which ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) were applied, it was found that the lowest MDA content was determined in the SA control groups at day 7th whereas the highest MDA content was determined in 75 ppm ER LFB groups at day 4th (Fig. 5).

Effect of ER LFB and 0.5 mM SA+ER LFB on antioxidant enzymes

In this study, POD activity was increased in both ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) groups compared with that in the control groups at 1st, 4th, and 7th days. The highest POD activity was found to be 14.51 U/mg protein in the 0.5 mM SA+ 300 ppm ER LFB groups on day 7 (Fig. 6). The SA pretreatment increased the POD activity under ER LFB stress.

The lowest GST activity was observed in the control groups. GST activity increased with each passing day. The highest GST activity was found to be 13.52 U/mg protein in the 0.5 mM SA+ 300 ppm ER LFB groups at day 7th (Fig. 7).

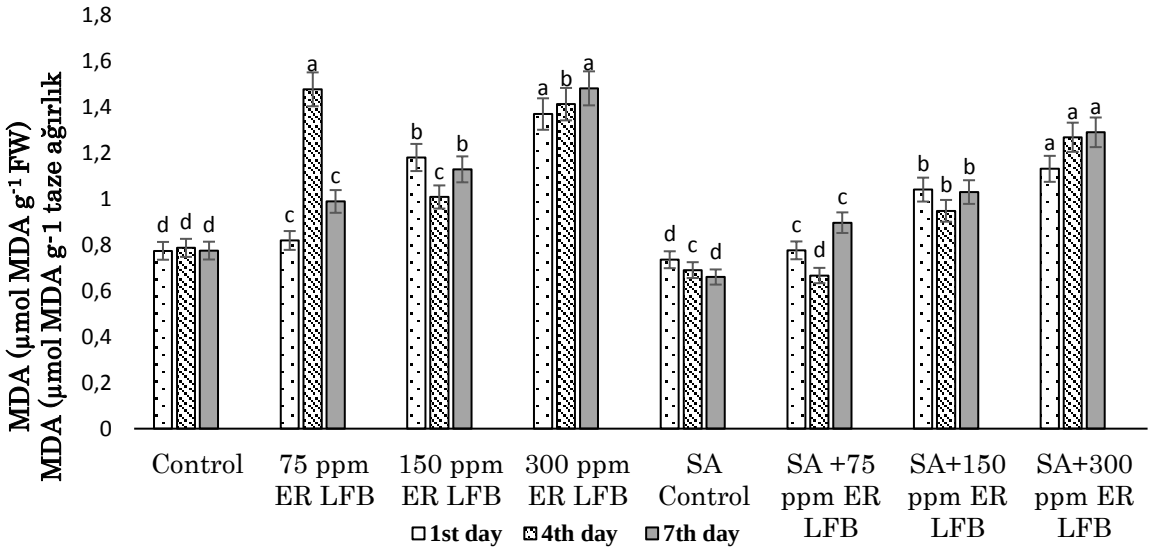

Figure 5. Changes in MDA in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ ER LFB.
Şekil 5. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki MDA değişimi

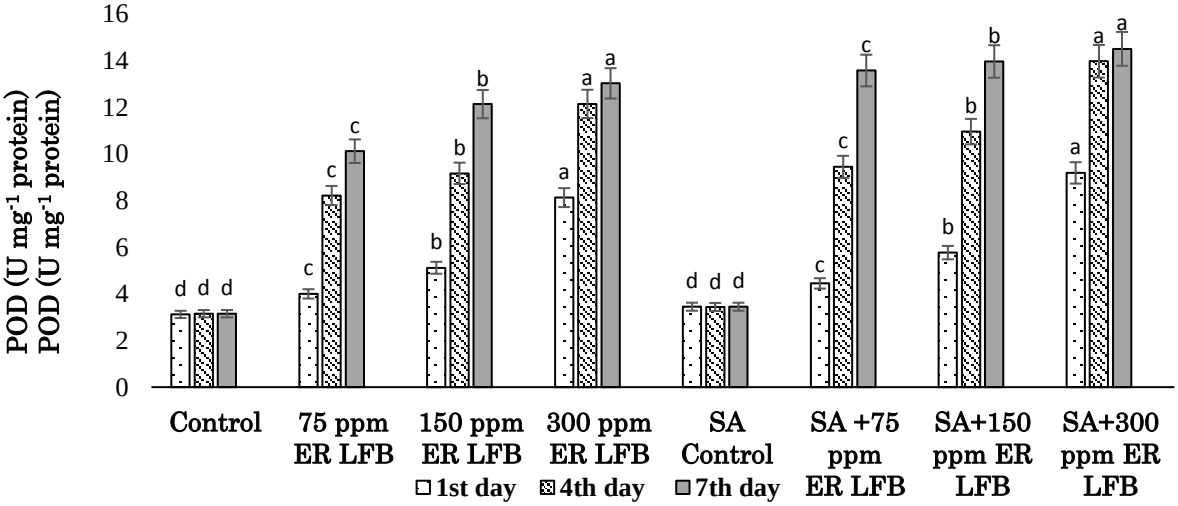

Figure 6. Changes in POD in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ ERLFB.
Şekil 6. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki POD değişimi

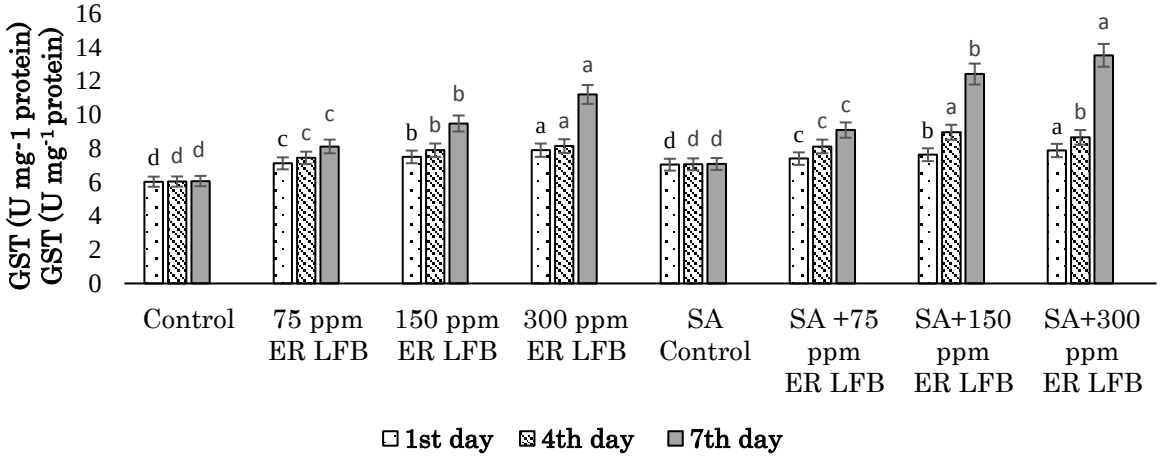

Figure 7. Changes in GST in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ ERLFB.
Şekil 7. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki GST değişimi

The lowest GR activity was found to be 0.14 U/mg protein in the 300 ppm ER LFB groups at day 7th and the highest GR activity was found to be 0.55 U/ mg protein in the SA control groups at day 1st (Fig. 8).

The GSH content was increased in both ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) groups compared with that in control groups. The highest GSH content was found to be 1,99 U/mg protein in the 0.5 mM SA+ 300 ppm ER LFB groups at day 7th (Fig. 9).

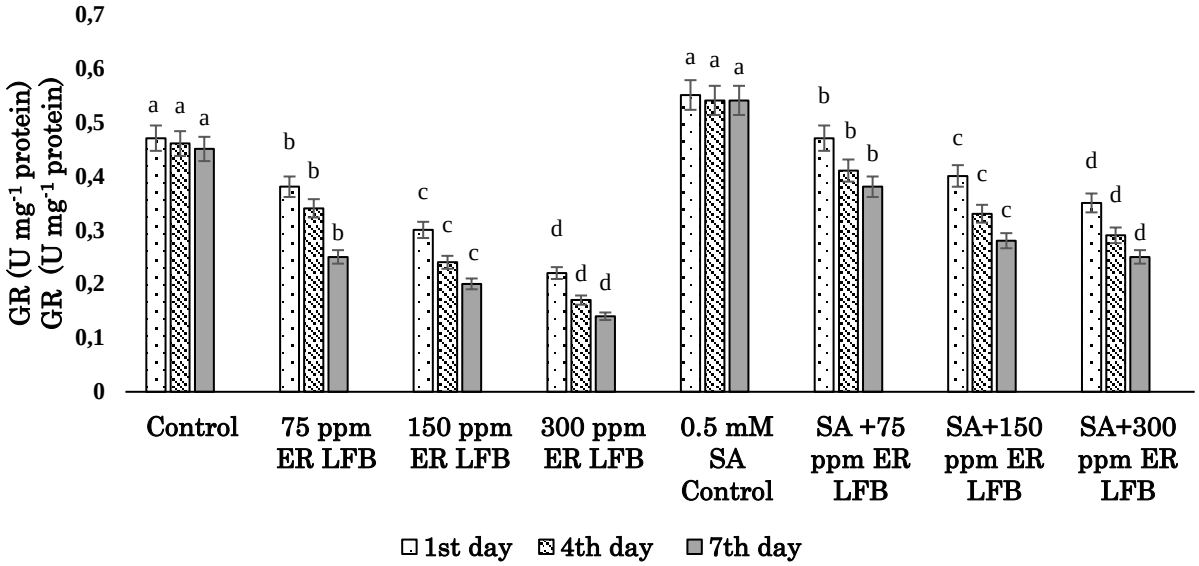

Figure 8. Changes in GR in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ER LFB
Şekil 8. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki GR değişimi

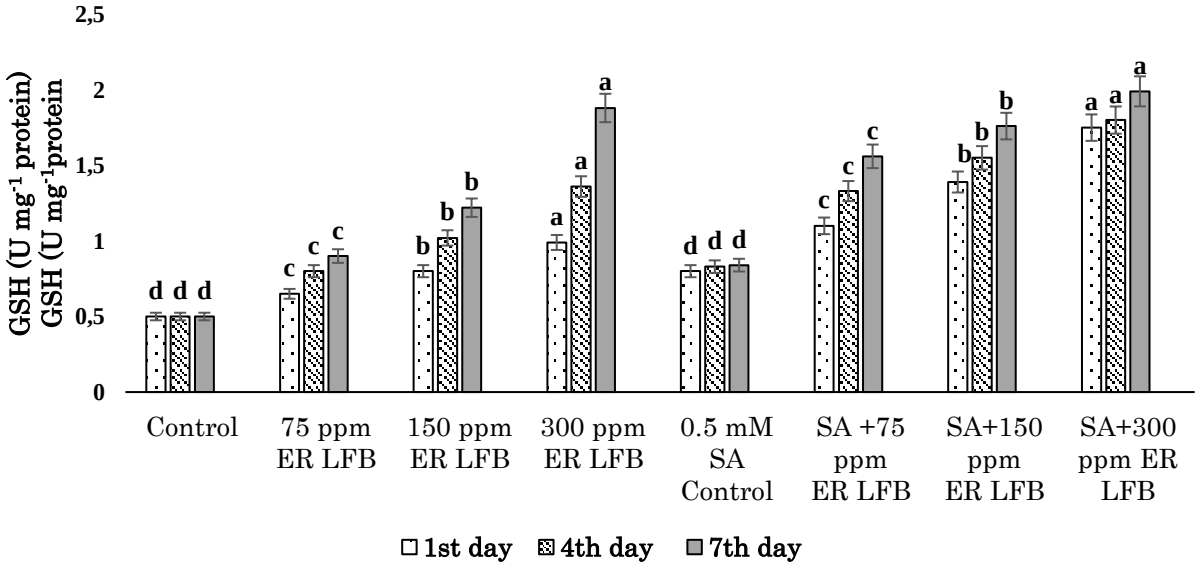

Figure 9 Changes in GSH in L. minor exposed to different concentrations of ER LFB and 0.5 mM SA+ER LFB.
Şekil 9. Farklı konsantrasyonlarda ER LFB ve 0.5 mM SA+ER LFB'ye maruz kalan L. minor'daki GR değişimi

DISCUSSION

Extensive application of synthetic dyes creates considerable environmental pollution. Removal of these dyes are difficult because of the complex chemical structures and synthetic origins (Sharma et al., 2007; Cicek et al., 2012). *L. minor* is widely used as experimental model system for ecotoxicological studies (Pomati et al., 2004; Razinger et al., 2007; Parlak, 2016). SA plays an important role in induction of plant defense against a variety of biotic and abiotic stresses (War et al., 2011; El-Beltagi et al., 2017, Klessig et al., 2018). In this study, it is observed that protective role of 0.5 mM SA on oxidative stress caused by ER LFB in *L. minor*.

The degradation of Chl pigments may finally decline photosynthetic efficiency in plants (Upadhyay and Panda, 2005; Parlak, 2016). Mouzaki-Paxinou et al. (2016) reported that after application of tritosulfuron, Chl and Car content were decreased in *L. minor*. After metribuzin application, there was a decrease in pigment content in high concentration treatments on day 3 and 5. Car protect photosynthetic apparatus against various harmful environmental factors (Strzałka et al., 2003). In our study, pigment content decreased compared to control groups (Fig. 1-4). Chlorophyll destruction might be related with chlorophyllase enzyme.

MDA is a commonly used as a significant biomarker of oxidative stress. Hou et al. (2007) reported that MDA content increased gradually with the increased concentration of copper in *L. minor*. In our study, it was observed that exogenous SA application generally increased the MDA content (Fig. 5).

POD is involved in many processes in plants including pathogen defense, wound healing (Lu et al., 2016; Pandey et al., 2017; Zahidi et al., 2018) and cell development (Criqui et al., 1992). In this study, POD activity was increased in both ER LFB (75, 150 and 300 ppm) and 0.5 mM SA+ER LFB (75, 150 and 300 ppm) groups compared with that in the control groups (Fig. 6). Similar to our findings, War et al. (2011) reported that POD activity was significantly higher sprayed with 1.5 mM SA in the chickpea (*Cicer arietinum* L.) plants. The reason of this increase may be related with metabolism in the induction of antioxidant activity by SA.

GSTs plays a role in the reduction of damage caused by pathogens (Lieberherr et al., 2003; Shahrtash, 2013). GR has a central role in maintaining the reduced state of the GSH pool under stress (Jozefczak et al., 2012). Teisseire and Guy (2000) found that GST and GR activities were inhibited by the high copper sulfate concentrations in *L. minor*. Lu et al., (2018) showed that application of Cd reduced GR activity in *L. minor*. The treatment of SA reduced the effect of Cd. GSH is an important molecule in oxidative stress (Miteva et al., 2010). Tatar et al. (2017) reported that, the GSH and GSSG levels in *L. minor* L. were found high in adapted group compared to the control group ($p<0.05$). In our study, the highest GSH and GST activities were determined after 0.5 mM SA + 300 ppm ER LFB groups at 7th day (Fig 7, 8). GR activity was decreased at 7th day in ER LFB groups (Fig. 9). Here we can say that SA is more effective on antioxidant system.

CONCLUSIONS

In conclusion, ER LFB dyestuff increased oxidative stress in *L. minor* and pretreatment with SA to reduce the injures which resulted from ER LFB treatment. Pigmentation was generally reduced by ER LFB and SA+ER LFB treatment according to control. Application of 0.5 mM SA+ 300 ppm ER LFB altered POD, GST and GSH activities compared to other days. Exogenous SA application generally increased the GR and MDA content compared to control and ER LFB groups. The data obtained from this study suggest that SA confers tolerance to ER LFB stress in *L. minor*.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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