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Exploring the defense strategies of benzalkonium chloride exposures on the antioxidant system, photosynthesis and ROS accumulation in *Lemna minor*

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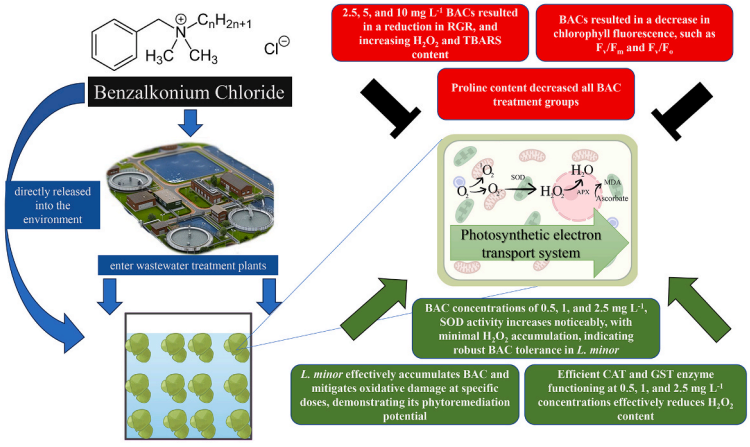
HIGHLIGHTS

- *L. minor* demonstrated elevated levels of H_2O_2 and TBARS after BAC exposure.
- Growth parameters decreased at 2.5, 5 and 10 mg L^{-1} BAC.
- Antioxidant activity increased under low-dose BAC applications.
- *L. minor* exposed to low-dose BAC applications maintain the redox state.

GRAPHICAL ABSTRACT



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ABSTRACT

With the advent of technological advancements post the industrial revolution, thousands of chemicals are introduced into the market annually to enhance different facets of human life. Among these, pharmaceutical and personal care products (PPCPs), including antibiotics and disinfectants, such as benzalkonium chlorides (BACs), are prominent. BACs, often used for surface and hand disinfection in high concentrations or as preservatives in health products such as nasal sprays and eye drops, may present environmental risks if they seep into irrigation water through prolonged exposure or improper application. The primary objective of this study is to elucidate the tolerance mechanisms that may arise in *Lemna minor* plants, known for their remarkable capability to accumulate substances efficiently, in response to exogenously applied BACs at varying concentrations. The study applied six different concentrations of BACs, ranging from 0.25 to 10 mg L^{-1}. The experimental period spanned seven days,

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during which the treatments were conducted in triplicate to ensure reliability and reproducibility of the results. It was observed that low concentrations of BACs (0.25, 0.5 and 1 mg L^{-1}) did not elicit any statistically significant changes in growth parameters. However, higher concentrations of BACs (2.5, 5, and 10 mg L^{-1}) resulted in a reduction in RGR by 20%, 28%, and 36%, respectively. Chlorophyll fluorescence declined significantly at BAC doses of 5 and 10 mg L^{-1}, with F_v/F_m ratios decreasing by 9% and 15%, and F_v/F_o ratios by 40% and 39%, respectively. Proline content decreased in all treatment groups, with a 46% reduction at 10 mg L^{-1} BAC. TBARS and H_2O_2 contents increased proportionally with BAC dosage, showing the highest increases of 30% and 40% at 10 mg L^{-1}, respectively. The noticeable increase in SOD enzyme activity at BAC concentrations of 0.5, 1, and 2.5 mg L^{-1}, with increases of 2.7-fold, 2.2-fold, and 1.7-fold respectively, along with minimal accumulation of H_2O_2, suggests that *L. minor* plants have a strong tolerance to BAC. This is supported by the efficient functioning of the CAT and GST enzymes, especially evident at the same concentrations, where increased activities effectively reduce the buildup of H_2O_2. In the AsA-GSH cycle, although variations were observed between groups, the contribution of the GR enzyme to the preservation of GSH content by recycling GSSG likely maintained redox homeostasis in the plant, especially at low concentrations of BACs. The study revealed that *L. minor* effectively accumulates BAC alongside its tolerance mechanisms and high antioxidant activity. These results underscore the potential for environmental cleanup efforts through phytoremediation.

1. Introduction

Benzalkonium chlorides (BACs) encapsulate a category of positively charged surface-active agents, forming a subset within quaternary ammonium compounds (QACs). BAC functions as a cationic surfactant, holding counterparts with a variety of alkyl chain lengths, extending from C8 to C18 (Pereira and Tagkopoulos, 2019). Surfactants find application in diverse domestic and industrial settings owing to their distinctive physicochemical characteristics (Collivignarelli et al., 2019). On the other hand, pharmaceutical and personal care products (PPCPs) generate numerous emerging pollutants annually, despite their intended purpose of combating human diseases and enhancing societal well-being (Chakraborty et al., 2023). Currently, experts in marine and freshwater resources are directing their attention towards PPCPs that enter the aquatic environment (Tong et al., 2022). Besides, the durability of the altered goods within a setting raises significant worries regarding the sustainability of the environment and the well-being of ecosystems. Primarily due to the increased use of PPCPs, substantial levels of these compounds, originating predominantly from urban or industrial sources and household wastewater, may find their way into municipal wastewater treatment plants (MWTPs) or be directly released into the environment (Camacho-Muñoz et al., 2014). The possibility exists that PPCPs might infiltrate drinking water through MWTPs, posing a potential health hazard to humans, animals, aquatic organisms, and the plant ecosystem. Significantly, the amounts of PPCPs in reclaimed water vary from ng L^{-1} to μg L^{-1}, influenced by the physical and chemical characteristics of PPCPs and how the treatment systems are run (Christou et al., 2017). Nowadays, PPCPs are often found at significant levels in both water and land environments due to their constant release from the disposal of processed city sewage, biosolids, and animal waste (Liu et al., 2020b).

To cope with this, various remediation methods have been proposed. Historically, bioremediation has been a common choice for surfactant removal. Presently, available remediation techniques span physical, chemical, biological, and membrane methods (Siyal et al., 2020). Researchers have conducted numerous studies investigating the removal of PPCPs from the environment, exploring topics such as their commonness, harmful environmental effects, and methods of destruction in both urban wastewater and agricultural settings (Kar et al., 2020; Liu et al., 2020a; Osuoha et al., 2023; Yuan et al., 2020). Moreover, the emergence of issues associated with the release of BAC, has prompted consideration of strategies to mitigate its presence before it enters wastewater treatment systems.

Alternative treatment methods, often used in conjunction with traditional biological processes, have shown promise in enhancing the removal of BAC from wastewater. For instance, research by Carbajo et al. (2016) demonstrated that increasing the ozone dosage to 18 mg L^{-1} resulted in a substantial 65% reduction in BAC concentrations. This suggests that ozonation, a powerful oxidation process, can be a valuable tool in BAC degradation. Further exploration of advanced oxidation processes (AOPs) has yielded additional promising results. Hong et al. (2017) investigated the use of ferric-activated persulfate and hydrogen peroxide combined with iron(II). These AOPs proved highly effective, achieving over 90% and over 80% BAC removal, respectively, within a 60-min timeframe. These findings highlight the potential of AOPs to rapidly and significantly break down BAC. Integrating alternative methods with existing biological treatment processes offers a multi-pronged approach to address BAC contamination in wastewater. Another method involves leveraging the capabilities of *Pseudomonas spp.*, key BAC-degrading organisms found in various environments (Moghadam et al., 2023). Biological processes can be disrupted due to decreased metabolic activity, impacting microorganisms' ability to treat wastewater effectively and generate biogas (Chen et al., 2018; Flores et al., 2015). However, prolonged exposure to BAC can induce tolerance and process recovery through resistance development or biodegradation, as shown in studies with membrane bioreactors and biological denitrogenation systems (Chen et al., 2018; Hajaya and Pavlostathis, 2012). *Pseudomonas spp.* have been identified as one of the most prevalent BAC-degrading organisms, isolated from environments such as recycled activated sludge, contaminated river sediment, and soil (Ertekin et al., 2016; Khan et al., 2015). Employing biological treatment methods offers advantages like lowering operational costs and promoting environmentally sustainable practices. Moreover, biological treatment methods offer advantages such as reduced costs and environmental sustainability. Phytoremediation, a subset of bioremediation, employs plants to remediate polluted environments (Kurade et al., 2021). Various plant species are utilized to extract contaminants from affected media, encompassing both terrestrial and aquatic sources. Chosen plants have the capacity to accumulate a wide array of pollutants, whether specific or broad in scope (Kafle et al., 2022). Among these, duckweed (*Lemna minor*) stands out as particularly significant; it holds the distinction of being the tiniest angiosperm or flowering plant within the plant kingdom (Ekperusi et al., 2019). Duckweed, a diminutive floating monocotyledonous plant, forms a dense canopy in nutrient-rich freshwater and brackish environments. Comprising one or a few leaves, referred to as fronds, and lacking a stem while having a single root or rootlet, this plant reproduces vegetatively by directly dividing to produce separate individual plants (Xu et al., 2023). In economic terms, it can grow indefinitely when provided with appropriate nutrients, light, and water, producing an unlimited supply of duckweed that can be utilized at any time. Recognized for their straightforward cultivation and robust bioremediation capabilities, *L. minor* has found extensive use in addressing diverse chemical pollutants (Ekperusi et al., 2019). Supalkova et al. (2008) demonstrated the effectiveness of *L. minor* in eliminating cisplatin from wastewater. Reinhold and Saunders (2006) recorded the successful eradication of

3-fluorophenol and 3-trifluoromethylphenol by *L. minor*. Amy-Sagers et al. (2017) noted a higher absorption of sucralose compared to fluoxetine by *L. minor*, particularly at elevated concentrations. In a comparative study by Reinhold et al. (2010) on the phytoremediation of emerging organic pollutants, using two duckweed species, *L. minor* and *L. punctata* displayed a comparable removal pattern for substances like ibuprofen, fluoxetine, triclosan, and 2,4-D (2,2,4-dichlorophenoxyacetic acid) over a 9-day period. Both plant species exhibited the capacity to eliminate roughly half of the pollutants in constructed wetlands.

The preceding research conducted by Richter et al. (2016) utilized *Lemna* plants to investigate the phytotoxicity and uptake of BACs, while Khan et al. (2018), focusing on *Lactuca sativa* and *Lepidium sativum*, documented a significant reduction in plant dry weight following a 12-day exposure period, with *L. sativa* experiencing a 68% reduction and *L. sativum* a 75% reduction. Simultaneously, evident toxicity symptoms, encompassing necrosis, chlorosis, and wilting, were observed. Despite the various BAC removal methods currently employed, including biological degradation and advanced oxidation processes, a significant knowledge gap persists regarding the efficacy of phytoremediation techniques in effectively removing BAC from contaminated environments. Further research is needed to elucidate the mechanisms involved in plant uptake, metabolism, and potential degradation of BAC, as well as to identify plant species that exhibit high efficiency in BAC removal. This understanding could pave the way for sustainable and eco-friendly strategies to mitigate BAC pollution. However, there remains a persistent ambiguity surrounding the comprehensive understanding of the physiological and biochemical alterations induced by BACs in plants, including the observation of toxicity symptoms such as necrosis, chlorosis, and wilting, and their subsequent implications on photosynthetic parameters. Furthermore, more research is needed to explore the mechanisms underlying detoxification and phytoremediation potential in *L. minor* when confronted with different levels of BAC exposure. The limited understanding of tolerance mechanisms underscores the necessity for broader investigations in this area. Consequently, the present study was undertaken to comprehensively evaluate the effects of exogenous BAC exposure on *L. minor*. plants, expanding upon existing knowledge from previous research and elucidating the tolerance mechanism by monitoring various physiological and biochemical parameters. This evaluation included the assessment of oxidative stress markers such as thiobarbituric acid reactive substances (TBARS) and H_2O_2 content, as well as the activity levels of both enzymatic and non-enzymatic antioxidant systems. Additionally, the efficiency of the photosynthetic apparatus was examined to determine the impact of BAC on plant growth and overall health. By analyzing these diverse parameters, this study aimed to provide a detailed understanding of the complex interplay between BAC exposure and plant responses, ultimately contributing to the development of effective phytoremediation strategies by identifying potential BAC accumulation mechanisms and evaluating the phytoremediation potential of *L. minor*.

2. Material and methods

2.1. Plant material and experimental design

Duckweed (*Lemna minor* L.) cultures were cultivated in a hydroponic Hoagland solution under tightly controlled environmental conditions. The seedlings were transferred to a half-strength Hoagland solution and cultivated under controlled growth chamber conditions, featuring a 16/8-h light/dark regime at 24 °C, 70% relative humidity, and a photosynthetic photon flux density of 350 μmol m^{-2} s^{-1}. Hoagland solution, according to Jie et al. (2008). protocol, the Hoagland solution comprised 2.5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 0.78 mM KH_2PO_4, 2 mM $MgSO_4$, 29.6 μM H_3BO_3, 10 μM $MnSO_4$, 50 μM FeEDTA, 1.0 μM $ZnSO_4$, 0.05 μM H_2MoO_4, and 0.95 μM $CuSO_4$. The concentrations of benzalkonium chlorides (BACs) used in the study were selected based on previous research by Khan et al. (2018). The chosen concentrations included BACs at 0.25, 0.5, 1, 2.5, 5, and 10 mg L^{-1}. Following a seven-day treatment period, the plants were harvested for further analysis, with the study conducted in triplicate to ensure accuracy and comprehensiveness (Jie et al., 2008).

2.2. Extraction of BAC from the plant and analysis by LC-MS

To extract BAC from plant tissues, the method developed by Díez et al. (2016) was adapted with certain modifications. Since BAC was applied to plants at very low concentrations, around 20 g of fresh plant sample was weighed for the extraction. Weighed samples from both control and BAC-applied were homogenized by grinding with a mortar in liquid nitrogen, then transferred into falcon tubes of appropriate volume. The samples were treated with a sufficient volume of acetonitrile containing 1% high purity acetic acid and gently mixed for 15 min. Afterwards, in order to increase the rate of BAC passing into the organic phase, QuEChERS extraction salt (Part Number:5982–6755, Agilent, USA) containing 6 g $MgSO_4$ and 1.5 g NaAcetate was added to all samples and mixing was continued for 5 min. The mixtures were centrifuged at 4000 rpm for 5 min to separate the phases and the supernatant was taken into a separate test tube and evaporated at room temperature in a nitrogen atmosphere. The remaining pellet was dissolved in 2 mL of ultrapure water and passed through a syringe filter containing a nylon membrane with a pore size of 0.45 μm and placed in a capped glass vial with a volume of 1.5 mL, making it ready for chromatographic analysis. Standard BAC was obtained and diluted with ultrapure water at different rates to create calibration solutions to be used in the chromatographic analyzes to determine the BAC uptake in the plant samples.

A Shimadzu (Kyoto, Japan) LCMS-8040 liquid chromatograph-mass spectrometer (LC-MS) system equipped with a Raptor Biphenyl (2.7 μm, 100 mm × 2.1 mm) column was used to analyze the BAC concentration in the samples. The mobile phase gradient was as follows: Started with 100% 10 mM ammonium formate dissolved in ultrapure water for 0–0.5 min and increased to 100% 10 mM ammonium formate dissolved in LC-MS grade methanol over 3.5 min, held for 3 min, and decreased to 100% 10 mM ammonium formate dissolved in ultrapure water over 3 min, with a constant flow rate of 0.45 mL min^{-1}. The column thermostat was set at 37 °C and the sample injection volume was 1.0 μL. The temperatures of the desolvation line and heat block in the MS were 250 and 400 °C, respectively. The flow rates of the nebulizing and drying gases were 3 and 15 L min^{-1}, respectively. The full MS scan mode was performed.

2.3. Analysis of physiological parameters

Physiological parameters, including the relative growth rate (RGR) of the leaves, and osmotic potential (Ψ_Π), were recorded. RGR was determined following previously established procedures (Hunt et al., 2002), and Ψ_Π was measured using a Vapro Vapor Pressure Osmometer 5600 (Santa-Cruz et al., 2002). For the determination of proline (Pro) content, 500 mg of fresh leaf samples were collected in triplicate. The samples were extracted following the method described by Chandrakar et al. (2016), utilizing ninhydrin reagent. The Pro content was then calculated from the absorbance values recorded at 520 nm, using a standard curve of L-proline.

2.4. Recording data for chlorophyll a fluorescence parameters and OJIP test

A portable fluorometer, specifically the Handy PEA (Plant Efficiency Analyser, Hansatech Instruments Ltd., Norfolk, UK), was utilized to assess key photosynthetic parameters. This device facilitated the measurement of the maximal quantum yield of PSII photochemistry (F_v/F_m), providing insights into the physiological state of the photosynthetic apparatus through the ratio of photosynthetic apparatus (F_o/F_m).

Additionally, it helped determine the potential photochemical efficiency via the potential photochemical efficiency (F_v/F_o) ratio. Detailed descriptions for the estimated parameters can be found in Supplementary File S1. The radar plots visually represent the mean parameter values for various treatments in leaves.

2.5. Analysis of oxidative stress biomarkers

Determination of H_2O_2 content was measured according to Liu et al. (2010). Leaves were homogenized in cold acetone and centrifuged. The supernatant was mixed with titanium reagent and then ammonium hydroxide was added to precipitate the titanium-peroxide complex. The reaction mixture was centrifuged. The pellet was washed with cold acetone and dissolved. The absorbance of the solution was measured at 410 nm. H_2O_2 concentrations were calculated using a standard curve prepared with known concentrations of H_2O_2.

Lipid peroxidation (thiobarbituric acid reactive substances (TBARS) content) was determined according to Rao and Sresty (2000). TBARS concentration of leaf samples was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

2.6. Analysis of enzyme/non-enzyme antioxidants

0.5 g leaf samples were extracted in Tris-HCl (25 mM Tris, 1% Triton-X100, pH: 7.4) and centrifuged at 14000 g for 30 min. Supernatants were collected, and total protein contents were measured by the Bradford (1976) method.

For SOD (EC 1.15.1.1) isozyme activity, samples were subjected to non-denaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). Total SOD activity assay was based on the method of Beauchamp and Fridovich (1971). CAT isozymes were detected according to Woodbury et al. (1971). Total CAT (EC 1.11.1.6) activity was estimated according to the method of Bergmeyer (1970). The isozymes and enzyme activity of POX (EC 1.11.1.7) were based on the method described by Seevers et al. (1971) and Herzog and Fahimi (1973), respectively. NADPH oxidase (NOX) isozymes were identified as described by Sagi and Fluhr (2001). NOX (EC 1.6.3.1) activity was measured according to Jiang and Zhang (2002). The enzyme/isozyme activities of glutathione S-transferase (GST, EC 2.5.1.18) and glutathione peroxidase (GPX, EC 1.11.1.9) were determined (Hossain et al., 2006; Ricci et al., 1984).

Electrophoretic APX separation was performed according to Mittler and Zilinskas (1993). APX (EC 1.11.1.11) enzyme activity was measured according to Nakano and Asada (1981). GR (EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976). Isozymes compositions of GR were determined by native PAGE analysis (Hou et al., 2004).

Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was assayed by the method of Miyake and Asada (1992). Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was measured according to Dalton et al. (1986). Total and reduced ascorbate (AsA) contents were done according to the method of Dutilleul et al. (2003) with modifications. The oxidized form of ascorbate (DHA, dehydroascorbate) was measured using the formula DHA = Total AsA-Reduced AsA. The glutathione (GSH) was assayed according to Paradiso et al. (2008). Oxidized glutathione (GSSG) was determined after the removal of GSH by 2-vinylpyridine derivatization. GSH redox state (%) was determined by calculating the ratio of GSH to total glutathione (GSH + GSSG) according to Shi et al. (2013).

Gels stained for SOD, CAT, POX, APX, GR, GST, and NOX activities were photographed with the Gel Doc XR + System and then analyzed with Image Lab software v4.0.1 (Bio-Rad, California, USA). Known standard amounts of enzymes (0.5 units of SOD and 0.2 units of CAT and POX) were loaded onto gels. For each isozyme set/group, the average values were significantly different at $p < 0.05$ using Tukey's post-test.

2.7. Statistical analysis

The information on statical analysis was given in Supplementary File S1.

3. Results

BAC concentrations determined as a result of 3 replicate LC-MS analysis of the plant samples to which BAC was applied at different concentrations are given in Table 1. When the control sample, which was not exposed to BAC, was examined, it was confirmed that BAC was not detected. However, BAC was detected in the plant samples treated with 0.25 and 0.50 mg L^{-1} BAC, indicating its presence within the plant. However, there is a significant and proportional increase in the BAC levels detected in the plant samples to which BAC was applied at 1 mg L^{-1} and above. Chromatographic analysis data in Table 1 indicate that approximately similar levels of BAC accumulation occurred in the plant tissue at both 5 and 10 mg L^{-1} (3.93 and 3.68 μg 100 g^{-1}) BAC applications, suggesting a plateau in the plant's capacity for BAC accumulation. These results demonstrate that BAC, particularly at concentrations of 1 mg L^{-1} and above, was absorbed and translocated within the plant.The LC-MS chromatogram of the plant sample to which 10 mg L^{-1} BAC was applied is given in Fig. 1. As can be seen from the relevant figure, the retention time of BAC is 4 min. The chromatograms of other plant samples to which BAC was applied are given in Supplementary File S2.

Upon the application of BACs to *L. minor* plants, discernible variations in RGR were noted. Specifically, in the groups subjected to lower BAC concentrations of 0.25, 0.5, and 1 mg L^{-1}, no statistically significant alteration in RGR was observed when compared to the control group, suggesting these concentrations did not impact plant growth rate. Conversely, higher dose groups, namely BAC 2.5, BAC 5, and BAC 10, exhibited a notable dose-dependent decline in RGR, with reductions of 20%, 28%, and 36%, respectively (Fig. 2A). This indicates that higher BAC concentrations increasingly inhibited plant growth rate. Further investigation into the impact of various BAC treatments unveiled noteworthy distinctions in the osmotic potential (Ψ_Π) of *L. minor* leaves (Fig. 2B). Lower concentrations of 0.25 and 0.5 mg L^{-1} BAC did not elicit any discernible change in Ψ_Π, suggesting minimal impact on cellular water balance at these levels. However, the application of BAC treatments at higher concentrations of 1, 2.5, 5, and 10 mg L^{-1} resulted in adverse effects, with dose-dependent negative increases observed in Ψ_Π. The most significant change was observed in the BAC 10 group, which exhibited a notable 48% decrease in Ψ_Π compared to the control, indicating a severe disruption of osmotic balance and potential cellular dehydration. The repercussions of varying concentrations of BACs on the *L. minor* species were further elucidated through an examination of their distinct impacts on proline (Pro) content (Fig. 2C). All concentrations of BACs demonstrated a discernible decrease in Pro content, a key osmoprotectant involved in stress response. The most substantial reduction was observed at the highest BAC concentration of 10 mg L^{-1}, with a decrease of 46% compared to the control group, suggesting a compromised ability to cope with osmotic stress induced by BAC exposure.

In comparison to the control group, plants subjected to BACs exhibited noticeable effects on various parameters related to the

Table 1
BAC uptake of the plant samples as a result of LC-MS analysis.

BAC applied (ppm)	BAC uptake (μg/100g plant)
Control	–
0.25	0.22 ± 0.20
0.50	0.51 ± 0.17
1.00	0.70 ± 0.18
2.50	1.68 ± 0.18
5.00	3.93 ± 0.19
10.00	3.68 ± 0.18

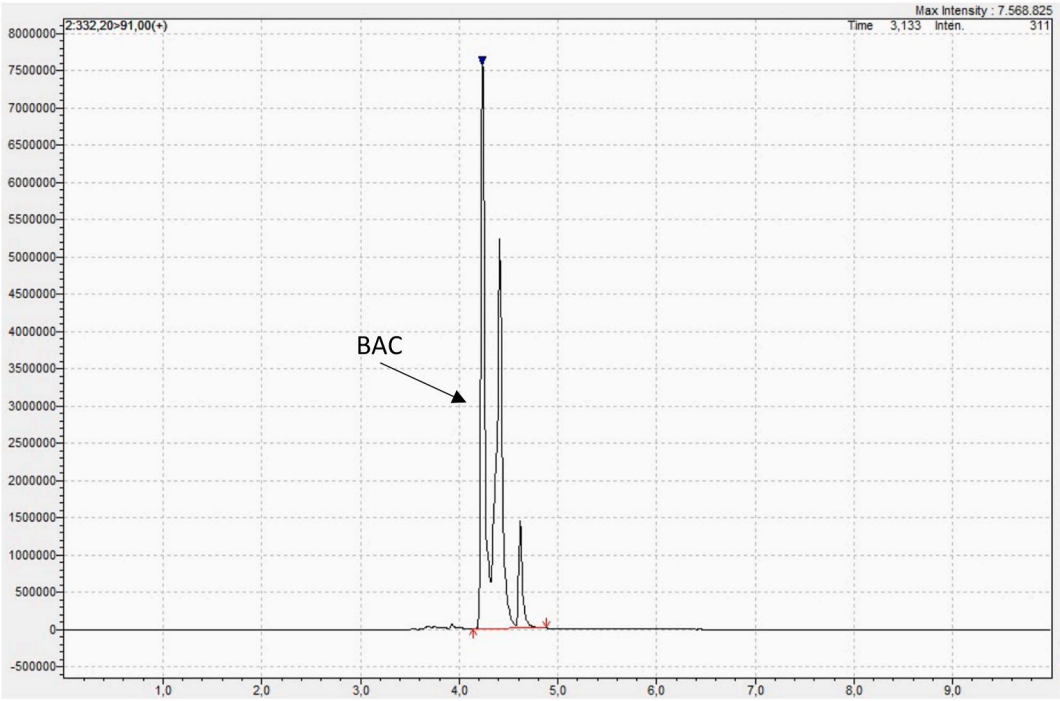

Fig. 1. The LC-MS chromatogram of the plant sample to which 10 mg L^{-1} BAC was applied.

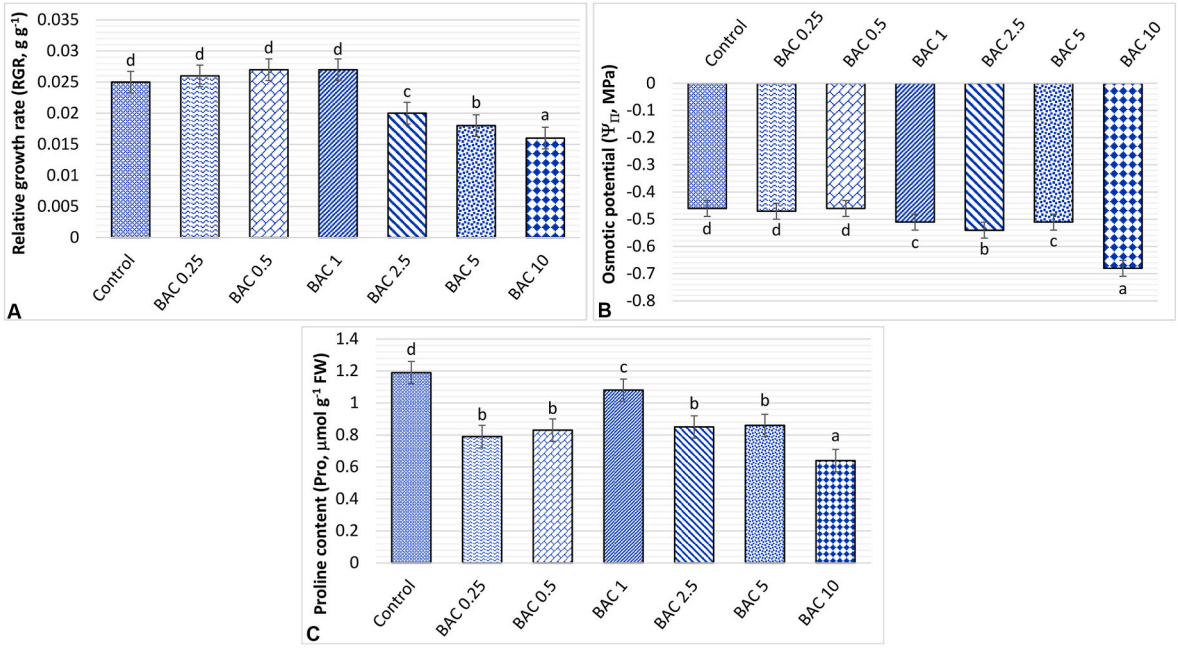

Fig. 2. The relative growth rate (RGR, **A**), osmotic potential (Ψ_{Π}, **B**), and proline content (Pro, **C**) in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

photosynthetic apparatus, including F_v/F_m, F_v/F_o, and F_o/F_m values (Fig. 3). Notably, the maximum quantum yield of photosystem II (F_v/F_m) was adversely impacted by all applications of BACs, resulting in a dose-dependent decrease compared to the control group. The most significant reduction was observed at the highest BAC concentration of 10 mg L^{-1}, with a decrease of 15% (Fig. 3A), indicating a substantial impairment of photosynthetic efficiency. Similarly, the efficiency of the water-splitting complex on the donor side of photosystem II (F_v/F_o) exhibited a decline with increasing doses of exogenously applied BACs. The most substantial reductions were recorded at concentrations of 5 and 10 mg L^{-1}, where decreases of 40% and 39% were respectively noted (Fig. 3B), suggesting a disruption of electron transport and potential damage to the photosynthetic machinery. Conversely, despite variations in minimum fluorescence (F_o/F_m) values depending on the dose of BACs, it was noted that they increased compared to the control group across all experimental groups (Fig. 3C). This increase in F_o/F_m might indicate a disruption of energy transfer within the photosynthetic apparatus or an accumulation of inactive chlorophyll molecules due to BAC-induced stress.

Radar graphs were generated utilizing OriginPro 2022, with the

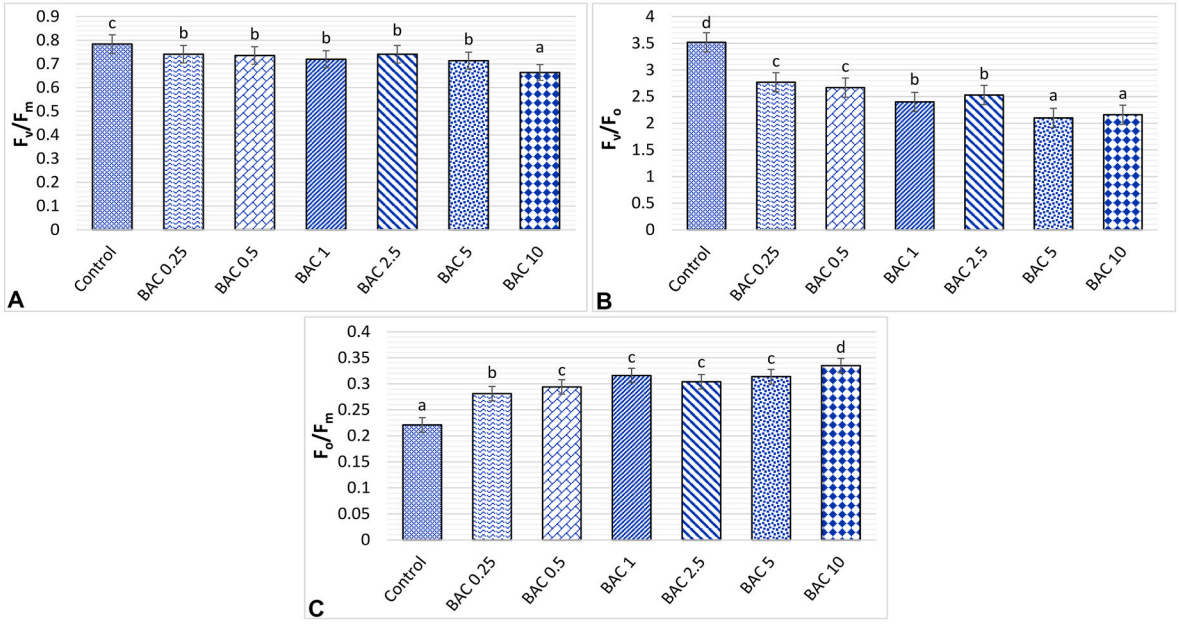

Fig. 3. The maximal quantum yield of PSII photochemistry (F_v/F_m, A), potential photochemical efficiency (F_v/F_o, B) and physiological state of the photosynthetic apparatus (F_o/F_m, C), in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

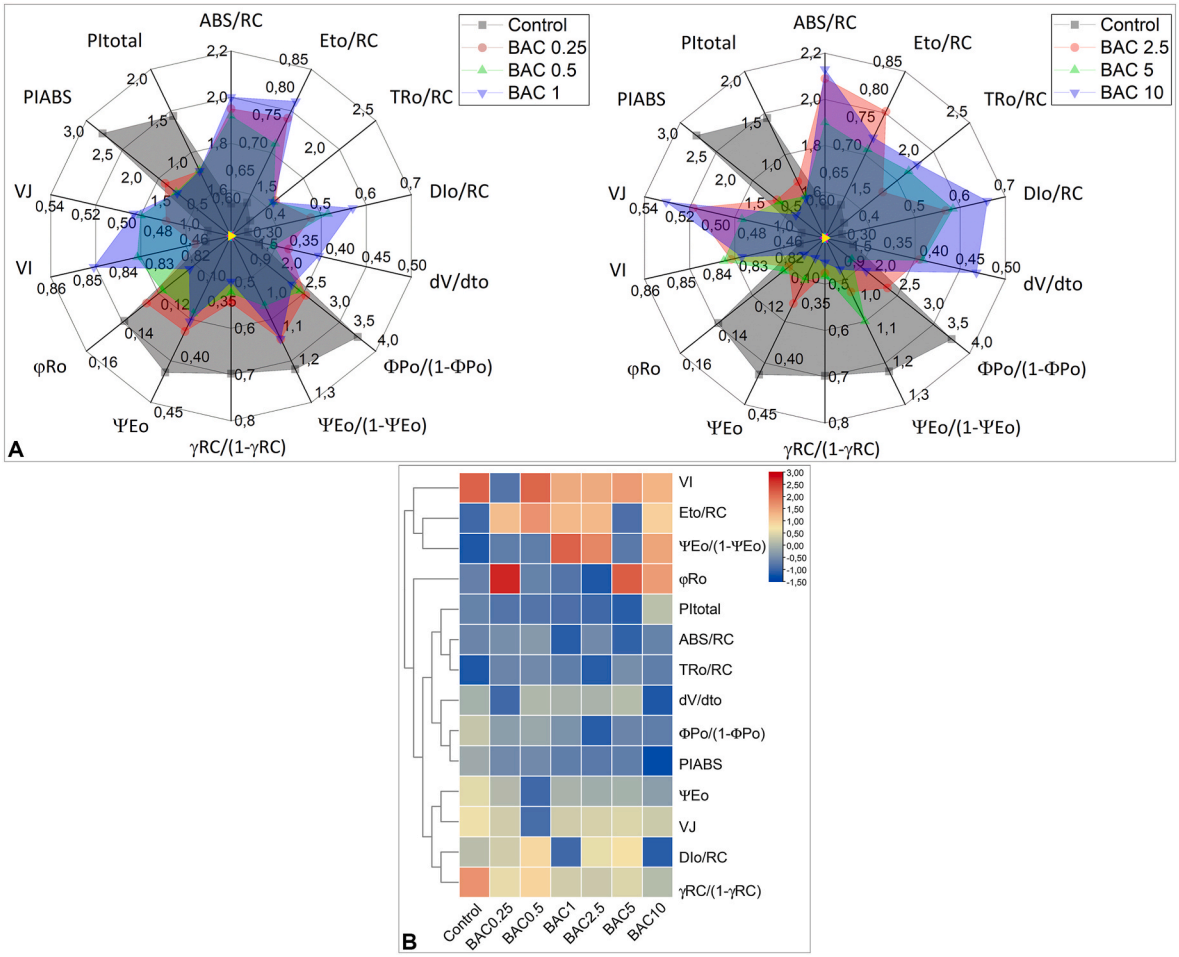

Fig. 4. OJIP transient radar plots (A) and cluster map of differences and similarities (Heat Map) (**B**), in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

treatment groups depicted in Fig. 4A. The exogenous application of varying doses of BACs resulted in increased values of ABS/RC, ET_o/RC, TR_o/RC, DI_o/RC, RE_o/RC, dV/dt_o, V_J and V_I. This suggests a dose-dependent intensification of light absorption, electron transport, and overall excitation pressure on the photosystem. Conversely, parameters such as $\Phi P_o/(1-\Phi P_o)$, $\Psi E_o/(1-\Psi E_o)$, $\gamma RC/(1-\gamma RC)$, ΨEo, ϕR_o, PI_{ABS}, and PI_{total} exhibited reductions in *L. minor* subjected to BACs. This indicates a decline in the efficiency of energy transfer, trapping, and conversion within the photosynthetic apparatus, likely due to BAC-induced stress and damage. Additionally, these findings are visually represented in Fig. 4B, where the differences in OJIP fluorescence transient induced by BACs applications are unveiled through cluster heat map analysis. The heat map highlights the differential responses of various photosynthetic parameters to increasing BAC concentrations, providing a comprehensive overview of the complex interplay between BAC exposure and plant physiology.

Compared to the control group, plants exposed to BACs exhibited dose-dependent effects on hydrogen peroxide (H_2O_2) and thiobarbituric acid reactive substances (TBARS) content, indicators of oxidative stress and lipid peroxidation, respectively (Fig. 5). Low-dose treatments of BACs at 0.25, 0.5, and 1 mg L^{-1} did not result in any statistically significant changes in H_2O_2 or TBARS content, suggesting minimal oxidative damage at these concentrations. However, H_2O_2 content increased significantly with the exogenously applied highest doses of BACs at 2.5, 5, and 10 mg L^{-1}. The most substantial increment was observed in the 10 mg L^{-1} BAC treatment, showing a 40% increase compared to the control (Fig. 5A). This indicates a heightened production of reactive oxygen species (ROS) and potential cellular damage at higher BAC concentrations. Similarly, TBARS content, a marker of lipid peroxidation and membrane damage, increased with the application of BACs at concentrations of 2.5, 5, and 10 mg L^{-1} in *L. minor* plants (Fig. 5B). This suggests that higher BAC doses induce oxidative stress, leading to lipid peroxidation and potential disruption of cellular membranes.

Fig. 6A elucidated the detection of six total SOD isozymes in *L. minor*, represented by two bands for Mn-SOD and four bands for Fe-SOD. The application of lower BAC doses (0.25, 0.5, and 1 mg L^{-1}) resulted in a dose-dependent augmentation of total SOD activity, as illustrated in the figure. Notably, the most substantial increase was observed at the 0.5 mg L^{-1} BAC treatment, exhibiting a 2.7-fold rise compared to the control group, suggesting a stimulated antioxidant response to mitigate oxidative stress. However, at higher BAC doses of 5 and 10 mg L^{-1}, no statistically significant change in SOD activity was detected in *L. minor* (Fig. 6B). This indicates a potential saturation or even suppression of the antioxidant defense system at these elevated BAC concentrations, possibly due to the overwhelming oxidative stress or direct inhibition of SOD enzymes.

The gel analysis (Fig. 6C) unveiled the presence of four CAT isozymes (CAT1-4) in *L. minor*, with their activity being differentially affected by BAC treatments. Notably, lower BAC doses (0.25 and 0.5 mg L^{-1}) stimulated CAT activity, with increases of 41% and 22%, respectively, compared to the control. This suggests a potential upregulation of the antioxidant defense system at these concentrations to counteract BAC-induced oxidative stress. Conversely, the highest BAC dose (10 mg L^{-1}) did not elicit a significant change in overall CAT activity, indicating a potential saturation or even downregulation of this antioxidant enzyme at such high stress levels. Interestingly, intermediate BAC concentrations (1, 2.5, and 5 mg L^{-1}) resulted in a dose-dependent decrease in CAT activity, with reductions of 19%, 26%, and 17%, respectively, compared to the control (Fig. 6D). This suggests a complex, non-linear response of CAT activity to BAC exposure, with an initial stimulation at lower doses followed by a decline at higher concentrations.

As illustrated in Fig. 7A, four bands corresponding to POX isozymes, designated as POX1-4, were observed. Application of a BACs dose at 0.25 mg L^{-1} resulted in a slight 9% decrease in POX activity compared to the group without BAC treatment. Conversely, higher BAC doses (0.5, 1, 2.5, 5, and 10 mg L^{-1}) induced a dose-dependent increase in POX activity (Fig. 7B). Notably, BAC applications at 1 and 2.5 mg L^{-1} led to a pronounced upregulation of POX1 and POX4 isozymes, as evidenced by the increased band densities on the native PAGE gel. This suggests a selective activation of specific POX isozymes in response to moderate BAC-induced stress. Furthermore, native PAGE analysis revealed the presence of seven GST isozymes (GST1-7) in *L. minor* (Fig. 7C). All BAC doses tested induced an increase in total GST activity, indicating a general upregulation of this detoxification enzyme system. The most substantial increase in GST activity was observed with the 0.5 mg L^{-1} BAC treatment, displaying a 2.6-fold rise compared to the control group (Fig. 7D).

The gel electrophoresis analysis conducted in this study elucidated the presence of five distinct NOX isozymes, denoted as NOX 1–5, within the *L. minor* plant (Fig. 8A). The results unveiled that the NOX enzyme activity remained unaffected by BAC at 0.25, 5 and 10 mg L^{-1}. In contrast, the intermediate BAC concentrations (0.5, 1, and 2.5 mg L^{-1}) exerted a pronounced impact on the NOX enzyme activity, eliciting a noteworthy increase of 2.7-fold, 2.9-fold, and 1.9-fold, respectively, in comparison to the non-BAC treatment group (Fig. 8B). The upregulation of NOX2 and NOX4 isozymes, as evidenced by the increased band intensities, likely contributed to this enhanced activity. This suggests a specific induction of certain NOX isozymes in response to moderate BAC stress, potentially leading to increased ROS production and signaling. As illustrated in Fig. 8C, gel analysis for observing GPX isozyme activity revealed four GPX bands in the *L.minor* leaves (GPX1–4). The lowest BAC dose (0.25 mg L^{-1}) did not significantly alter GPX activity, suggesting a minimal impact on this antioxidant enzyme at low stress levels. In contrast, BAC doses of 0.5, 1, 2.5, and the higher dose of 10 mg L^{-1} induced increases of 13%, 15%, 8%, and 9%, respectively (Fig. 8D). Particularly noteworthy was the effect of BAC at 5 mg L^{-1} on GPX activity, exhibiting a pronounced and statistically significant increase of 31% compared to the group without BAC application.

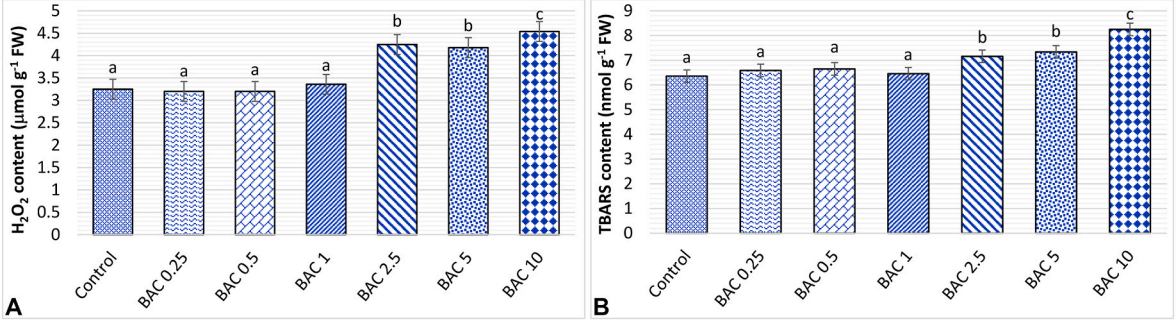

Fig. 5. The changes of hydrogen peroxide content (H_2O_2, **A**) and lipid peroxidation level (TBARS content, **B**) in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

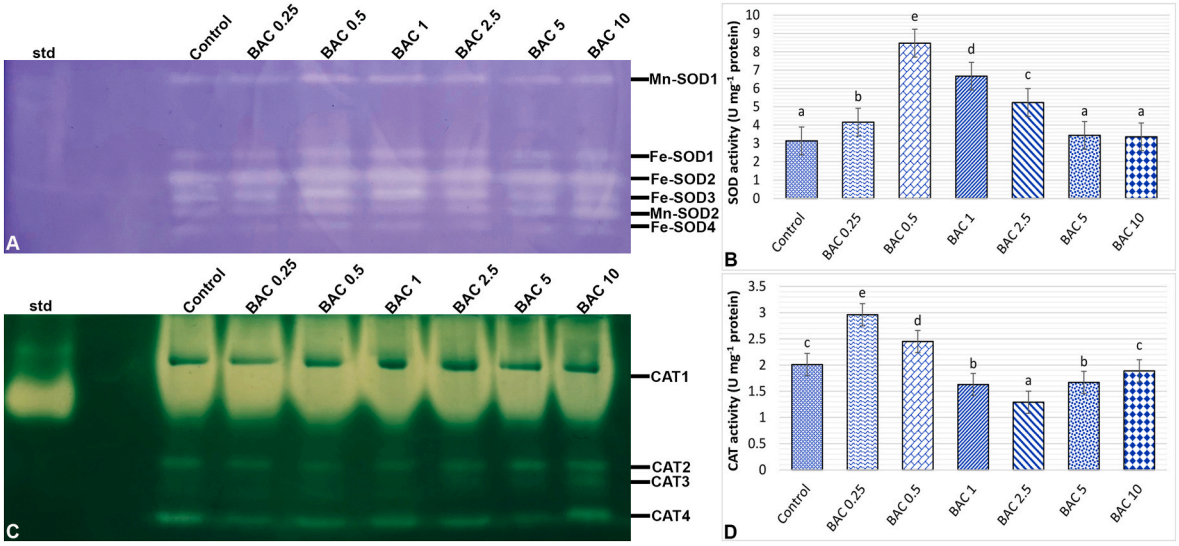

Fig. 6. Relative band intensity of different types of superoxide dismutase isoenzymes (SOD, **A**) and SOD activity (**B**), relative band intensity of different types of catalase isoenzymes (CAT, **C**) and CAT activity (**D**) in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

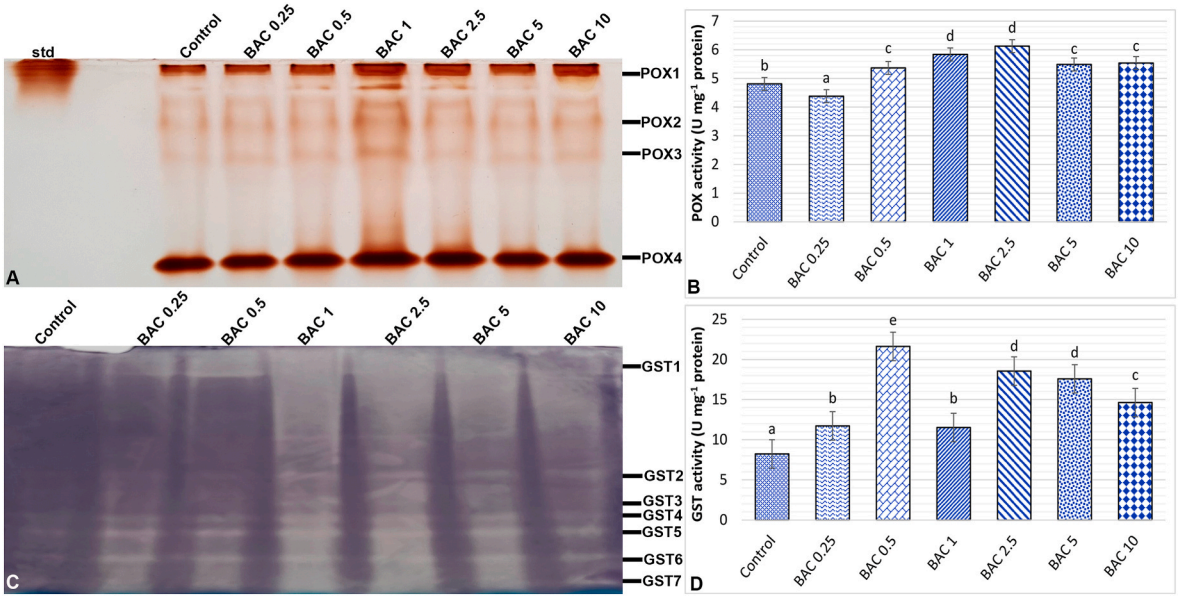

Fig. 7. Relative band intensity of different types of peroxidase isoenzymes (POX, **A**) and POX activity (**B**), relative band intensity of different types of glutathione S-transferase (GST, **C**), GST activity (**D**) in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

In L. *minor* plants, as depicted in Fig. 9A, a single APX isozyme was observed. Notably, lower and higher doses of BAC 0.25, 0.5, and 10 mg L^{-1} did not significantly affect APX activity, suggesting a potential resilience of this enzyme to both low and high levels of BAC-induced stress. However, doses of 1, 2.5, and 5 mg L^{-1} induced APX activity, with the highest effect observed at 2.5 mg L^{-1}, resulting in a 1.5-fold increase compared to the control, as illustrated in Fig. 9B. This indicates a specific stimulation of APX in response to moderate BAC stress levels, likely as part of the antioxidant defense mechanism. As depicted in Fig. 9C, gel analysis aimed at observing GR isozyme activity revealed the presence of five GR bands in the leaves of *L. minor* (GR1–5). The application of exogenous BAC at 0.25 mg L^{-1} resulted in a 37% reduction in total GR activity compared to the non-BAC treatment. Conversely, BAC applications at 0.5, 1, and 2.5 mg L^{-1} led to an increase in GR activity, with the most substantial rise observed in the BAC 0.5 mg L^{-1} group, exhibiting a 3.4-fold increase (Fig. 9D). However, higher BAC doses 5 and 10 mg L^{-1} did not induce statistically significant changes in GR activity, suggesting a possible saturation or downregulation of this enzyme at higher stress levels.

In L. *minor* plants, Fig. 10A illustrated that MDHAR activity statistically changed with the application of BACs depending on the doses. Low doses of BACs (0.25 and 0.5 mg L^{-1}) reduced MDHAR activity, indicating that even small amounts of BACs can negatively affect this enzyme's function. However, intermediate doses (1, 2.5, and 5 mg L^{-1}) increased MDHAR activity, with the highest induction in the BAC 5 mg L^{-1} group, exhibiting a 3.7-fold increment. This suggests that moderate BAC levels might stimulate a protective response in the plants. Interestingly, the highest BAC dose at 10 mg L^{-1} reduced MDHAR activity by 26% compared to the non-BAC treatment, demonstrating a threshold beyond which BACs become harmful (Fig. 10A). As illustrated in

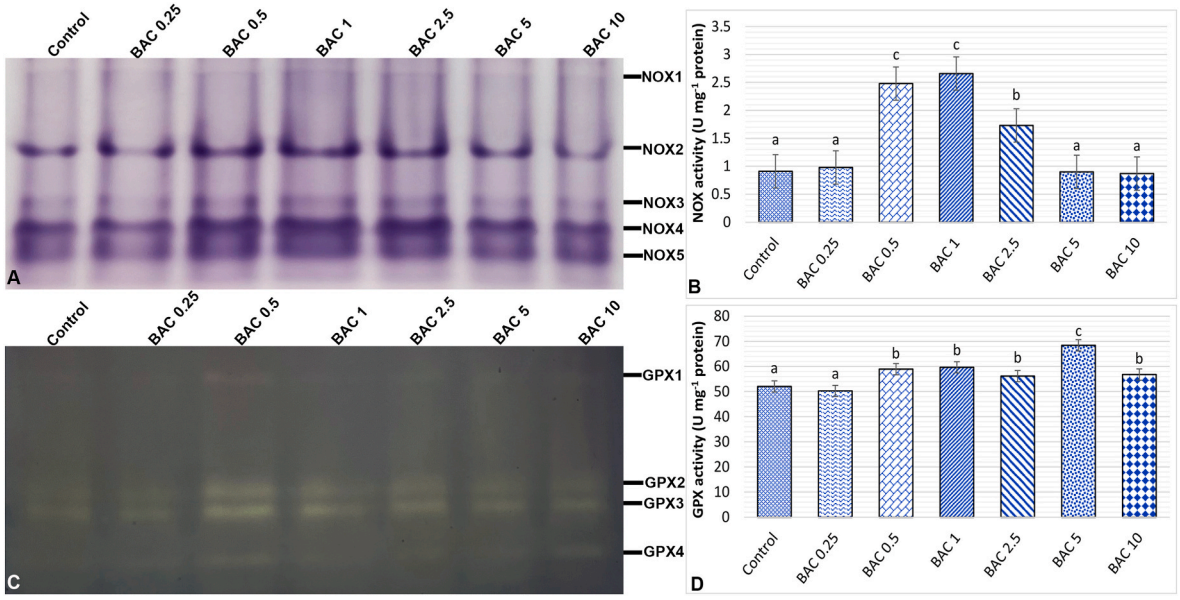

Fig. 8. Relative band intensity of different types of NADPH oxidase isoenzymes (NOX, **A**), NOX activity (**B**), relative band intensity of different types of glutathione peroxidase activity (GPX, **C**) and GPX activity (**D**) in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

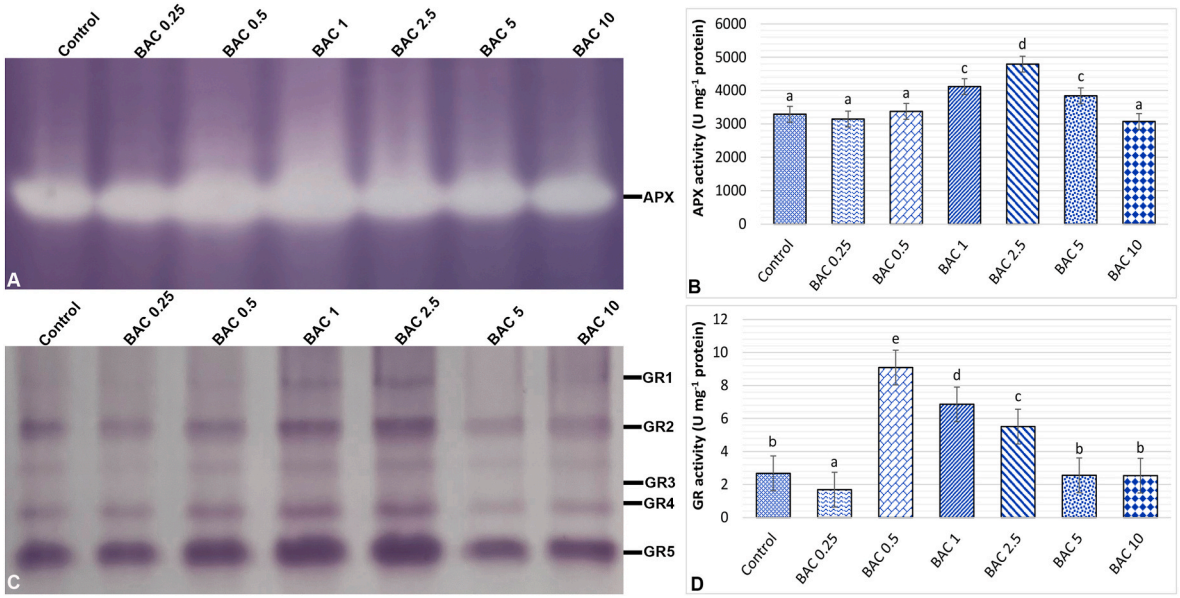

Fig. 9. Relative band intensity of different types of ascorbate peroxidase (APX, **A**), APX activity (**B**), relative band intensity of different types of glutathione reductase activity (GR, **C**) and GR activity (**D**) in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

Fig. 10B, BACs effect on *L.minor* plant different effect on DHAR activity. It was determined that DHAR activity decreased in all applications of BACs compared to the control group. The highest decrease was observed to be 3.4-fold at the 1 mg L^{-1} BAC application. This consistent decrease across various BAC concentrations highlights the susceptibility of DHAR activity to BAC exposure. Differences in tAsA content were observed based on BAC concentrations. Low-dose applications of BAC 0.25 and BAC 0.5 led to an increase in tAsA content by 19% and 21%, respectively (Fig. 10C). There was no statistically significant difference observed in BAC 1 and high-dose BAC 10 applications compared to the control group. However, 2.5 and5 mg L^{-1} BAC applications significantly reduced tAsA content by 32% and 38%, respectively, suggesting a detrimental effect of moderate BAC levels on tAsA content. DHA content exhibited an increase with all BAC applications. The most substantial increases were observed with BAC 1, BAC 5, and BAC 10 mg L^{-1} applications, with rates determined to be 2.4, 2.4, and 2.2-fold, respectively, compared to the control group (Fig. 10D). This indicates that BAC exposure leads to elevated DHA levels, potentially reflecting oxidative stress. BAC applications at doses of 0.25, 0.5, and 1 mg L^{-1} which are considered low BAC concentrations, did not elicit any changes in GSH content. Conversely, an increase in BAC concentration correlated with a decrease in GSH content, with the highest reduction observed in the BAC 10 mg L^{-1} group, reaching 35% (Fig. 10E). This suggests that higher BAC levels deplete GSH, impairing the plant's antioxidant defense. BAC applications at doses of 0.25 and 0.5 mg L^{-1} did not induce any significant changes in GSSG content compared to the control group.

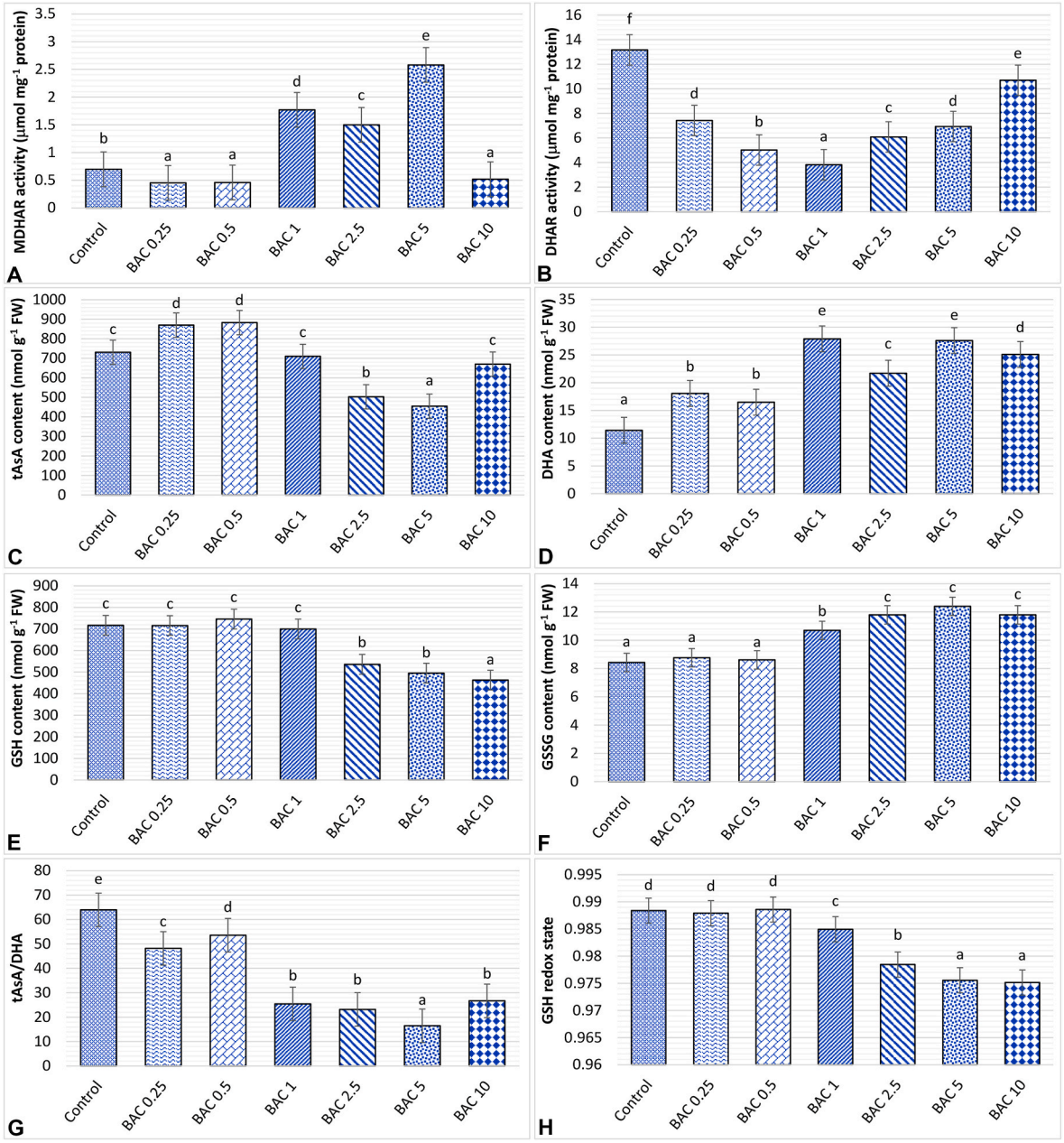

Fig. 10. The monodehydroascorbate reductase activity (MDHAR, **A**), dehydroascorbate reductase activity (DHAR, **B**), total ascorbate content (tAsA, **C**), dehydroascorbate content (DHA, **D**), glutathione content (GSH, **E**), oxidized glutathione content (GSSG, **F**), tAsA/DHA (**G**) and GSH redox state (**H**) in benzalkonium chloride (BAC, 0.25, 0.5, 1, 2.5 and 5 mg L^{-1}) applied *L.minor* plants. All data obtained were subjected to a one-way analysis of variance (ANOVA). Differences were considered to be significant at $p < 0.05$.

Conversely, concentrations of BACs 1, 2.5, 5, and 10 mg L^{-1} resulted in increases in GSSG content, with increments of 27%, 40%, 47%, and 40%, respectively (Fig. 10F). These findings indicate that higher BAC concentrations lead to oxidative stress, as reflected by the increased GSSG levels. It has been observed that tAsA/DHA exhibits a minor decline with low-dose applications of BACs 0.25 and 0.5 mg L^{-1}. As the concentration of BACs doses increased, the reduction in tAsA/DHA became more pronounced, with the most significant decrease recorded at 74% in the BAC 5 mg L^{-1} application (Fig. 10G). This substantial decline suggests a shift towards oxidative stress with higher BAC doses. GSH/GSSG exhibited no statistically significant change at low BAC concentrations (0.25 and 0.5 mg L^{-1}). Low-dose applications of BACs 0.25 and 0.5 mg L^{-1} do not appear to significantly impact GSH redox status. However, it has been observed that as the doses of BACs increase, albeit gradually, there is an effect on GSH redox status, leading to progressively higher decreases (Fig. 10H). This indicates that higher BAC levels impair the plant's redox balance, potentially compromising its antioxidant defense mechanisms.

4. Discussion

4.1. The response of L. minor concerning its relative growth rate (RGR), proline accumulation, and osmotic potential in the presence of BACs

The global rise in population and personal needs drives a surge in PPCP residues. Increased demand for hygiene products and widespread use of human and veterinary drugs contribute to this growth. PPCPs encompass diverse substances like hormones, antibiotics, and disinfectants (Dey et al., 2019). Excessive medication consumption, easy access to hygiene products, and agricultural activities lead to significant

introduction of micropollutants into wastewater treatment plants (da Silveira Barcellos et al., 2022). Concentrations vary widely, from ng dm^{-3} to μg dm^{-3}, with drinking water levels much lower than raw wastewater (Patel et al., 2019; Richardson and Ternes, 2018). The COVID-19 pandemic has further increased disinfectant use worldwide (Dewey et al., 2021), notably BACs, commonly used for surface and hand disinfection in high concentrations or as preservatives in health products like nasal sprays and eye drops (Short et al., 2021). The BACs in irrigation water has been observed to have a detrimental impact on the biomass and growth of plants when compared to control conditions (Khan et al., 2018; Richter et al., 2016). RGR exhibited a slight increase with the application of BACs doses at 0.25, 0.5, and 1 mg L^{-1}. However, the observed reduction in RGR content with increasing concentrations of BACs (2.5, 5, and 10 mg L^{-1}) in wastewater indicates a potential limitation in the efficacy of *L. minor* as a phytoremediator under higher BACs doses. The observed reduction in RGR with increasing BAC concentrations may be attributed to several underlying mechanisms. One possibility is the disruption of cellular processes crucial for plant growth and development. BACs, as cationic surfactants (Do et al., 2024), can interact with and damage cell membranes, leading to impaired nutrient uptake, photosynthesis, and overall metabolic activity. Additionally, BACs may induce oxidative stress in plants, generating reactive oxygen species that cause cellular damage and inhibit growth.

This finding underscores the importance of understanding the plant's response to varying levels of BACs in wastewater, especially in the context of phytoremediation strategies aimed at mitigating environmental pollution. The findings presented here align with those reported by Richter et al. (2016), whose study similarly indicated that different concentrations of BACs adversely impacted the yield, average growth rate of leaf number, and overall growth inhibition in *L. minor* plants. In another analogous study by Khan et al. (2018), a significant reduction in plant dry weight was noted in lettuce plants with escalating BAC doses. This observation led to the interpretation that this relationship could be associated with a decrease in essential plant nutritional elements.

To investigate the impact of BACs on plant stress response and osmoprotection mechanisms, Pro concentration, a key amino acid involved in these processes (Spormann et al., 2023), was monitored in *L. minor*. Contrary to expectations, Pro concentration decreased across all BAC concentrations, suggesting that Pro may not play a protective role in mitigating BAC-induced stress in this plant species. This decrease in Pro concentration could be attributed to several underlying mechanisms. BACs may disrupt Pro biosynthesis by inhibiting key enzymes, such as pyrroline-5-carboxylate synthase, or by downregulating gene expression related to Pro synthesis. Alternatively, BACs could enhance Pro catabolism by activating proline dehydrogenase (PDH) (Lebreton et al., 2020), leading to increased Pro breakdown and reduced cellular Pro levels. Furthermore, the observed negative correlation between Pro concentration and osmotic potential, particularly at the highest BAC dose of 10 mg L^{-1}, indicates that *L. minor* struggles to maintain osmotic balance under BAC stress. This inability to regulate osmotic pressure, potentially due to impaired Pro accumulation, may contribute to the observed decline in growth parameters at this dose. These findings align with previous research by Christou et al. (2016), who reported either unchanged or decreased Pro content in alfalfa plants exposed to various PPCPs, suggesting a limited role for Pro in mitigating stress induced by these compounds. In conclusion, the observed decrease in Pro concentration and its negative correlation with osmotic potential suggest that *L. minor* may have limited capacity to utilize Pro for osmoprotection and stress adaptation in the presence of BACs. This highlights the need for further research to elucidate the complex interactions between BACs, Pro metabolism, and plant stress response mechanisms to develop effective phytoremediation strategies for BAC-contaminated environments.

4.2. Photosynthetic apparatus-related response of L.minor in the presence of BACs

The fluctuations observed in biomass ratios are intricately linked to various facets of photosynthesis. Parameters such as chlorophyll fluorescence or electron transport properties serve as reliable and pivotal early indicators of physiological stress, shedding light on the intricate mechanisms underlying plant responses to environmental stimuli (Muhammad et al., 2021). In this study, chlorophyll fluorescence parameters were adversely impacted across various concentrations of BACs. The application of BACs caused a slight decrease in F_v/F_m but notably induced a significant decrease in the F_v/F_o ratio, especially at the highest BAC concentration. These observed negative effects on F_v/F_m and F_v/F_o parameters are likely attributable to BACs' interference with electron transport and reaction centers within the PSII regions, potentially leading to the disruption of the oxygen-evolving complex (Gupta, 2020). Under stressful conditions, chloroplasts, the primary sites of photosynthesis in plant cells, are particularly susceptible to damage (Basu et al., 2021). Changes in the values of F_v/F_m and F_v/F_o in *L. minor* plants exposed to high concentrations of BAC may be indirectly related to the impact of the electron transport chain system (ETC). Several factors contribute to this phenomenon: First, disruption of photochemical reactions in PSII may have occurred, disrupting the normal flow of electrons and leading to the accumulation of energy intermediates and reductants (Zavafer and Mancilla, 2021). Second, the inadequate internal tolerance mechanism and limited phytoremediation capabilities in *L. minor* may render the plant susceptible to the deleterious effects of excess ROS (Gomes et al., 2022). As emphasized by Foyer and Hanke (2022), the specific combination of excess energy intermediates, reductants, and high oxygen levels within the chloroplast creates an ideal environment for the production of ROS during unforeseen environmental fluctuations. In conclusion, our study observed an increase in H_2O_2 content in *L. minor* plants exposed to high BAC concentrations, which may be associated with potential damage to PSII.

The analysis of fast fluorescence transitions of O-J-I-P through the JIP assay becomes indispensable for assessing the condition of plants subjected to environmental stresses. When exposed to high-intensity actinic light, typically exceeding 200 W m^{-2} or 2500 μmol photons m^{-2} s^{-1}, photosynthetic organisms manifest Chl fluorescence signals characterized by distinct alterations (Strasser et al., 2004). These changes offer valuable insights into the structure and functionality of the photosynthetic apparatus, enabling researchers to scrutinize variations within the plant, particularly concerning PSII. In our study, it was revealed that all BACs treatments led to an increase in ABS/RC, ET_o/RC, TR_o/RC, DI_o/RC, and RE_o/RC. This elevation in parameter values may potentially result in heightened energy losses within the electron transport chain. Such losses could stem from inadequate electron conduction between PSII and PSI, consequently disrupting the efficient flow of electrons (Shanker et al., 2022). Furthermore, in line with these findings, parameters such as $\Phi P_o/(1-\Phi P_o)$, $\Psi E_o/(1-\Psi E_o)$, $\gamma RC/(1-\gamma RC)$, PI_{ABS}, and PItotal exhibited reductions in *L. minor* plants subjected to BACs. This collective decrease in parameter values indicates a significant slowdown in electron flow from PSII to PSI, suggesting a notable alteration in the dynamic interaction of the photosynthetic machinery (Dąbrowski et al., 2023).

4.3. Response of L. minor to biochemical parameters in the presence of BACs

The redox state inside a plant cell undergoes continuous oxidation and reduction processes (Mittler et al., 2022). Adverse environmental conditions and biological factors can disturb this balance, affecting different physiological and biochemical functions. Such disruptions can hinder plant growth and development, particularly in challenging and prolonged conditions (Kumar, 2020). This disturbance can lead to oxidative stress, harming crucial cellular components like proteins,

enzymes, nucleic acids, carbohydrates, and lipids (Mansoor et al., 2022). Our study investigated the impact of BACs on oxidative stress in *L. minor*, as measured by TBARS and H_2O_2 levels. At low BAC concentrations, TBARS and H_2O_2 content remained unchanged, suggesting that *L. minor* possesses effective mechanisms to mitigate oxidative damage under these conditions. However, it was found that elevated concentrations of BACs (2.5, 5, and 10 mg L^{-1}) induced oxidative damage, likely due to the plant's inadequate tolerance mechanisms. This suggests that the plant's antioxidant defense mechanisms become overwhelmed at these concentrations, leading to ROS-induced damage to lipid membranes and the formation of malondialdehyde (MDA), a byproduct of lipid peroxidation (Sachdev et al., 2023). In a previous study, it was discovered that exposure to 10 mg L^{-1} concentrations of diclofenac, sulfamethoxazole, trimethoprim, or 17α-ethinylestradiol resulted in heightened lipid peroxidation in alfalfa leaves (Christou et al., 2016). Another investigation revealed that applying PPCPs to cucumber plants led to the simultaneous occurrence of ROS production, membrane damage, and lipid peroxidation. The authors underscored this phenomenon as indicative of oxidative damage induced in the plants by increasing doses of the applied compounds (Sun et al., 2018). These findings highlight the concentration-dependent effects of BACs and other pollutants on plant oxidative stress. At higher concentrations, the antioxidant systems in plants like *L. minor* may become insufficient, leading to oxidative damage and impaired phytoremediation capacity.

The effectiveness of the antioxidant-based detoxification system is pivotal in safeguarding plants against elevated levels of ROS and empowering them to endure adverse conditions (Sachdev et al., 2022). Antioxidants operate by neutralizing the detrimental effects of ROS through scavenging mechanisms, inhibiting oxidation processes by forming chelate complexes with metals, modulating the activation or deactivation of enzymes, and facilitating the repair and elimination of ROS-induced damages (Dumanović et al., 2021). Throughout the duration of exposure to BACs, *L. minor* plants displayed a significant induction of both enzymatic and non-enzymatic antioxidant systems. SOD stands as a pivotal antioxidant enzyme present in nearly all oxygen-exposed living cells (Sadiq, 2023). Its principal function revolves around shielding cells from the detrimental effects instigated by superoxide radicals ($O_2^{\bullet -}$), which emerge as by-products of oxygen metabolism (Rajput et al., 2021). In our investigation, we observed an elevation in SOD activity across all BACs doses in *L. minor* plants, with the most substantial increments noted at BACs concentrations of 0.5, 1, and 2.5 mg L^{-1}. Previous studies have demonstrated contrasting effects on SOD activity in response to various stressors: an increase in activity following exposure to paracetamol (An et al., 2009a) and PPCPs in cucumber plants (Sun et al., 2018), whereas a decrease in activity was observed in wheat seedlings exposed to triclosan and galaxolide (An et al., 2009b). This variability in SOD response likely reflects the complex interplay between the intensity of oxidative stress and the plant's capacity to activate antioxidant defenses (Garcia-Caparros et al., 2021). A decrease in SOD activity may indicate that the oxidative burden overwhelms the enzyme's capacity, leading to a decline in its protective function. Additionally, our study revealed a positive correlation between H_2O_2 content and CAT activity in groups exposed to BACs doses other than the higher concentrations (2.5, 5, and 10 mg L^{-1}), while a negative correlation was observed in the BAC 1 mg L^{-1} application group. This negative correlation between increasing H_2O_2 content and the enzymatic activities of CAT and SOD may also suggest potential modifications in other cellular mechanisms involved in H_2O_2 scavenging, such as alterations in the ascorbate redox state and POX activity. Consequently, despite the surge in SOD activity in response to the 1 mg L^{-1} BAC application, the maintenance of H_2O_2 content at control levels could be attributed to heightened activities of POX, GPX, APX, and GR enzymes. A prior study by Christou et al. (2016). similarly reported a negative correlation between increasing H_2O_2 content and CAT and SOD enzymatic activity in alfalfa plants subjected to various pharmaceutical active compounds (PhACs) at different doses. In conclusion, *L. minor* may activate a multi-faceted antioxidant response to counteract BAC-induced oxidative stress. The observed upregulation of SOD activity and the complex interplay between H_2O_2 content and antioxidant enzyme activities could highlight the plant's adaptability and resilience in the face of environmental challenges.

In stressful environments, plant cells rely on plasma membrane NOX (also known as respiratory burst oxidase homologs, RBOH), a key ROS producer, for signaling (Angelos and Brandizzi, 2018). Belonging to the NOX family, this enzyme facilitates ROS signaling alongside class III peroxidases and cell wall oxidases. NADPH oxidase, like all NOX members, transports electrons across membranes to reduce oxygen to $O_2^{\bullet -}$, crucial for mediating plant responses to environmental stress via ROS pathways (George et al., 2023; Hu et al., 2020). In the study, a significant increase in NOX activity was observed in *L. minor* plants following the application of BACs at concentrations of 0.5, 1, and 2.5 mg L^{-1}. However, no change in NOX activity was noted at concentrations of 0.25, 5, and 10 mg L^{-1}. This concentration-dependent response suggests a non-linear relationship between BAC exposure and NOX activation, potentially influenced by factors such as receptor saturation or feedback mechanisms, and may be related to H_2O_2 production. Although treatments with BACs at 0.5 and 1 mg L^{-1} increased NOX activity, they did not lead to H_2O_2 accumulation. It is noteworthy that the activation of cytosolic Ca^{2+} and Ca^{2+}-binding proteins can initiate the activation of NOX/RBOH at the N-terminal (Hu et al., 2020). This activation results in NOX-mediated reactive oxygen species (ROS) production, subsequently activating plasma membrane Ca^{2+} channels (Kurusu et al., 2015). Consequently, this process may serve as a signaling mechanism, further elevating cytosolic Ca^{2+} levels and triggering the activity of enzymatic antioxidants. This elevated Ca^{2+} concentration triggers the activity of enzymatic antioxidants such as SOD and CAT, which mitigate oxidative stress by detoxifying ROS, thereby maintaining cellular homeostasis and protecting against oxidative damage (Raina et al., 2021). Consistent with our study, SOD activity was found to increase at BAC concentrations of 0.5, 1, and 2.5 mg L^{-1}.

GPX and APX are vital components of the cellular antioxidant defense system, each with unique enzymatic properties. GPX, a non-heme peroxidase enzyme, facilitates the reduction of H_2O_2 to water (H_2O) and oxygen (O_2) (Rajput et al., 2021). On the other hand, APX belongs to the class I heme-peroxidase enzymes and utilizes protoporphyrin as its prosthetic group (Sachdev et al., 2022). These distinct enzymatic properties allow GPX and APX to play crucial roles in cellular antioxidant defense mechanisms. While GPX primarily targets the neutralization of H_2O_2, APX contributes to peroxide detoxification by utilizing ascorbate as a reducing agent. In our study, increases in GPX activity were observed in *L. minor* plants at BACs concentrations other than 0.25 mg L^{-1}. Similarly, APX activity showed a statistically significant increase, except for BACs 0.25 and 10 mg L^{-1} high-dose applications. The differential responses in GPX and APX activities among the groups suggest a fine-tuned regulatory mechanism. These enzymes likely work synergistically to maintain H_2O_2 levels close to those in control plants, preventing excessive accumulation of H_2O_2. By doing so, they enhance the oxidative stress tolerance mechanism in *L. minor*, ensuring cellular homeostasis and promoting plant survival under varying BAC concentrations. This balance between H_2O_2 production and detoxification is crucial for mitigating oxidative damage and supporting the plant's adaptive response to environmental stressors. Huber et al. (2016) reported that PPCPs applied to *Typha latifolia* plants increased APX activity. In another study, Sun et al. (2018) demonstrated that APX and GST activities increased in parallel with increasing doses of exogenously applied PPCPs, suggesting that the APX and GST enzyme families may play a crucial role in the conversion and conjugation of PPCPs in plants. GR is a another crucial enzyme in cellular antioxidant mechanisms, using NADPH to maintain the reduced form of GSH, an important antioxidant (Kunert and Foyer, 2023). In plants, the AsA-GSH cycle involves enzymes like MDHAR and DHAR (Chauhan et al., 2022). MDHAR converts monodehydroascorbate (MDHA) to ascorbate (AsA), while

DHAR regenerates AsA from dehydroascorbate (DHA) using GSH, balancing cellular redox and antioxidant levels by recycling key components (Rajput et al., 2021). The maintenance of AsA-GSH homeostasis in plants following exposure to PPCPs has not been extensively documented to date. Previous studies, such as those by Sun et al. (2018), Bartha et al. (2014), (Zhang et al. (2016), Farkas et al. (2007)), have reported glutathione conjugation with various PPCPs in plant systems. These include diclofenac, 8:2 fluorotelomer alcohol, and chlortetracycline, each demonstrating different properties. In our study, we observed a significant increase in GR activity in *L. minor* plants at BAC doses of 0.5, 1, and 2.5 mg L^{-1}. Particularly noteworthy was the substantial increase in GR activity in the 0.5 and 1 mg L^{-1} treatments, indicating that GR, an integral component of the AsA-GSH cycle, acts as an effective ROS scavenger. Furthermore, to maintain GSH content, GSSG may have been efficiently recycled by the GR enzyme in the presence of the reductant NADPH, which is essential for cellular function (Cohen et al., 2020), thereby preserving GSH content in *L. minor* under BAC doses of 0.25, 0.5, and 1 mg L^{-1}. Similarly, to uphold the GSH/GSSG ratio, GR catalyzes the reduction of disulfide GSSG (dimeric form) to GSH (monomeric form), thus maintaining the redox state of the cell (Rajput et al., 2021). The GSH/GSSG content was sustained at BAC doses of 0.25 and 0.5 mg L^{-1}, ensuring survival by preserving the GSH redox state and maintaining tolerance against elevated BAC levels.

5. Conclusion

In conclusion, the study demonstrates that *L. minor* exhibits toxic effects in response to increasing doses of BACs applications. The concentration of 2.5, 5 and 10 mg L^{-1} BACs led to an increase in H_2O_2 and TBARS contents, ultimately hindering the RGR. The increase in oxidative stress markers such as H_2O_2 and TBARS indicates a strong oxidative stress response, which correlates with the observed decrease in RGR. This suggests that higher BAC concentrations exacerbate oxidative damage, impairing the plant's growth. Moreover, the photosynthetic efficiency parameters, F_v/F_m and F_v/F_o, decreased across all BAC treatments, indicating that BAC exposure detrimentally affects the photosynthetic machinery. The decline in these photosynthetic parameters was directly proportional to the reduction in RGR, highlighting the critical impact of BACs on the overall physiological health of *L. minor*. This oxidative damage serves as an early indicator of plant response to PPCPs introduced into agricultural ecosystems. Moreover, given the detoxifying properties of robust phytoremediation plants *L. minor*, they may serve as reliable indicators of environments contaminated with PPCPs. While the application of 0.5 mg L^{-1} BAC notably enhances SOD activity, the maintenance of H_2O_2 content at control levels, resulting in suppressed TBARS content close to control levels, leads to increased activities of CAT, POX, GST, and GR enzymes within the same BAC groups. This coordinated response may be indicative of a regulatory mechanism aimed at efficiently managing oxidative stress induced by BAC exposure. It is worth noting that the accumulation of BACs in *L. minor* plants increased proportionally with the dosage level. However, given the relatively short lifespan of accumulating plants *L. minor*, it is important to recognize that compounds accumulated within these plants will be reintroduced into the water upon plant death. Consequently, regular collection and safe disposal of these plants become imperative as a viable solution to prevent the re-release of accumulated contaminants back into the environment.

CRediT authorship contribution statement

Fevzi Elbasan: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Busra Arikan-Abdulveli:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Ceyda Ozfidan-Konakci:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Evren Yildiztugay:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **İsmail Tarhan:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Berfin Çelik:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

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