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Physiological and biochemical tolerance responses of the estuarine saltmarsh plant *Limbarda crithmoides* L. under acetaminophen exposure: Insights into plant–pollutant interactions

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ABSTRACT

Pharmaceutical contamination in estuarine and coastal environments is a growing concern due to continuous urban discharges and limited removal in conventional wastewater treatment. Acetaminophen, a frequently detected analgesic in surface and estuarine waters, was selected as a model compound to investigate its physiological effects on the native saltmarsh plant *Limbarda crithmoides* L. Rooted explants were exposed to acetaminophen (0.1, 1.0, and 2.0 mg/L) in liquid culture for 7, 14, and 21 days. Removal from solution was monitored by HPLC-DAD and GC–MS, while pigment levels, oxidative stress markers (MDA), osmoprotectants (proline, proteins, sugars), and secondary metabolites (phenolics, flavonoids, tannins, shikimic acid, PAL activity) were quantified in roots and shoots. *L. crithmoides* showed moderate tolerance to acetaminophen exposure, maintaining photosynthetic pigments and stable stress markers. Early responses involved osmoprotectant accumulation and controlled oxidative damage, followed by the activation of antioxidant and phenolic pathways at later stages, suggesting metabolic acclimation to sustained stress. Root tissues displayed lower oxidative damage and higher proline accumulation than aerial parts, indicating spatial differentiation of stress responses. Despite continuous exposure, plants preserved functional integrity and biochemical homeostasis. These results reveal key physiological and biochemical mechanisms underlying the tolerance limits of a native estuarine species to pharmaceutical stress, providing insight into plant–pollutant interactions and the potential role of saltmarsh vegetation in the functioning of contaminated coastal ecosystems.

1. Introduction

Contaminants of Emerging Concern (CECs) are increasingly reported in coastal and estuarine environments, where their persistence, occurrence as complex mixtures, and poorly defined safety thresholds threaten the functioning of transitional ecosystems and human health (Petrović et al., 2003; Petrie et al., 2015; United States Environmental Protection Agency (US-EPA), 2012; Yadav et al., 2021). This heterogeneous group includes pharmaceuticals, personal care products, pesticides, and per- and polyfluoroalkyl substances (PFAS), many of which resist conventional treatment and may display additive or synergistic toxic effects. Reflecting the growing policy relevance of CECs in Europe, the Urban Wastewater Directive has been revised with reinforced requirements for the reduction of priority substances (Council Directive 91, 2024).

Pharmaceuticals are among the most problematic CECs because they are continuously introduced into aquatic environments via municipal effluents and other urban discharges, with incomplete removal by conventional wastewater treatment plants (WWTPs) (Mompelat et al., 2009; Trần et al., 2018). Among these compounds, acetaminophen (paracetamol) is one of the most widely used analgesics and antipyretics worldwide and has been frequently detected in surface, estuarine, and coastal waters across different regions (United States Environmental Protection Agency (US-EPA), 2012; Trần et al., 2018; Mohammed et al., 2021). Regional monitoring programs have similarly reported acetaminophen among the most recurrent pharmaceutical contaminants in

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southern European estuaries, including the Guadiana (Bebianno et al., 2017). Its high solubility and relatively low sorption potential facilitate transport through aquatic systems, while its continuous input from wastewater effluents leads to recurrent detection from nanogram to microgram per litre levels (United States Environmental Protection Agency (US-EPA), 2012; Trần et al., 2018). This global occurrence and environmental persistence make acetaminophen a relevant model compound for assessing the physiological and biochemical stress responses of coastal vegetation and for understanding plant–pollutant interactions in estuarine systems.

Conventional WWTPs are not specifically designed for the removal of pharmaceutical micropollutants, and their limited efficiency has motivated the search for cost-effective, nature-based approaches to complement existing treatment systems, particularly in saline or brackish settings typical of estuaries (De la Cruz et al., 2012; Kumar et al., 2022; Saravanan et al., 2021). Salt-tolerant plants from coastal saltmarshes exhibit traits such as salinity and waterlogging tolerance, robust root systems, osmoprotectant accumulation, and resilience to oxidative stress—attributes that support ecosystem functioning and can contribute to pollutant attenuation in saline wetlands (Oliveira et al., 2014; Kumar et al., 2019; Zhao et al., 2020). Nevertheless, their potential to withstand or attenuate pharmaceutical contamination remains underexplored and has so far focused on a limited number of species (Dordio et al., 2011; Turcios and Papenbrock, 2021).

Limbarda crithmoides L. (syn. *Inula crithmoides*), a perennial species widely distributed along Atlantic–Mediterranean and North African saltmarshes, thrives in intertidal, saline, and even metal-rich substrates, and shows tolerance to oxidative stress with a strong capacity to accumulate bioactive secondary metabolites (Zurayk et al., 2001; Ghabriche et al., 2017; Custódio et al., 2024; Rodrigues et al., 2024). Importantly, this species is also consumed locally as a wild edible plant ("golden samphire"), with young shoots and leaves used fresh, cooked, or pickled (Lima et al., 2021), which further highlights the need to understand how pharmaceutical pollutants may influence its physiology and potential safety.

This study established an in vitro experimental framework to (i) characterise the physiological and biochemical responses of *L. crithmoides* to acetaminophen exposure and (ii) assess its capacity to tolerate and modulate stress under controlled conditions. The results provide mechanistic insight into tolerance to pharmaceutical contamination in saltmarsh species and their potential ecological roles in estuarine and coastal environments affected by emerging pollutants.

2. Material and methods

2.1. Chemicals and equipment

Most solvents used for analytical procedures were obtained from Sigma-Aldrich (Germany), while methanol and ethanol were supplied by VWR (Belgium) and AGA (Sweden), respectively. Bovine serum albumin (BSA) and Bradford reagent were purchased from TCI (India), and sulphuric acid from Merck (Germany). Plant agar and Murashige and Skoog (MS) basal medium were obtained from Duchefa Biochemie (The Netherlands), and D(+)-sucrose from Labkem (Spain).

Regarding equipment, a pH meter (pHenomenal®) and ultrasonic bath (Ultrasonic Cleaner USC-TH) were provided by VWR (Belgium). A freeze dryer (FreeZone, 6 L) was provided by Labconco (USA), and absorbance readings were performed using an EZ Read 400 microplate reader (Biochrom, Austria) and BioTek Synergy 4 (ThermoFisher, USA).

2.2. Plant material and in vitro culture conditions

In vitro-derived shoots of *L. crithmoides* were obtained from previously established micropropagation cultures, developed according to Rodrigues et al. (2023). Four-week-old shoots, approximately 4–5 cm in height with 6–8 leaves, were selected, and any pre-formed roots were carefully removed to standardize the rooting phase. The individual shoots were then transferred to test tubes containing 6 mL of liquid MS basal medium (Murashige and Skoog (1962)), supplemented with 2 % (w/v) sucrose, and adjusted to pH 5.7–5.8 prior to autoclaving. Cultures were maintained under controlled conditions (25 ± 2 °C; 16/8 h light/dark photoperiod; light intensity of 56 μmol $m^{-2}{\cdot}s^{-1}$) to promote root development in a liquid-phase system for 4 weeks. The rooted plantlets obtained from this stage were subsequently used for the *Plant–Pollutant Interaction Assay*.

2.3. Plant-pollutant interaction assay

Rooted shoots, measuring approximately 8–10 cm in height, 10–12 leaves, and with roots approximately 5–6 cm long, were individually transferred to 6 mL of liquid MS basal medium supplemented with acetaminophen at concentrations of 0.1, 1.0, and 2.0 mg/L. A control group with no added contaminant was included. Acetaminophen stock solutions were prepared in ultrapure water, producing a 1000 mg/L master stock that was subsequently sterilized through a 0.20 μm syringe filter. A sterile 100 mg/L working stock was then prepared from this solution and aseptically added to the autoclaved, cooled MS medium to obtain final concentrations of 0.1, 1.0, and 2.0 mg/L. Cultures were maintained under standard growth conditions and sampled at 7-, 14-, and 21-days post-exposure. At each time point, the growth medium, roots, and aerial parts were collected separately and stored at −20 °C for subsequent analysis. For each treatment and sampling time, ten individual plants were cultured under identical conditions and harvested together to form one composite sample representative of that treatment. From this pooled material, three technical replicates (n = 3) were prepared and analyzed independently for each biochemical parameter.

2.4. Acetaminophen quantification in culture media

2.4.1. Solid-Phase Extraction (SPE)

Culture media samples were processed using SPE with Supra-poly HLB cartridges (3 mL, 30 μm; PerkinElmer, France). The cartridges were preconditioned with 7.5 mL of methanol followed by 7.5 mL of distilled water. Samples, previously adjusted to pH 7, were percolated through the SPE cartridges. These were then air-dried under vacuum for 15 min. Acetaminophen was eluted with 5 mL of methanol, which was subsequently evaporated to dryness at 30 °C using a rotary evaporator. The dried residues were redissolved in 1.0 mL of distilled water and filtered through a 0.45 μm PVDF syringe filter.

2.4.2. High-Performance Liquid Chromatography (HPLC)

The concentration of acetaminophen in the culture medium was determined using a Knauer Smartline HPLC system (Germany), equipped with a vacuum degasser (E4320V2), quaternary pump (EA4300V1), and a diode array detector (E4350). Sample analysis was carried out on a reverse-phase TSKgel ODS-100V column (5 μm, 4.6 × 150 mm; Tosoh Bioscience). The mobile phase consisted of acetonitrile (solvent A) and 0.1 % (v/v) phosphoric acid in water (solvent B), applied using the following elution program: 0–10 min isocratic (60:40 A:B), followed by a linear gradient to 25:75 A:B from 10 to 15 min. The flow rate was 1.0 mL/min, the injection volume was 20 μL, and detection was performed at 243 nm.

The mobile phase was filtered using nylon membrane filters (0.45 μm, Ø 47 mm; Branchia). Samples were filtered through 0.45 μm PVDF syringe filters (Ø 13 mm; Branchia) prior to injection. Acetaminophen was identified by retention time and UV spectral profile and quantified based on a calibration curve ($y = 27.074x + 43.533$; $R^2 = 0.9991$) using standard solutions ranging from 0 to 80 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were 3.15 μg/mL and 9.55 μg/mL, respectively.

2.4.3. Gas Chromatography Mass Spectrophotometry (GC-MS)

GC-MS analyses were performed with an Agilent Technologies system (USA) constituted by a 6890 Network Gas Chromatograph System, equipped with an Agilent 7683 autosampler, coupled to an Agilent 5973 Network Mass Selective Detector. All data were recorded, and instrumental control was performed using MS ChemStation software (E.02.00.493). The injections volume was 2 μL in splitless mode (pressure 5.52 psi), with the injector at 280 °C. A capillary column Mega 5MS (30.0 m × 0.25 mm × 0.25 μm; 5 % phenyl, 95 % methylpolysiloxane, Mega - Legnano, Italy) was used for the GC analysis, along with helium as the carrier gas, in constant flow of 0.7 mL/min. In full-scan mode, oven temperature was programmed starting at 90 °C (hold 2 min) and then increasing to 315 °C (20 °C/min), achieving a total run time of approximately 18.25 min. The transfer line temperature was 300 °C, the quadrupole analyser temperature was 200 °C, and the ion source temperature was 250 °C. A solvent delay of 5 min was selected. Electron ionization (70 eV) with an ionization current of 34.6 μA and a multiplier voltage of 1800 V were used. In the full-scan mode, a range of masses between 40 and 450 Da was selected.

2.5. Physiological and biochemical analysis

2.5.1. Photosynthetic pigments

Chlorophyll *a*, chlorophyll *b*, and carotenoids were extracted from 25 mg of fresh plant tissue using 4 mL of methanol, followed by sonication. Absorbance was measured at 470, 653, 666, and 750 nm using a spectrophotometer, and pigment concentrations were calculated according to the equations described by Lichtenthaler (1987).

2.5.2. Oxidative stress markers

Malondialdehyde (MDA) was extracted from 100 mg of fresh plant tissue using 1 mL of 0.1 % (w/v) trichloroacetic acid (TCA) and centrifuged at 12 000×*g* for 15 min. MDA content was quantified using the thiobarbituric acid (TBA) assay, following the method of Heath and Packer (1968), with modifications for improved accuracy as described by Hodges et al. (1999).

2.5.3. Osmoprotectants

For extraction, 250 mg of fresh plant material were homogenised in 5 mL of 80 % (v/v) ethanol and placed in an ultrasonic bath for 30 min at 80 °C. Free proline content was determined using the acid–ninhydrin method described by Bates et al. (1973). Soluble protein concentration was quantified according to the Bradford assay, using bovine serum albumin (BSA) as standard (Bradford, 1976). Total soluble sugar content was determined by the phenol–sulphuric acid method described by Dubois et al. (1956).

2.5.4. Secondary metabolites

Phenolics, flavonoids, and tannins were extracted as described in Section 2.5.3. Total phenolic content was determined using the Folin–Ciocalteu method according to Velioglu et al. (1998). Flavonoid content was quantified following the method of Pirbalouti et al. (2013), based on the formation of an $AlCl_3$–ethanol complex. Tannin content was determined using the DMACA method described by Li et al. (1996), with modifications by Zou et al. (2011).

Shikimic acid (SA) was extracted from 100 mg of fresh tissue using 2 mL of 0.25 M hydrochloric acid and centrifuged at 20 000 g for 15 min at 4 °C. Quantification was done by periodate oxidation according to Alzandi and Naguib (2020). For the determination of phenylalanine ammonia-lyase (PAL) activity, 20 mg of fresh plant material were extracted in 1 mL of 0.1 M sodium borate buffer (pH 8.8) and centrifuged at 20,000 g for 20 min at 4 °C. Enzyme activity was determined using a modified protocol of Rösler et al. (1997). Briefly, 100 μL of the extract was mixed with 500 μL L-phenylalanine (10 mM), and 1.4 mL of sodium borate buffer. After 1 h incubation at 37 °C, the absorbance was read at 290 nm. Specific PAL activity (μmol CAE/mg protein/min) was calculated using total soluble protein content obtained through the Bradford assay (BSA standard), which was used as the denominator for activity normalization.

2.6. Statistical analyses

The results were expressed as mean ± standard deviation (SD), and all statistical analyses were performed using the XLSTAT package in Microsoft Excel. Each value represents the mean of three technical replicates (n = 3) obtained from aliquots of a composite sample prepared by pooling ten plants from the same treatment and sampling time. This composite sampling approach was adopted to minimise intra-treatment variability inherent to in vitro assays.

The Shapiro–Wilk test was used to verify data normality. A two-way ANOVA was then conducted with Concentration (0, 0.1, 1.0, and 2.0 mg/L) and Time (7, 14, and 21 days) as fixed factors. When significant main or interaction effects were detected, Tukey's post-hoc test ($p < 0.05$) was applied to identify pairwise differences. The complete ANOVA outputs, including F-values, degrees of freedom, and p-values for all parameters, are presented in Tables S1 and S2 (Supplementary Materials).

3. Results

3.1. Quantification of acetaminophen in nutrient solutions

A progressive decrease in acetaminophen concentration was observed in the culture medium across all treatments over the 21-day period (Fig. 1a). In the 0.1 mg/L condition, 92.8 % of the compound was removed after 7 days, and complete removal (100 %) was achieved by day 14. At higher concentrations, removal was also highly efficient: 98.30 ± 0.10 % (1.0 mg/L) and 99.5 % (2.0 mg/L) on day 7; 96.9 % (1.0 mg/L) and 99.2 % (2.0 mg/L) on day 14. By day 21, removal rates remained high, reaching 98.2 % and 99.3 % in the 1.0 and 2.0 mg/L treatments, respectively (Fig. 1b). GC–MS was used to confirm the removal of acetaminophen detected by HPLC-DAD (Table 1).

In the 1.0 and 2.0 mg/L treatments, residual acetaminophen was still detectable at days 7 and 14, but only in trace amounts. The molecular ion corresponding to acetaminophen was observed, although with weak intensity and without clear confirmation of the base ion. This suggests the presence of residual compound near the detection limit. By day 21, no acetaminophen was detected at any concentration. In the 0.1 mg/L treatment, no acetaminophen was observed at any time point. These results corroborate the HPLC-DAD quantification, supporting the conclusion that *L. crithmoides* effectively removed acetaminophen from the culture medium within the tested timeframe. The weak GC–MS signals at early time points in higher concentrations indicate that the compound was nearly depleted by day 14 and fully removed by day 21.

3.2. Plant responses

3.2.1. Photosynthetic pigment determination

Total chlorophyll in aerial parts was highest in the control at day 7 (0.87 mg/g FW), and decreased progressively with increasing acetaminophen concentration, reaching 0.31 mg/g FW at 2.0 mg/L. However, significant reductions of the total chlorophyll from the control were only found in the higher concentrations (1 and 2 mg/L, $p < 0.05$) By day 14, chlorophyll content increased significantly in 1.0 and 2.0 mg/L (0.93 and 1.12 mg/g FW, respectively), while it declined significantly in control and 0.1 mg/L ($p < 0.05$). On day 21, levels increased significantly in the 0.1 mg/L group but decreased significantly in the 1.0 mg/L and 2.0 mg/L treatment (Fig. 2a, $p < 0.05$). In roots, chlorophyll content was consistently low across all treatments, ranging between 0.26 and 0.63 mg/g FW, with only minor fluctuations throughout the experiment (Fig. 2c). On Day 7, chlorophyll levels varied among treatments, with 1.0 mg/L showing the lowest values, significantly below

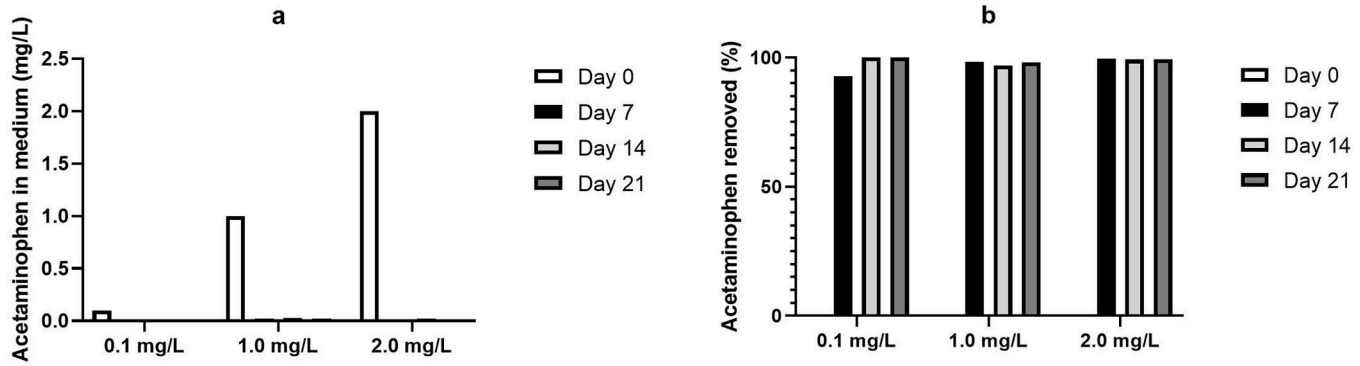

Fig. 1. Acetaminophen concentration (mg/L) in MS medium (a) and removal percentage (%) by *L. crithmoides* (b) across treatments (0.1, 1.0, and 2.0 mg/L) and incubation periods (7, 14, and 21 days). Values represent mean ± SE of three technical replicates (n = 3) obtained from a composite sample of 10 plants per treatment.

Table 1
GC–MS analysis of acetaminophen-spiked culture media across treatment concentrations (0.1, 1.0, and 2.0 mg/L) and incubation times (days 7, 14, and 21).

Concentration	Day 7	Day 14	Day 21
0.1 mg/L	Nd	Nd	Nd
1.0 mg/L	Trace ion *m/z* 151*	Trace ion *m/z* 151*	Nd
2.0 mg/L	Trace identified*	Trace identified*	Nd

Nd: Not detected. *: below the quantification limit.

both 0.1 and 2.0 mg/L ($p < 0.05$). By Day 14, chlorophyll peaked under the 1.0 and 0.1 mg/L treatments, while the 2 mg/L treatment remained similar to the control. On day 21 showed a significant increase in the acetaminophen treatments (0.1–2 mg/L) compared to the control, with the highest levels depicted in the roots exposed to 0.1 mg/L (0.52 mg/g FW).

Carotenoid levels in aerial tissues followed a similar pattern. Initial values ranged from 0.05 to 0.11 mg/g FW across treatments, with significant lower levels at higher acetaminophen concentrations (1.0 and 2.0 mg/L) ($p < 0.05$). A significant increase occurred on day 14 in 1.0 and 2.0 mg/L (0.11 and 0.13 mg/g FW, respectively), followed by a

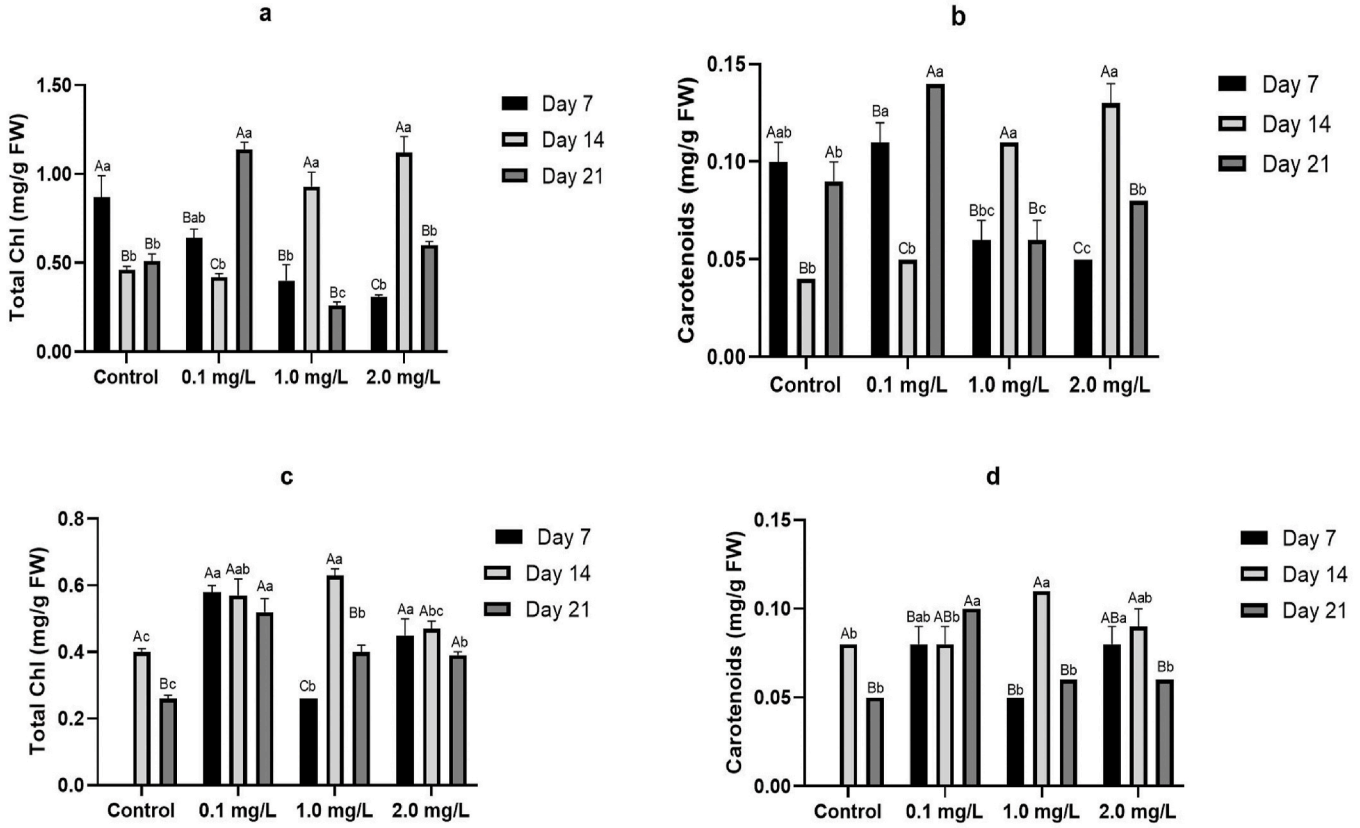

Fig. 2. Total chlorophyll (mg/g FW) and total carotenoids (mg/g FW) of *L. crithmoides* aerial parts (a-b) and roots (c-d) across treatments (0.1, 1.0, and 2.0 mg/L) and incubation periods (7, 14, and 21 days). Values represent mean ± SE of three technical replicates (n = 3) obtained from a composite sample of 10 plants per treatment. Columns marked with different uppercase letters (A–C) indicate significant differences between incubation times; lowercase letters (a–c) indicate differences between treatments within each time point (Two-way ANOVA with factors Concentration and Time, Tukey test, $p < 0.05$). Shared letters denote no significant difference.

significant decline on day 21 (Fig. 2b; $p < 0.05$). In contrary, the control and 0.1 mg/L showed a significant decrease at day 14 followed by a significant increase on day 21 ($p < 0.05$). Root carotenoid content remained more stable, with a slight increase observed on day 14, especially at 1.0 mg/L, before returning to near-initial values (Fig. 2d, $p < 0.05$). The 0.1 mg/L treatment showed a further increase on day 21, significantly different from the values measured on day 7 ($p < 0.05$)

3.2.2. Oxidative stress markers

Malondialdehyde (MDA) levels in 20 % TCA extracts of aerial and root tissues varied with both acetaminophen concentration and incubation time. In aerial tissues, MDA content remained relatively stable across treatments, with no significant differences from the control. An exception was observed on day 14, where plants treated with 0.1 mg/L showed a significant lower MDA level (14.66 µmol/g FW) compared to the control (22.06 µmol/g FW) ($p < 0.05$). In control plants, MDA increased significantly over time, from 16.88 on day 7–24.29 µmol/g FW on day 21 ($p < 0.05$). In the 0.1 and 1.0 mg/L treatments, MDA reached maximum values on day 21 (23.34 and 31.02 µmol/g FW, respectively), which was significantly different from day 7 and day 14 for 1.0 mg/L. At 2.0 mg/L, MDA levels did not show significant variation over time (Fig. 3a).

In root tissues, MDA content was generally higher on day 7 across treatments, with a significant decrease observed over time only in the 1.0 mg/L group ($p < 0.05$). At this concentration, MDA decreased significantly from 25.77 µmol/g FW on day 7–19.84 and 20.85 µmol/g FW on days 14 and 21, respectively. At the end of the experiment, MDA levels were consistently lower in roots than in aerial parts (Fig. 3b).

3.2.3. Osmoprotectants determination

The levels of free proline, soluble proteins, and total soluble sugars in *L. crithmoides* were assessed in aerial and root tissues following ethanol extraction. In aerial tissues, proline content peaked on day 7 at 1.0 mg/L (2.22 µmol/g FW), compared to 1.65 µmol/g FW in the control ($p < 0.05$), whereas the 0.1 mg/L treatment showed significant lower levels (1.16 µmol/g FW). On day 14, values increased further in all plants, reaching 2.64 µmol/g FW at 2.0 mg/L ($p < 0.05$), except for 1.0 mg/L where levels remained stable. By day 21, proline levels decreased across all treatments returning to their initial values, except 1.0 mg/L where the decrease is lower than the control but not significantly (Fig. 4a). In roots, proline levels were higher overall in the treated plants, with values exceeding 4.5 µmol/g FW at 1.0 mg/L on day 7. Even though no significant changes were observed in the control with increased incubation time, the treated plants showed a significant progressive decline with increased incubation times ($p < 0.05$) except for 2.0 mg/L where day 14 showed the highest levels of free proline (4.08 65 µmol/g FW, $p < 0.05$), followed by a significant decline on day 21 (Fig. 5a).

Soluble protein content in aerial tissues showed highest levels on day 7, reaching 1.67 mg/g FW at 0.1 mg/L. Protein levels significantly declined by day 14 in all groups ($p < 0.05$), except for 1.0 mg/L where levels remained stable throughout time. On day 21, a recovery in protein content was observed in the 0.1 and 2.0 mg/L treatments, while control values decreased significantly further (Fig. 4b, $p < 0.05$). In roots, protein levels were initially high in all treatments (e.g., 4.28 mg/g FW in the control on day 7). On day 14, protein content remained relatively stable in the 0.1 and 1.0 mg/L treatments but decreased significantly in the 2.0 mg/L group ($p < 0.05$). By day 21, protein content declined across all treatments ($p < 0.05$), most notably in the 0.1 mg/L group (Fig. 5b), except for control where levels remained stable throughout time.

Soluble sugar content in aerial tissues increased significantly in all treatments compared to control, reaching highest levels at 1.0 mg/L (6.85 mg/g FW). On day 14, values remained high at 1.0 mg/L (5.94 mg/g FW), whereas a significant increase was depicted in control, 0.1 and 2.0 mg/L. By day 21, sugar levels declined significantly in all groups (Fig. 4c, $p < 0.05$), except for 1.0 mg/L. In root tissues, sugar significantly increased in the treated groups, particularly at 1.0 mg/L on day 7 (13.81 mg/g FW), followed by a gradual decrease over time ($p < 0.05$). At 2.0 mg/L, sugar content dropped to 5.10 mg/g FW by day 21 (Fig. 5c).

3.2.4. Secondary metabolism keys

The phenolic, flavonoid and tannin content in ethanol extracts in both aerial parts and roots of *L. crithmoides* were assessed (Fig. 6). In aerial tissues, phenolic content showed a delayed but progressive accumulation in treated plants (Fig. 6a and c). On day 7, levels were comparable between control and 0.1 mg/L, ranging from 0.52 to 0.51 mg/g FW. However, 1.0 and 2.0 mg/L showed a significant increase ($p < 0.05$). By day 14, increases were observed at 0.1 and 2.0 mg/L (0.78 and 0.87 mg/g FW, respectively) ($p < 0.05$), while control and 1.0 mg/L showed similar values to day 7. On day 21, maximum values were recorded for all groups, ranging from 0.71 to 1.38 mg/g. In roots, only the duration of the incubation time had a significant effect. Phenolic levels were initially higher in the control (1.62 mg/g FW), and either remained stable or decreased over time, particularly in the 2.0 mg/L group, which declined from 1.36 to 0.96 mg/g FW between days 7 and 21 ($p < 0.05$).

Flavonoid content in aerial parts declined progressively and significantly in treatments, with values from dropping from 1.79 mg/g FW in the control to 1.50 and 1.29 mg/g FW in 1.0 and 2.0 mg/L, respectively ($p < 0.05$). Over time in the control and 0.1 mg/L groups ($p < 0.05$), with values dropping from around 1.7 to 1.0 mg/g FW and 1.69 to 0.83 mg/g FW, respectively. At 1.0 and 2.0 mg/L, flavonoid levels decreased significantly on day 14 ($p < 0.05$) and increased significantly again by

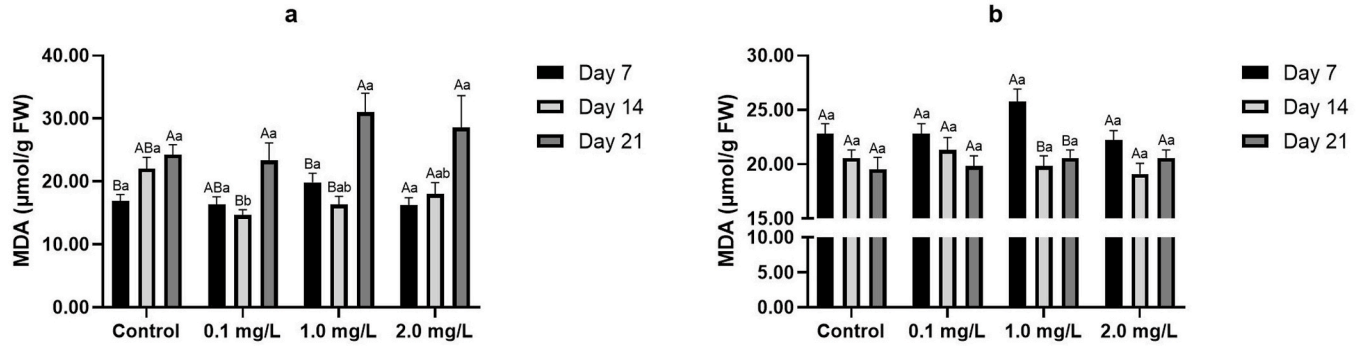

Fig. 3. Malondialdehyde (MDA) content (µmol/g FW) of *L. crithmoides* aerial parts (a) and roots (b) across treatments (0.1, 1.0, and 2.0 mg/L) and incubation periods (7, 14, and 21 days). Values represent mean ± SE of three technical replicates (n = 3) obtained from a composite sample of 10 plants per treatment. Columns marked with different uppercase letters (A–B) indicate significant differences between incubation times; lowercase letters (a–b) indicate differences between treatments within each time point (Two-way ANOVA with factors Concentration and Time, Tukey test, $p < 0.05$). Shared letters denote no significant difference.

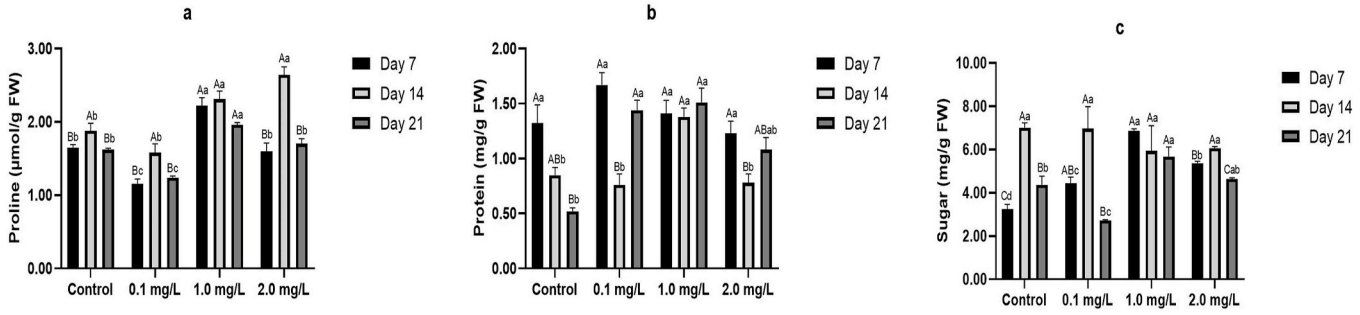

Fig. 4. Free proline content (µmol/g FW), total soluble protein (mg/g FW) and total soluble sugars (mg/g FW) of *L. crithmoides* aerial parts (a-c) across treatments (0.1, 1.0, and 2.0 mg/L) and incubation periods (7, 14, and 21 days). Values represent mean ± SE of three technical replicates (n = 3) obtained from a composite sample of 10 plants per treatment. Columns marked with different uppercase letters (A–C) indicate significant differences between incubation times; lowercase letters (a–b) indicate differences between treatments within each time point (Two-way ANOVA with factors Concentration and Time, Tukey test, $p < 0.05$). Shared letters denote no significant difference.

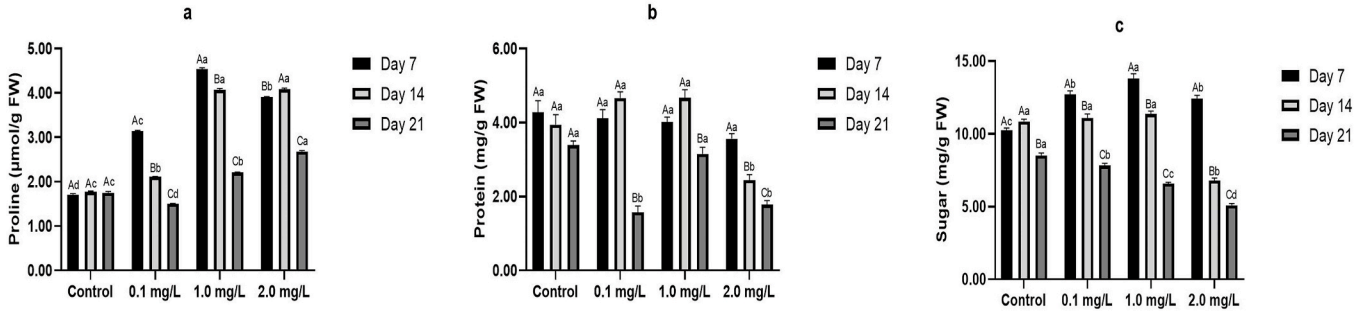

Fig. 5. Free proline content (µmol/g FW), total soluble protein (mg/g FW) and total soluble sugars (mg/g FW) of *L. crithmoides* roots (a-c) across treatments (0.1, 1.0, and 2.0 mg/L) and incubation periods (7, 14, and 21 days). Values represent mean ± SE of three technical replicates (n = 3) obtained from a composite sample of 10 plants per treatment. Columns marked with different uppercase letters (A–C) indicate significant differences between incubation times; lowercase letters (a–b) indicate differences between treatments within each time point (Two-way ANOVA with factors Concentration and Time, Tukey test, $p < 0.05$). Shared letters denote no significant difference.

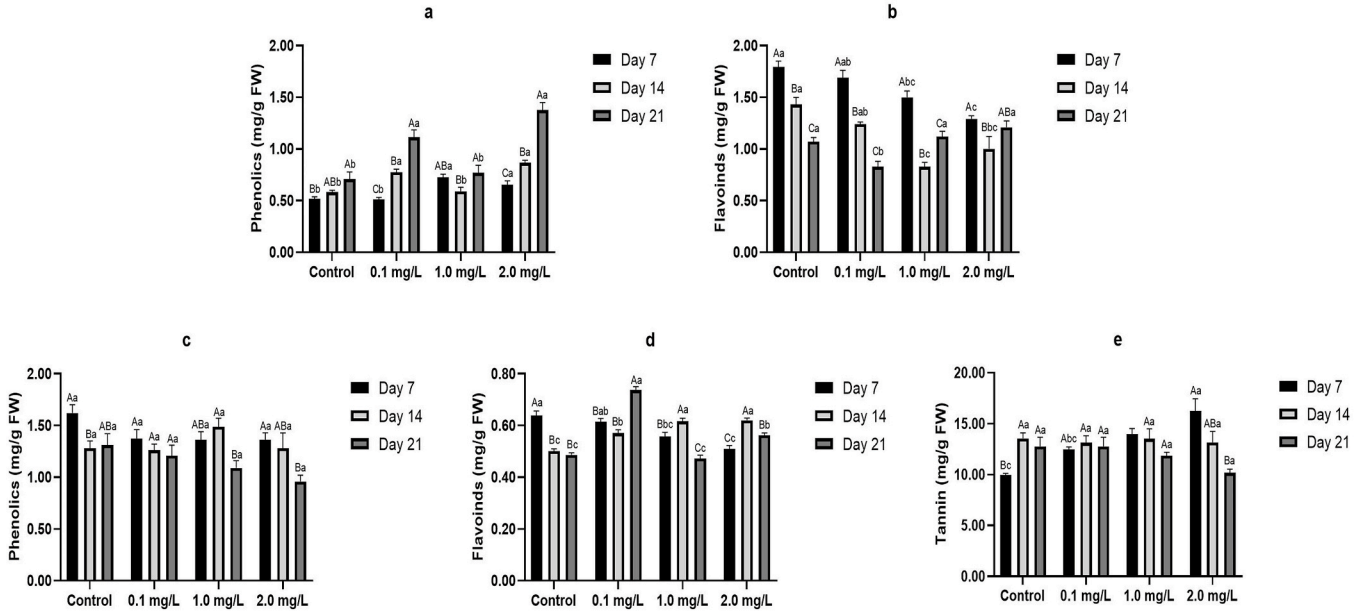

Fig. 6. Total phenolic content (mg/g FW), total flavonoid content (mg/g FW) and total tannin content (mg/g FW) of *L. crithmoides* aerial parts (a-b) and roots (c-d) across treatments (0.1, 1.0, and 2.0 mg/L) and incubation periods (7, 14, and 21 days). Values represent mean ± SE of three technical replicates (n = 3) obtained from a composite sample of 10 plants per treatment. Columns marked with different uppercase letters (A–C) indicate significant differences between incubation times; lowercase letters (a–b) indicate differences between treatments within each time point (Two-way ANOVA with factors Concentration and Time, Tukey test, $p < 0.05$). Shared letters denote no significant difference.

day 21 ($p < 0.05$), reaching 1.21 mg/g FW at 2.0 mg/L (Fig. 6b), however remaining lower than on day 7. In roots, flavonoid levels were lower overall. Just like in the aerial parts, on day 7 a significant and progressive decline is observed in the treatments from 0.64 mg/g FW in control to 0.56 and 0.51 mg/g FW in 1.0 and 2.0 mg/L ($p < 0.05$). In the control, levels decreased significantly from day 7 to day 21 (0.64–0.49 mg/g FW, respectively; $p < 0.05$). At 0.1 mg/L, values increased progressively, peaking at 0.74 mg/g FW on day 21, significantly different from day 7 ($p < 0.05$). In the higher concentrations, a peak was observed on day 14, followed by a significant decrease (Fig. 6d; $p < 0.05$).

Tannin content was detected only in root tissues. On day 7, treated plants showed a significant and progressive increase in the higher concentrations (13.96 and 16.24 mg/g FW). Control plants exhibited a significant increase from day 7 to day 14, reaching 13.56 mg/g FW ($p < 0.05$). A similar pattern was observed at 0.1, however here this pattern was not significant. At 2.0 mg/L, tannin content peaked earlier, with 16.24 mg/g FW on day 7, followed by a significant decline to 10.17 mg/g FW by day 21 (Fig. 6e; $p < 0.05$).

Shikimic acid (SA) levels in aerial tissues, fluctuated around 19 mg/g FW. On day 7, a significant increase is observed in 0.1 mg/L (18.96 mg/g FW; $p < 0.05$), however, 1.0 mg/L showed a significant increase (19.57 mg/g FW; $p < 0.05$) compared to the control. In the control, there is significant decrease on day 14 ($p < 0.05$) followed by a significant increase on day 21 ($p < 0.05$). Whereas, on day 21 at 2.0 mg/L a significant decrease is depicted in the SA levels (18.91 mg/g FW) (Fig. 7a). In roots, SA levels also remained more stable, with control values ranging between 18.82 and 18.91 mg/g FW and treated groups showing similar trends (Fig. 7c).

Phenylalanine ammonia-lyase (PAL) activity increased significantly in aerial parts treated with higher concentrations (1.62 and 2.02 37 μmol/mg protein/min; $p < 0.05$). In control and 0.1 mg/L a significant increase was observed on day 14 (3.43 and 4.37 μmol/mg protein/min; $p < 0.05$), followed by a significant decrease on day 21 ($p < 0.05$). In the 1.0 and 2.0 mg/L treatment, PAL activity continued to rise significantly until day 21 (2.76 and 3.71 μmol/mg protein/min; $p < 0.05$) (Fig. 7b). In roots, PAL activity remained more stable with significant fluctuations observed in 1.0 and 2.0 mg/L. The treated plants showed a significant higher activity than the control, particularly 1.0 mg/L (1.05 μmol/mg protein/min; $p < 0.05$). In the control, day 14 showed the highest significant activity (1.01 μmol/mg protein/min; $p < 0.05$), followed by a significant decline on day 21 (0.89 μmol/mg protein/min; $p < 0.05$). This decline from day 14–21 was also depicted in 0.1 mg/L ($p < 0.05$) (Fig. 7d).

To provide a unified interpretation of the temporal dynamics observed under acetaminophen exposure, Table 2 summarizes the major response patterns at Days 7, 14, and 21 across the main functional categories. The table integrates statistically supported trends together with their biological meaning, presented separately for aerial parts and roots. Rather than detailing individual parameters, the summary highlights the dominant physiological processes underlying early stress perception, mid-term acclimation, and late-phase responses, offering a clear overview of organ-specific strategies over time.

4. Discussion

4.1. Quantification of acetaminophen in nutrient solutions

This study demonstrated that *L. crithmoides* effectively attenuates acetaminophen concentrations in nutrient solutions under controlled in vitro conditions. The rapid and near-complete disappearance of the compound across all tested levels (0.1–2.0 mg/L) indicates that this species can influence acetaminophen dynamics in aqueous media.

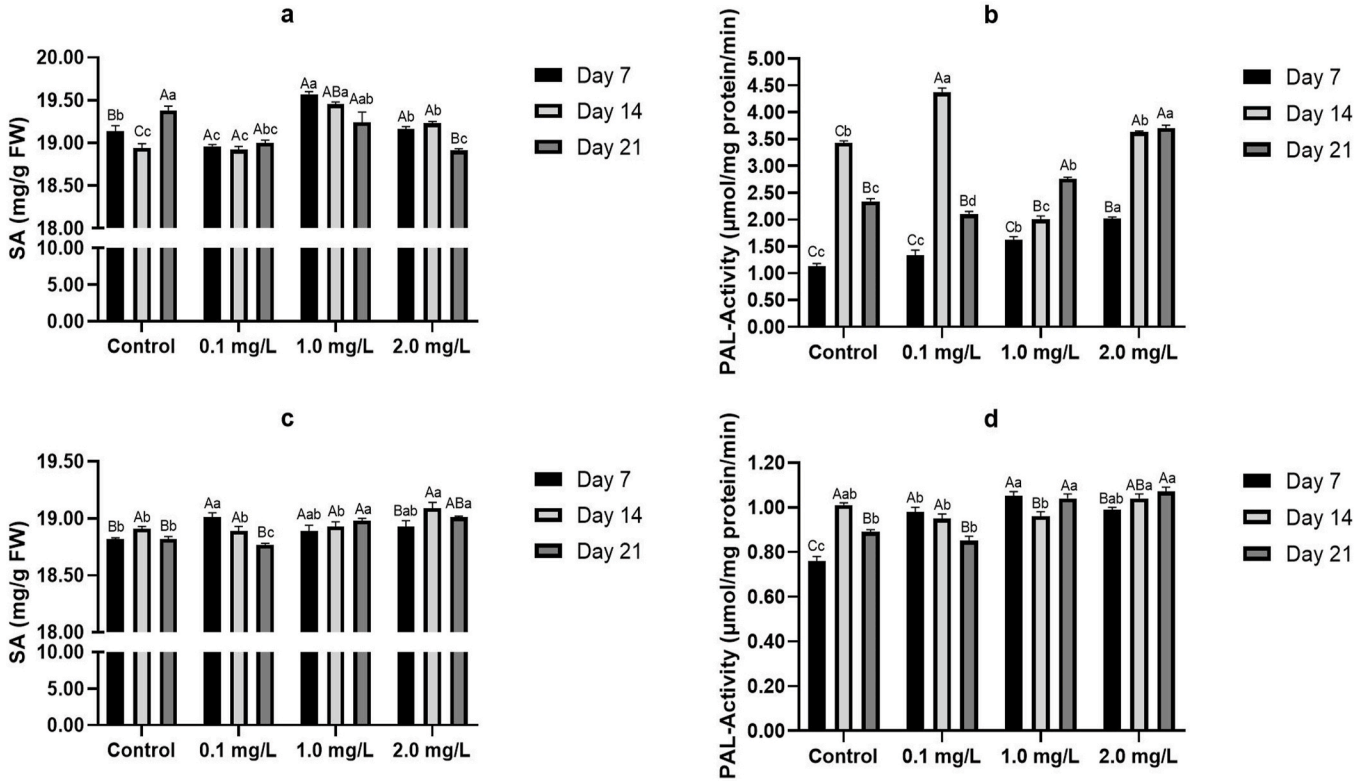

Fig. 7. Shikimic acid (SA) content (mg/g FW) and PAL-activity (μmol/mg protein/min) of *L. crithmoides* aerial parts (a-b) and roots (c-d) across treatments (0.1, 1.0, and 2.0 mg/L) and incubation periods (7, 14, and 21 days). Values represent mean ± SE of three technical replicates (n = 3) obtained from a composite sample of 10 plants per treatment. Columns marked with different uppercase letters (A–B) indicate significant differences between incubation times; lowercase letters (a–c) indicate differences between treatments within each time point (Two-way ANOVA with factors Concentration and Time, Tukey test, $p < 0.05$). Shared letters denote no significant difference.

Table 2
Summary of the major functional responses of *L. crithmoides* exposed to acetaminophen (0–2 mg/L) over 21 days. Trends reflect statistically supported changes across parameters measured within each functional category. Aerial parts and roots are shown separately to highlight organ-specific patterns of early stress perception (Day 7), mid-term (Day 14), and late responses (Day 21).

Category	Day 7 – Early response		Day 14 – Mid response		Day 21 – Late response	
	Aerial parts	Roots	Aerial parts	Roots	Aerial parts	Roots
Photosynthetic performance (chlorophylls, carotenoids)	Pigments reduced at higher doses → early photosynthetic stress in shoots.	Pigment levels low with only minor differences → roots largely insensitive at this stage.	Clear pigment recovery in treated shoots → effective acclimation of photosynthesis.	Slight pigment increase in some treatments → stable performance.	Pigments decline again at higher doses; low dose partly maintained → late photosynthetic cost.	Pigments generally slightly higher in treated roots than control → root photosynthetic status remains stable or mildly stimulated.
Oxidative balance (MDA)	MDA similar to control in shoots → oxidative damage still controlled.	Highest MDA values → roots show an early oxidative signal at contaminant entry.	MDA stable or reduced in shoots → oxidative stress well contained.	MDA decrease at 1 mg/L → mitigation of early oxidative stress in roots.	MDA rises in shoots, especially at 0.1–1 mg/L → emerging late imbalance.	MDA mostly stable, with slight rise only at highest dose → moderate final oxidative burden in roots.
Osmotic and primary metabolic adjustment (proline, soluble sugars, proteins)	Proline and sugars strongly increased in shoots (especially 1–2 mg/L) → strong early osmotic/ROS buffering; proteins still relatively high.	Very high proline and sugars in treated roots → roots act as main osmoprotectant reservoir.	Proline and sugars remain high in most shoot treatments → protective pools maintained during acclimation, although proteins decline.	Proline and sugars start to decrease; proteins reduced at 2 mg/L → early buffering in roots begin to be used up.	Proline, sugars and proteins generally decline in shoots → partial exhaustion of primary osmoprotective pools.	Sugars and proteins decline further but remain higher than in shoots → roots retain residual osmotic protection despite metabolic cost.
Phenylpropanoid/ secondary metabolism (phenolics, flavonoids, tannins, PAL; shikimic acid precursor)	Phenolics already higher at 1–2 mg/L and flavonoids reduced in shoots → early antioxidant mobilization; shikimic acid pool stable.	High basal phenolics and early tannin peak at 2 mg/L → strong constitutive and structural antioxidant capacity in roots.	Phenolics and PAL activity rise in shoots, especially at some doses → active engagement of the phenylpropanoid pathway; shikimic acid remains stable.	Root phenolics generally stabilize or begin to decrease; tannins highest in control/0.1 mg/L → shift from strong reinforcement to maintenance.	Phenolics reach maximum levels, and PAL remains high in shoots, with partial flavonoid recovery; shikimic acid only slightly reduced at 2 mg/L → sustained late antioxidant effort.	Root phenolics and tannins decline and PAL stabilizes → reduced root investment in secondary defenses after the initial peak.

Although this suggests an active plant–pollutant interaction, the data, derived solely from the nutrient solution, cannot distinguish whether the reduction results from uptake and internal transformation by the plant or from adsorption of the compound onto root surfaces. Because the experiments were conducted under sterile in vitro conditions, microbial degradation can be ruled out. Further studies quantifying acetaminophen and its metabolites within plant tissues are therefore needed to confirm the removal mechanisms involved. Although previous applications of *L. crithmoides* in constructed wetland systems reported substantial reductions in nutrient and organic loads from port effluents (Oliveira, 2021), the behaviour of pharmaceutical contaminants had not been addressed. The present results therefore provide the first evidence that this estuarine species can influence the fate of acetaminophen in aqueous media, supporting its potential ecological role in contaminant attenuation within saltmarsh environments.

Few studies have examined acetaminophen uptake by plants. *Alternanthera* spp. cultivated in mesocosm wetlands removed up to 89 % of acetaminophen (100 mg/L) after 35 days (Mohammed et al., 2021). Under hydroponic conditions, *Lupinus albus* achieved complete elimination within 2–4 days at 0.1–0.2 mM, while *Hordeum vulgare* and *Phragmites australis* showed lower or inconsistent responses (Kotyza et al., 2010). Despite the lower concentrations tested here, *L. crithmoides* displayed >92 % reduction at 0.1 mg/L and >98 % at 1.0–2.0 mg/L within seven days, reaching near-complete disappearance by day 14. Such rapid attenuation suggests efficient uptake, sequestration, or biotransformation within plant tissues rather than passive sorption.

Nevertheless, it remains necessary to determine whether acetaminophen is fully mineralized, enzymatically transformed into less toxic intermediates, or compartmentalized within plant tissues. Clarifying these processes under environmentally relevant conditions will be essential for understanding the contribution of saltmarsh vegetation to contaminant cycling and attenuation in estuarine ecosystems.

4.2. Plant responses

The physiological and biochemical responses of *L. crithmoides* to acetaminophen exposure revealed a temporally structured stress adaptation strategy, characterized by an initial oxidative signal followed by metabolic adjustments that included the modulation of photosynthetic performance, osmoprotectant accumulation, and secondary metabolism. These responses illustrate a coordinated balance between damage containment and activation of defense pathways over time and across organs.

A reduction in photosynthetic pigments observed on day 7, particularly at higher acetaminophen concentrations, reflects early oxidative impairment of chloroplasts. Similar pigment reductions have been reported in *Lactuca sativa* and *Zea mays* under acetaminophen stress, linked to ROS-mediated inhibition of chlorophyll biosynthesis and PSII activity (Leitão et al., 2021; Kudrna et al., 2020; Opriş et al., 2020). At this early stage, MDA levels remained low, suggesting that oxidative stress was present but effectively buffered.

This protective capacity was likely mediated by early accumulation of proline and soluble sugars, known to stabilize proteins and membranes, chelate ROS, and support osmotic balance (Szabados and Savouré, 2010; Slama et al., 2015). A comparable pattern was reported in *Hordeum vulgare*, where ROS accumulation under acetaminophen exposure was not accompanied by MDA elevation, due to efficient antioxidant buffering (Soares et al., 2018).

By day 14, pigment levels recovered in aerial tissues even under continued exposure. This coincided with increased proline, sugars, and PAL activity, as well as enhanced phenolic accumulation, indicating mobilization of both primary and secondary defences. These responses suggest a successful acclimation phase supported by ROS-scavenging phenolics and compatible solutes. Similar recovery mechanisms have been documented in barley and lettuce under acetaminophen stress (Soares et al., 2018; Leitão et al., 2021), reinforcing this interpretation.

However, this acclimation likely involved a reallocation of metabolic resources. Between days 7 and 14, total protein content declined in aerial parts, which may reflect both diversion of nitrogen and energy toward antioxidant defenses and conversion of soluble proteins into structural components or stress-related enzymes involved in detoxification and cell protection. Such metabolic reprogramming is proteomic studies showing that stress-tolerant plants redirect protein turnover toward defense and repair pathways rather than primary growth (Kosová et al., 2018). By day 21, soluble sugars also declined, likely due to sustained utilization as energy substrates and carbon skeletons supporting antioxidant and phenolic biosynthesis. Together, these changes suggest not only a metabolic cost but also an adaptive redistribution of biochemical resources supporting tolerance and recovery under prolonged exposure. In roots, proline levels were generally higher than in aerial parts, especially at early timepoints, highlighting their role as primary stress sensors. Despite direct contact with acetaminophen, MDA levels in roots remained low throughout, potentially due to early antioxidant activation or limited internalization through anatomical barriers such as the endodermis (Chen et al., 2022). Additionally, root-specific accumulation of tannins suggests cell-wall fortification and xenobiotic sequestration mechanisms (Dixon et al., 2004).

By day 21, the protective capacity of aerial tissues appeared compromised: MDA levels rose sharply at higher acetaminophen concentrations, alongside reductions in pigments, proline, sugars, and proteins. This pattern reflects oxidative damage accumulation and exhaustion of metabolic reserves. PAL activity and total phenolics continued to rise, indicating ongoing attempts to enhance antioxidant defenses. However, the decline in flavonoids, despite the general phenolic increase, suggests preferential use or turnover of specific antioxidant pools (Shalaby and Horwitz, 2014).

Shikimic acid levels and PAL activity remained comparatively stable in roots but increased markedly in aerial parts, indicating spatially differentiated metabolic responses. The delayed oxidative stress in aerial tissues, combined with relatively stable root MDA levels, supports the hypothesis of translocation of acetaminophen or its metabolites from root to shoot via xylem. Similar spatial patterns have been reported in *Lactuca* and *Hordeum* under acetaminophen exposure, where roots act as initial contact points, but leaves develop stronger stress signatures over time (Leitão et al., 2021; Soares et al., 2018). Although this interpretation aligns with physiological and biochemical markers, confirmation of compound distribution would require direct quantification in shoot and root tissues.

In summary, the response of *L. crithmoides* to acetaminophen stress involved early osmoprotectant accumulation (proline, sugars) and moderate phenolic activation to buffer oxidative damage, followed by progressive antioxidant mobilization (PAL activity and total phenolics) and, at later stages, oxidative imbalance marked by pigment decline and elevated MDA in aerial tissues. This pattern indicates that, although the species exhibits a marked tolerance to acetaminophen, a physiological threshold may be reached under prolonged exposure, leading to partial oxidative damage. The coordinated behavior between roots and aerial parts suggests possible translocation of acetaminophen or its metabolites within the plant; however, this should be regarded as a working hypothesis, since no quantification of the compound or its transformation products in tissues was performed.

These temporally and spatially coordinated defenses illustrate the ability of a native saltmarsh species to tolerate pharmaceutical exposure and maintain metabolic functionality under controlled conditions. Nevertheless, the occurrence of late oxidative stress reveals that this tolerance is not absolute but rather reflects an adaptive balance between resistance and damage repair. This dual evidence of tolerance and pollutant attenuation underscores the ecological relevance of *L. crithmoides* as a resilient component of saltmarsh vegetation in estuarine environments subject to chemical stress. Furthermore, as *L. crithmoides* is also consumed as a wild edible plant ("golden samphire"), future studies should investigate tissue-level accumulation and metabolite transformation to clarify potential environmental implications of pharmaceutical exposure.

While this work provides a controlled demonstration of the physiological and biochemical responses of *L. crithmoides* to acetaminophen exposure, several limitations should be acknowledged. The experiments were performed under sterile in vitro conditions, which ensured reproducibility and isolation of plant responses but do not fully reflect the complexity of natural saltmarsh environments, where interactions with soil matrices and associated microbiota can influence contaminant fate. Moreover, internal concentrations of acetaminophen and its metabolites were not quantified, preventing direct confirmation of uptake and transformation processes. The exposure concentrations (0.1–2.0 mg/L), although representative of moderate contamination scenarios, were also selected to facilitate reliable analytical detection and quantification of acetaminophen and the associated biochemical markers with the available instrumental methods. Nevertheless, they may not encompass the full range of concentrations typically observed in situ (ng–µg/L), particularly under chronic or episodic pollution events. Finally, the absence of environmental fluctuations such as tides, variable salinity, and co-occurring stressors limits the extrapolation of these results to field conditions.

In addition, because *L. crithmoides* is a naturally resilient and widely distributed salt-tolerant species along the southern Portuguese coast, its responses may not represent those of more sensitive plant species. Comparative studies involving less tolerant taxa will therefore be important to evaluate the broader ecological relevance of the mechanisms identified here.

Future studies should therefore integrate non-sterile soil–plant systems, environmentally relevant pollutant levels, and longer exposure periods, while quantifying contaminant and metabolite levels in tissues. Such approaches will help bridge the gap between mechanistic understanding and the ecological role of saltmarsh vegetation in pharmaceutical attenuation under realistic conditions.

5. Conclusions

This study demonstrates that *L. crithmoides*, a salt-tolerant plant native to Atlantic–Mediterranean saltmarshes, can effectively remove acetaminophen in aqueous media under controlled in vitro conditions. The consistent reduction across all tested concentrations, together with the species' ability to maintain physiological performance through temporally structured stress responses, highlights its tolerance and metabolic resilience to pharmaceutical exposure, although signs of late oxidative imbalance indicate that this tolerance has physiological limits.

These findings provide the first direct evidence that a native coastal plant with both ecological and ethnobotanical value can withstand acetaminophen-induced stress while modulating physiological and biochemical defenses. Although this work was conducted under controlled conditions and did not quantify tissue burdens or transformation products, it establishes a mechanistic framework for understanding how pharmaceutical contaminants interact with saltmarsh vegetation.

Such resilience and attenuation capacity under controlled exposure suggest that *L. crithmoides* could contribute to natural or assisted contaminant attenuation processes in saline and estuarine environments, supporting the development of sustainable, nature-based strategies for mitigating pharmaceutical pollution. Future studies should verify the localization and transformation of acetaminophen and its metabolites within plant tissues, extend observations to environmentally relevant concentrations and exposure times, and evaluate the broader ecological implications of pharmaceutical stress on the structure and functioning of coastal vegetation.

CRediT authorship contribution statement

Hanne Hoornaert: Writing – original draft, Methodology,

Investigation, Formal analysis, Data curation. **Nuno Neng:** Methodology. **Luísa Custódio:** Resources, Conceptualization. **Maria João Rodrigues:** Writing – review & editing, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2025.109640.

Data availability

Data will be made available on request.

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