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Biochemical Changes in Aquatic Weeds in Response to Perchlorate Contamination, an *in vitro* Study

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ABSTRACT

The current study assessed the effect of perchlorate on four common aquatic weeds: *Pistia stratiotes*, *Eichhornia crassipes*, *Salvinia molesta* and *Spirodela polyrhiza*. Notably, *P. stratiotes* exhibited a significant reduction of 26.18% in a 1 ppm perchlorate solution. Bioorganic characterization revealed a decrease in the concentration of total reducing sugars, lipids, and chlorophyll across all four plants under perchlorate stress. Conversely, carotenoid content, a recognized antioxidant, increased in response to perchlorate exposure. The activity of antioxidant enzymes, including catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX), exhibited tissue-specific patterns. CAT was notably elevated in both shoots and roots of *P. stratiotes*, establishing it as the most crucial antioxidant enzyme under perchlorate stress. Variations in the concentrations of APX and GPX were observed in all plants, indicating distinct reactive oxygen species (ROS) scavenging mechanisms across different plant parts. The results indicate the role of CAT and other antioxidants in *P. stratiotes* to tolerate perchlorate stress through improved ROS management, shifts in antioxidant enzyme balance, adjustments in metabolic pathways, and potential changes in protein expression related to the stress response.

Keywords Perchlorate, Phytoremediation, *Pistia stratiotes*, Catalase, SDS-PAGE.

INTRODUCTION

Perchlorate (ClO_4^-) is a compound found both naturally and as a byproduct of human activities, constituting the majority (Sijimol *et al*. 2015). Widely utilized in defense, aerospace, and various industrial applications, ClO_4^- has raised environmental and health concerns (Li *et al.* 2022, Sijimol *et al.* 2015). Its impact extends to disrupting reproductive cycles, endocrine functions, immune systems, nervous systems, and thyroid activities in aquatic and terrestrial organisms, as well as causing reduced growth and damage to the photosystem and root system in plants (Li *et al.* 2022, Acevedo-Barrios and Olivero-Verbel 2021). With high stability, solubility, and mobility in water, ClO_4^- becomes a significant inorganic contaminant in groundwater and aquatic bodies, alarming levels of which have been detected in various environmental compartments (Li *et al.* 2022). Human and animal exposure to ClO_4^- primarily occurs through drinking

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contaminated water and ingesting polluted foods (Steinmaus 2016). Efforts to remediate ClO_4^- contamination involve physico-chemical and biological approaches (Fang and Naidu 2023, Nair *et al.* 2020). Biological methods like phytoremediation, including phytoaccumulation, phytodegradation, and rhizodegradation, allow for the uptake and accumulation of ClO_4^-, contributing to effective remediation (Sai *et al.* 2024). In response to ClO_4^- stress, plants activate a complex antioxidant defense system involving enzymes such as CAT and peroxidases, crucial for mitigating oxidative damage and maintaining cellular balance (Gupta and Soni 2015). The present *in vitro* study aims to evaluate the effect of ClO_4^- stress on four common aquatic weeds and the biochemical changes in these plants, thereby contributing essential knowledge to understanding ClO_4^- effects on aquatic plant biology.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical reagent grade. Potassium perchlorate (99.99%), Coomassie brilliant blue R-250 (technical grade, ~50%), ethylene diaminetetra acetic acid (EDTA 99.5%), glycine (≥99%), acrylamide (99.9%), bisacrylamide, ammonium persulfate (99%), N,N,N',N'-tetramethylethylenediamine (~99%), 2-mercaptoethanol (99%), bromophenol blue (ACS grade), Tris-HCl (99%), and dinitrosalicylic acid (DNS) were procured from Sigma-Aldrich (Millipore Sigma), Merck, United States. Sodium phosphate monobasic (99%), sodium phosphate dibasic (99%), potassium phosphate monobasic (99%), potassium phosphate dibasic (99%), sodium dodecyl sulfate (SDS 99.5%), sodium hydroxide (≥97%), glucose, copper sulfate (99.5%), sodium bicarbonate (99.5%), trichloroacetic acid (99%), Folin's reagent (AR grade), bovine serum albumin (BSA, 98%), chloroform (99%), methanol (99.8%), sodium chloride (99.9%), ninhydrin (99%), acetone (99%), guaiacol (≥99%), hydrogen peroxide (H_2O_2 30%), ferric chloride (98%), and potassium ferricyanide (98%) were obtained from Sisco Research Laboratories Pvt Ltd (SRL), India. Instruments used included a UV-VIS spectrophotometer (Systronics 108), a pH meter (Labtronics LT-10), a centrifuge (REMI CPR-24 Plus), a water bath (Sisco India 1 SIC-33(D)), an electrophoresis apparatus (Biotech Dual Page - Mini 05-03), electronic weighing balances (Shimadzu AUY 220), anion chromatograph (Dionex-1100), and a transilluminator (Biotech).

Young plants of *Pistia stratiotes*, *Eichhornia crassipes*, *Salvinia molesta* and *Spirodela polyrhiza* were collected from the Veli and Chakkai regions of Thiruvananthapuram, Kerala, and identified based on morphological characteristics. The plants were maintained in tap water in separate cement tanks at the Botanical Garden, University College, Thiruvananthapuram, and allowed to acclimatize for two weeks prior to the experiments.

Screening experiment of aquatic weeds for ClO_4^- removal

The initial screening of plants was conducted in 25-liter capacity plastic vessels. Each experimental setup comprised four vessels, with one serving as a control and three as samples. All vessels were filled with five liters of tap water. In the sample vessels, 5 ml of water was removed, and 5 ml of potassium perchlorate solution was added from a 1000 ppm stock solution, resulting in a final 1 ppm solution. One hundred grams of healthy plants, selected from the stock tank, were added to all four vessels. Before weighing, excess water was blotted away using a clean cloth. Throughout the experiment, the loss of water due to evapotranspiration was accounted for by recording the initial and final weights. The entire setup was arranged in low sunlight conditions. Water samples were collected from each vessel at 72-hour intervals, and residual ClO_4^- was measured. ClO_4^- removal was calculated after adjusting for water loss from the respective vessels. The experiment duration was 7 days. On the final day, the treated plants were weighed to determine any change in biomass. Subsequently, the plants were stored in plastic bags at 4°C for further analytical studies. ClO_4^- concentration was measured using ion chromatography IC (Dionex-1100) with AS 11 and AS 16 columns. The mobile phase consisted of Na_2CO_3:$NaHCO_3$ (1:4.8 mM) and 50 mM NaOH buffer for AS 11 and AS 16 columns, respectively, at a flow rate of 1.5 ml/min.

Variations in concentrations of primary metabolites and pigments

The variation in concentration of total reducing sugar (Miller 1959), protein (Lowry *et al.* 1951), and total lipids (Bligh and Dyer 1959) in the leaves and roots of plants were determined based on standard protocols. Additionally, the total chlorophyll and carotenoid content were estimated (Arnon 1949). Spirodela, being a minute plant, was used as such for the extraction.

Enzyme assays

The shoots and roots of plants were homogenized in 10 ml of 0.1 M potassium phosphate buffer (pH 7) containing 1 mM EDTA. The homogenate was filtered and centrifuged at 7400 rpm for 15 minutes at 4°C. The resulting supernatant served as the enzyme source for measuring the activities of CAT and APX. CAT activity (EC 1.11.1.6) was determined following the method of Aebi (1974), and APX activity (EC 1.11.1.11) was estimated using the method described by Nakano and Asada (1981). Guaiacol peroxidase (GPX) (EC 1.11.1.7) was isolated and assayed according to the procedures outlined by Ingham *et al.* (1998) and Goliber and Feldman (1989).

Qualitative analysis of catalase and SDS-PAGE

The quantitative analysis of catalase was conducted through zymography (Weydert and Cullen 2010). The separating and stacking gels had concentrations of 8% and 5%, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed (Laemmli 1970). The separating and stacking gels had concentrations of 12% and 5%, respectively. The protein markers used were lysozyme (14.3 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), egg albumin (45 kDa), and BSA (66 kDa).

RESULTS AND DISCUSSION

Variations in aquatic weeds during initial ClO_4^- removal screening

In response to ClO_4^- stress, *Pistia* and *Spirodela* exhibited changes in biomass. Conversely, *Eichhornia* and *Salvinia* showed no significant change in biomass after 7 days of ClO_4^- treatment in the experimental setup. Perchlorate-treated *Pistia* and *Spirodela* plants displayed an average biomass decrease of 30%, compared to 20% for the control group. Among the four plants, only *Pistia* exhibited a noteworthy reduction in ClO_4^- ion concentration, reaching up to 26.18% (Fig. 1). While *Spirodela* showed biomass variation similar to *Pistia*, it resulted in a maximum ClO_4^- ion reduction of 6.26%. *Pistia* demonstrated effective removal, eliminating 16% of ClO_4^- within the third day and 26% by the seventh day of treatment with a 1 ppm solution (Fig. 1). The other plants showed low significance in this regard. Phytoremediation, encompassing phytoaccumulation, phytodegradation, and rhizodegradation, proved successful for ClO_4^- removal by plants. Phytoaccumulation and rhizodegradation were identified as the modes of ClO_4^- remediation in *Pistia* (Beniah and Christian 2019). *Spirodela* displayed severe wilting and decay, leading to biomass loss, while *Eichhornia* showed root decay and shoot yellowing. In *Salvinia*, some decay of submerged fronds and yellowing of normal aerial shoots were observed. These changes may result from increasing ClO_4^- ion concentration due to evapotranspiration. This is supported by the observed increase in carotenoid pigment concentration and the reduction in total chlorophyll concentration in the present study. ClO_4^- on entering the plants chiefly accumulates in the leaf tissues resulting in tip burn and chlorosis. ClO_4^- is known to affect normal growth and chlorophyll concentration with previous reports on chlorosis and necrosis under ClO_4^- stress (Sahu and Kumari 2024). Variations in biomass under ClO_4^- stress were noted in rice seedlings, with leaf growth being most affected and root vigor decreasing, potentially due to oxidative damages induced by ClO_4^- stress (Xie *et al.* 2014). A linear relationship between ClO_4^- concentration and the inhibitory effect on growth in vegetal systems of *Phaseolus vulgaris* and *Zea mays* was observed by Acevedo-Barrios *et al.* (2024). The vegetative parts leaves, stems, and roots were affected by alterations in growth, chlorosis, size, shape, thickness, weakness, nodulation and loss of secondary roots, with more pronounced effects observed in the bean species, while the corn plants exhibited higher tolerance.

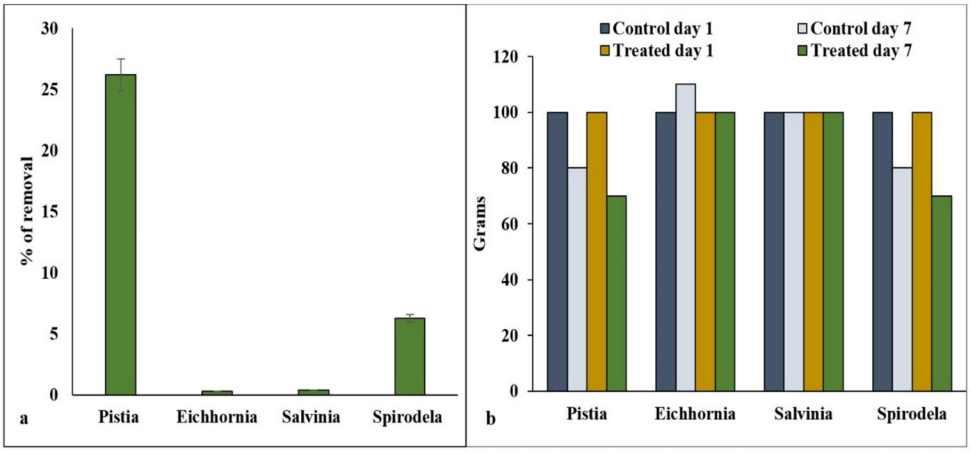

Fig. 1. A. Removal of ClO_4^- by the four selected aquatic weeds. B. Change in biomass of four aquatic weeds.

Variations in concentrations of primary metabolites and pigments

ClO_4^- induces physiological and biochemical stress in plants, potentially impacting metabolic processes (Acevedo-Barrios *et al.* 2024). Plants synthesize, convert, and store starch, proteins, and lipids, and alterations in their levels can directly relate to the plant life cycle. The concentration of total reducing sugar decreased in the presence of ClO_4^- in all plants, with the most significant variation observed in *Salvinia* shoots (2.43 mg/g) and *Spirodela* (1.86 mg/g) (Table 1). A Similar trend of decrease in reducing sugar content was reported in the leaf tissue of *Eucalyptus* under elevated ammonium Perchlorate concentrations (Gupta and Soni 2015). Protein concentration decreased with ClO_4^- treatment in the shoots of *Pistia, Salvinia* and *Eichhornia* (Table 1), with the most notable variation in *Pistia* shoots (0.76 mg/g) and *Salvinia* shoots (0.69 mg/g). In a similar study, the total soluble

Table 1. Variation in concentration of reducing sugar, protein, CAT, APX and GPX under ClO_4^- stress (C- Control and T- ClO_4^- treated), values given are mean ± SD of triplicate.

Sl. No.	Plants	Reducing sugar (mg/g tissue)	Protein (mg/g tissue)	CAT (Units/ min/mg protein)	APX (Units/ min/mg protein)	GPX (Units/ mg protein)
1	*Pistia* shoot ^{C}	1.91 ± 0.12	3.98 ± 0.41	22.44 ± 0.96	2.06 ± 0.08	6.84 ± 0.57
2	*Pistia* shoot ^{T}	1.14 ± 0.2	3.22 ± 0.37	27.01 ± 0.83	2.55 ± 0.12	4.53 ± 0.51
3	*Pistia* root ^{C}	0.35 ± 0.17	0.46 ± 0.29	47.99 ± 0.92	4.31 ± 0.53	23.76 ± 0.82
4	*Pistia* root ^{T}	0.26 ± 0.13	0.49 ± 0.38	58.54 ± 1.05	4.18 ± 0.51	22.32 ± 1.14
5	*Eichhornia* shoot ^{C}	3.61 ± 0.19	3.93 ± 0.66	14.63 ± 1.13	2.71 ± 0.04	5.82 ± 0.73
6	*Eichhornia* shoot ^{T}	3.07 ± 0.25	3.42 ± 0.17	14.11 ± 1.4	2.9 ± 0.06	6.44 ± 0.39
7	*Eichhornia* root ^{C}	1.98 ± 0.24	1.07 ± 0.11	24.74 ± 0.87	3.11 ± 0.28	14.27 ± 1.03
8	*Eichhornia* root ^{T}	1.56 ±0.18	0.98 ± 0.39	33.56 ± 1. 23	3.94 ± 0.34	9.47 ± 0.57
9	*Salvinia* shoot ^{C}	6.07 ± 0.67	3.52 ± 0.69	4.7 ± 0.935	2.55 ± 0.71	3.69 ± 0.67
10	*Salvinia* shoot ^{T}	3.64 ± 0.83	2.83 ± 0.21	5.84 ± 0.98	3.58 ± 0.13	2.56 ± 0.41
11	*Salvinia* submerged fronds ^{C}	2.42 ± 0.16	1.64 ± 0.18	13.17 ± 1.15	2.81 ± 0.39	5.51 ± 0.62
12	*Salvinia* submerged fronds ^{T}	1.28 ± 0.11	1.86 ± 0.15	5.54 ± 1.05	5.03 ± 0.76	7.29 ± 0.35
13	*Spirodela* ^{C}	5.66 ± 0.49	3.68 ± 0.31	12.15 ± 0.85	3.99 ± 0.53	4.81± 0.55
14	*Spirodela* ^{T}	3.8 ± 0.15	3.7 ± 0.29	11.08 ± 1.17	2.92 ± 0.37	–

Table 2. Variation in concentration of lipids, total chlorophyll, and carotenoids under ClO_4^- stress (C- Control and T- $ClO4^-$ treated), values given are mean ± SD of triplicate.

Sl. No.	Plants	Lipid (mg/g tissue)	Total chlorophyll (mg/g tissue)	Carotenoids (mg/g tissue)
1	*Pistia* C	0.25 ± 0.02	1.35± 0.016	0.33 ± 0.007
2	*Pistia* T	0.17 ± 0.016	1.07 ± 0.03	0.42 ± 0.01
3	*Eichhornia* C	0.41 ± 0.009	1.88 ± 0.011	0.19 ± 0.004
4	*Eichhornia* T	0.35 ± 0.013	1.3 ± 0.021	0.38 ± 0.012
5	*Salvinia* C	0.31 ± 0.011	1.68 ± 0. 015	0.42 ± 0.016
6	*Salvinia* T	0.24 ± 0.009	1.03 ± 0.014	0.48 ± 0.008
7	*Spirodela* C	0.45 ± 0.012	1.52 ± 0.023	0.39 ± 0.007
8	*Spirodela* T	0.3 ± 0.008	0.94 ± 0.019	0.5 ± 0.013

protein content in *Oryza sativa* leaves decreased under ClO_4^- stress (Xie *et al.* 2014). Lipid concentration varied significantly, with *Spirodela* showing the highest reduction (0.15 mg/g) and *Pistia, Eichhornia* and *Salvinia* exhibiting decreases of 0.08 mg/g, 0.06 mg/g, and 0.07 mg/g, respectively (Table 2).

The concentration of photosynthetic pigments exhibited variations in response to ClO_4^- treatment, with changes in chlorophyll content considered an indicator of environmental stress (Chen *et al.* 2015a). Total chlorophyll content decreased by 0.015, 0.032, 0.012 and 0.019 (mg/g) in *Pistia*, *Eichhornia*, *Salvinia* and *Spirodela*, respectively (Table 2). This decrease may result from the inhibition of enzymes associated with chlorophyll synthesis. In rice plants, decreased chlorophyll content under ClO_4^- stress led to biomass changes and retarded plant growth. Previous studies have shown damage to leaves and photosynthetic systems, leading to lower photochemical productivity and growth inhibition, aligning with the results of the present study (Sahu and Kumari 2024, Gupta and Soni 2015, Chen *et al*. 2015a). Studies by Zhang *et al.* (2024) revealed that in *Synechocystis* ClO_4^- mostly damaged the photosystems and the degree of inhibition was more in photosystem II than that of photosystem I. Carotenoids, a group of non-enzymatic antioxidants, increased in concentration after treatment in all four plants, with *Eichhornia* displaying a notable increase of 2.35 mg/g (Table 2). Despite lower levels of phytoremediation and biomass change in *Eichhornia*, the plant may be losing chlorophyll because of stress-related metabolism. Carotenoid content in *Pistia* and *Spirodela* increased by 1.42 mg/g and 1.52 mg/g after ClO_4^- stress.

Enzyme assays

Plants respond to oxidative stress by producing antioxidant enzymes to enhance their defense system and counteract the effects of powerful oxidizers like ClO_4^-, which can lead to retarded growth and plant death (Chen *et al.* 2015a). CAT, GPX and APX are crucial antioxidant enzymes involved in maintaining intracellular ROS concentration, with reports indicating their production under ClO_4^- stress (Gupta and Soni 2015, Chen *et al.* 2015a, Zhang *et al.* 2024). These enzymes play a key role in counteracting the toxicity of ROS, thereby increasing tolerance to various stress factors. Perchlorate-induced stress can impede photosynthesis and induce oxidative strain in plants (Zhang *et al.* 2024). The results of the antioxidant enzyme assays are given in Table 1. Under ClO_4^- stress, CAT activity increased in *Pistia* plants, with activities of 27.01 and 58.54 (U/min/mg protein) compared to control values of 22.44 and 47.99 (U/min/mg protein) in shoots and roots, respectively. Higher CAT activity was also observed in the treated roots of *Eichhornia* and shoots of *Salvinia*. Plants employed in phytoremediation can mobilize antioxidants like CAT for detoxification (Sharma *et al.* 2021). APX activity increased in *Pistia* shoots (from 0.09 to 0.13 U/min/mg protein), *Eichhornia* roots (from 0.21 to 0.37 U/min/mg protein), *Salvinia* shoots (from 0.07 to 0.17 U/min/mg protein), and submerged fronds compared to their respective control plant parts. However, *Spirodela* exhibited a significant decrease in APX activity. The enzyme activity was least affected in the roots of *Pistia* and shoots of *Eichhornia*. GPX activity was highest in the control roots of *Pistia*, and under ClO_4^- stress, GPX activity showed minimal difference

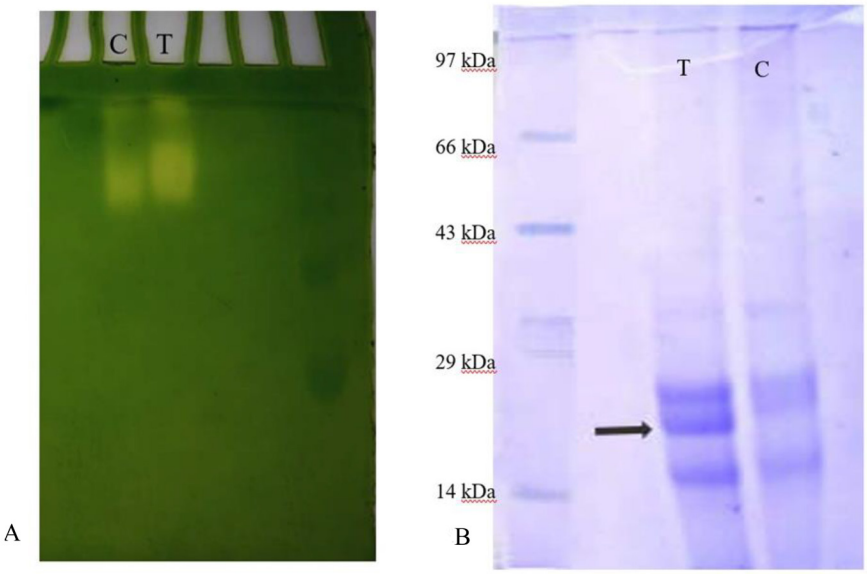

Fig. 2. A. Qualitative analysis CAT (zymogram), C- Control and T- ClO_4^- treated. B. SDS-PAGE of *Pistia*, arrow shows the extra protein band, C- Control and T- ClO_4^- treated.

in *Eichhornia* shoots. GPX activity was completely absent in *Spirodela* plants after ClO_4^- stress, indicating a minor role in response to ClO_4^- stress at lower concentrations.

According to Chen *et al*. (2015b), antioxidant enzymes play a significant role in mitigating ROS toxicity induced by ClO_4^-, with their effectiveness influenced by factors such as ClO_4^- concentration, exposure duration, plant species, and age of the plants. The magnitude of enzyme activity is plant-type-specific and appears to be highly tissue-specific. The results indicate fluctuating antioxidant enzyme activity in all four plants in the presence of ClO_4^-. Variations in enzyme activity may stem from the inhibition of enzyme synthesis, changes in the assembly of enzyme subunits under stress conditions, or degradation induced by peroxisomal proteases (Sharma *et al.* 2021). CAT and GPX, both involved in H_2O_2 decomposition, exhibited distinct patterns. CAT activity increased in *Pistia*, indicating efficient ROS scavenging and maintenance of elevated H_2O_2 levels under ClO_4^- stress. In contrast, APX activity appeared satisfying, while GPX activity decreased in *Pistia*, correlating with $ClO4^-$ reduction. The higher CAT activity observed in plants with minimal ClO_4^- remediation underscores the significance of these enzymes in overcoming oxidative stress due to environmental variations. In rice plants, CAT activity was found to be higher and dependent on both ClO_4^- concentration and exposure duration (Xie *et al.* 2014). Peroxidase emerged as the most sensitive antioxidant enzyme in *Oryza sativa* and *Eucalyptus* plants under ClO_4^- oxidative stress (Xie *et al.* 2014, Gupta and Soni 2015). Zhang *et al.* (2024) reported increased activities of the antioxidant's glutathione and superoxide dismutase in *Synechocystis* to scavenge injurious reactive oxygen species in response to ClO_4^- exposure.

Qualitative analysis of CAT and SDS-PAGE

Quantitative assays demonstrated an increase in CAT activity in *Pistia*, which was further assessed qualitatively through a zymogram study using the electrophoretic method. The zymogram revealed two clear zones in each well, with the first band located near the stacking gel. The second band in well 'T' shows a more pronounced clear zone compared to well 'C' indicating increased CAT activity under ClO_4^- stress (Fig. 2). Additionally, SDS-PAGE of extracts from ClO_4^- treated *Pistia* plants revealed a

new prominent band which is absent in the control, indicating the production of stress-related proteins due to ClO_4^- treatment with a molecular weight of approximately 20 kDa (Fig. 2).

CONCLUSION

Pistia, one of the common aquatic weeds in the fresh and brackish waters of Kerala, were identified as an effective candidate for ClO_4^- remediation. Physiological changes due to ClO_4^- stress was observed, concentrations of primary metabolites and pigments varied depending on the tissues and plants. The study highlighted the importance of antioxidants in aquatic plants for mitigating oxidative stress caused by ClO_4^- contamination. Further studies are necessary to identify the stress-related proteins and assess variations in ROS scavenging activities at elevated ClO_4^- concentrations to understand the actual tolerance mechanism in *Pistia.*

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