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Survival Strategies of *Synechocystis* sp. PUPCCC 64 under Pretilachlor Induced Oxidative Stress

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Abstract: Extensive use of pesticides in agriculture has deteriorated agri-ecosystems and has put human and livestock health at risk. Some cyanobacteria in pesticide-laden fields have evolved strategies to survive in such environments, continuing to contribute to soil fertility and are potential candidates for pesticide remediation. The present study was undertaken to understand the mechanism of pretilachlor tolerance in *Synechocystis* sp., an isolate from pesticide-contaminated rice fields. The half-maximal inhibitory concentration (IC_{50}) of pretilachlor for this organism was determined to be 12 mg L^{-1}. Exposure of the organism to the herbicide under laboratory conditions induced reactive oxygen species production and lipid peroxidation, which negatively affected chlorophyll a, photosynthesis and growth. These effects peaked at 48 h of exposure to the herbicide, after which their impact decreased, leading to resumption of growth. The organism managed herbicide-induced oxidative stress by inducing activities of superoxide dismutase (100% increase), glutathione peroxidase (189% increase), glutathione reductase (123% increase), maintaining high levels of glutathione, and by secreting exopolysaccharides. An interesting observation is that this organism is unique in having only a glutathione peroxidase/glutathione/glutathione reductase system to detoxify H_2O_2, as peroxidase and catalase activities did not increase on exposure to the herbicide. The organism coped with pretilachlor toxicity through two distinct, time-based mechanisms; one operating before and the other after 48 h of exposure. During the initial 48 h of exposure, herbicide tolerance is mediated by the activation of the antioxidant defense system, whereas after 48 h of exposure, reduced ROS production suggests the involvement of a different mechanism.

Keywords: Antioxidants, Cyanobacterium, Exopolysaccharides, Oxidative stress, Pretilachlor, Survival strategy, *Synechocystis*

Introduction

Since the green revolution, Indian farmers have heavily relied on chemical pesticides, leading to contamination of surface- and groundwater, harming aquatic ecosystems (Kanda et al., 2023). Repeated use of pesticides alters their degradation pattern, leading to their biomagnification and bioaccumulation in crops posing serious health risks to livestock and humans (Sondhia, 2014). Rice, a water-intensive staple crop grown mainly in tropical climates, faces significant yield loss due to the

growth of weeds, which are primarily controlled using herbicides (Ramprakash and Madhavi, 2019).

Pretilachlor[2-chloro-2',6'-diethyl-N(propyloxyethyl)acetanilide],an important member of chloroacetamide herbicides, is a pre-emergent and early post-emergent herbicide used in corn, soybeans and rice fields for the control of annual grasses and broad-leaved weeds (Kanda et al., 2023). Cyanobacteria, dominantly growing in rice fields, act as biofertilizers, enhance soil fertility by increasing phosphate content, improving water-holding capacity of soil, releasing growth-promoting hormones and fixing atmospheric nitrogen (Singh et al., 2013). However, excessive use of herbicides in crop fields severely impacts non-target microflora, can lead to long-term detrimental effects on aquatic ecosystems (Kanda et al., 2023). Although physical and chemical methods exist for pesticide removal, biological approaches are gaining attention for their sustainability. Freshwater phytoplanktons inhabiting polluted environments have evolved adaptive survival mechanisms and demonstrate significant potential to sequester and accumulate lipophilic pesticides from the ambient medium (Hamed et al., 2020). *Synechocystis* sp. PUPCCC 64, isolated from pesticide-contaminated rice fields, demonstrated significant tolerance to the herbicide pretilachlor under laboratory conditions, indicating adaptive tolerance mechanisms. Unravelling these mechanisms is essential for advancing sustainable agriculture and effective environmental management. The present study aimed to elucidate the survival mechanism of *Synechocystis* sp. PUPCCC 64 under pretilachlor stress, with the goal of harnessing its potential for bioremediation.

Materials and Methods

Chemicals

All analytical grade chemicals were obtained from Sigma Aldrich, USA and SD Fine-chem Limited, India. PRETGOLD (Pretilachlor 50% EC), manufactured by Star Chemicals, India was purchased from the local market. A stock solution of pretilachlor (100 mg L^{-1} in HPLC-grade water) was prepared and desired concentrations were obtained through serial dilutions.

Microorganism and culture conditions

Synechocystis sp. PUPCCC 64, utilised in this study, was previously isolated from pesticide-contaminated rice fields of Dera Bassi (30°58'72" N; 76°8'2" E), Punjab, India; purified and identified by our laboratory. The identification of the organism was based on its phenotypic characteristics and partial sequencing of the 16S rRNA gene (Singh et al., 2011). The cyanobacterial cultures were grown in modified Chu-10 medium in a culture room at 28±2°C under continuous illumination (44.5 µmol photons m^{-2} s^{-1}) for 14 h per day (Singh et al., 2013). The cultures were maintained in the active dividing phase by transferring to the fresh medium every 6 to 8 days.

Growth, generation time and tolerance limit towards pretilachlor

Minimum lethal concentration of pretilachlor for the organism was determined through preliminary experiments on wide range of concentrations (4-40 mg L^{-1}). It was observed that the minimum lethal concentration of pretilachlor was 24 mg L^{-1}. Thus, growth experiment was studied in 500 mL Erlenmeyer flasks containing 250 mL nutrient medium amended with graded concentrations (4-24 mg L^{-1}) of pretilachlor. Actively growing washed cultures were added to each flask to achieve a baseline cell density of 1×10^7 cells mL^{-1} and incubated under the above-stated conditions. Increase in cell population was measured using a Neubauer haemocytometer at 24 h interval with average of 20 counts.

Generation time of the organism was calculated as per the equations given below:

Specific growth rate constant (μ) = $(lnN_2\text{-}lnN_1)/T_2\text{-}T_1$

Generation Time (g) = $1/\mu$

N_1 = number of cells at time T_1

N_2 = number of cells at time T_2

$T_2–T_1$= time interval in hours.

The half-maximal inhibitory concentration (IC_{50}) of pretilachlor was determined from the growth data.

Determination of chlorophyll *a*

Cell pellets of 5 mL growth experiment cultures were obtained, washed, centrifuged and suspended in 5 mL of 80% acetone overnight at 4°C. After centrifugation, absorbance of the supernatant was noted at λ_{660}, λ_{645} to determine chlorophyll *a* (Chl*a*) content (Holms, 1954). Simultaneously, washed cell pellets were dried at 60°C for 12 h to determine the dry biomass weight (mg mL^{-1}) of the culture, which was subsequently used for normalisation of Chl*a* content.

Rate of photosynthesis

Actively dividing stock cultures were inoculated in 250 mL flasks containing 100 mL nutrient medium, without and with IC_{50} dose of pretilachlor. At regular intervals, a thick cell suspension was transferred to a dissolved oxygen measuring vessel fitted with an oxygen electrode connected to an oxygen analyzer (5300A, YSI Bioanalytical Products, USA). Increase in dissolved oxygen was recorded for 10 min after illuminating the vessel with light (90 µmol photons m^{-2} s^{-1}). Chl*a* content in the vessel was measured as discussed above. The rate of photosynthesis is expressed as µmol O_2 evolved mg^{-1} Chl*a* min^{-1}.

Scanning electron microscope (SEM) analysis

Pretilachlor treated and untreated cells of test organism were used for SEM analysis (JSM, 6510LV, JEOL, Japan) by following the method of Sachu et al. (2021).

Exopolysaccharide staining

Cultures were concentrated by centrifugation, stained with 1% (w/v) alcian-blue solution prepared in 3% (v/v) acetic acid for 10 min following Pippo et al. (2013) and observed under a light microscope.

Oxidative stress and response of antioxidants

Cell-free crude extract preparation

At the desired time, the washed cell pellet was suspended in 0.1 M phosphate buffer (pH 7.8, unless otherwise stated) and sonicated at 4°C to obtain cell-free crude extract. After centrifugation at 10,000 *g* at 4°C, the supernatant was collected and its protein content was determined. The extract was used for the determination of reactive oxygen species (ROS), reduced glutathione (GSH) content, activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR).

Determination and visualization of ROS production

The method of Elstner and Heupel (1976) was followed to evaluate ROS production. Two mL of cell-free extract was mixed with 3 mL phosphate buffer (65 mM, pH 7.8) and 0.1 mL hydroxylamine hydrochloride (10 mM) and incubated at room temperature for 30 min, and then 0.2 mL α-naphthylamine (3 mM) and 0.2 mL sulphanilamide (8.5 mM) were added. After 10 min, the absorbance of the solution was noted at λ_{530}. A standard curve was prepared by using sodium nitrite.

Production of intracellular ROS in pretilachlor-exposed *Synechocystis* sp. was visualized using fluorescence probe 2',7'-dichlorofluorescin diacetate (DCFH-DA). Five µL of thick cell suspension was suspended in 5 µM DCFH-DA solution and kept in the dark for about one hour in a shaker (200 rpm) (Ling et al., 2011). Using a fluorescence microscope (Leica DM 4000 B LED), fluorescence (excitation at λ_{485}; emission at λ_{530}) produced within the cells was observed, and images were captured with an attached camera.

Determination of Lipid peroxidation

The method of Yin et al. (2008) was followed to determine the extent of lipid peroxidation in terms of malondialdehyde (MDA) equivalents. At the desired time, washed cell suspension was sonicated in 5 mL of 1% trichloroacetic acid (TCA) and centrifuged. To 1 mL extract, 2 mL of 0.6% thiobarbituric acid prepared in 20% TCA

were added, then heated at 95°C for 15 min and transferred to an ice bath. The absorbance of the supernatant obtained after centrifugation was noted at λ_{532}. The absorbance was also recorded at λ_{600} for subtraction from the absorbance at λ_{532} to nullify non-specific turbidity. The extinction coefficient of 155 mM^{-1} cm^{-1} was used to calculate MDA content.

Assay of superoxide dismutase (EC 1.15.1.1)

SOD activity was determined following the method of Beauchamp and Fridovich (1971). The assay mixture contained 1.1 mL phosphate buffer (0.1 M; pH 7.8), 0.2 mL methionine (30 mg mL^{-1}), 0.2 mL Na_2CO_3 (105 mg mL^{-1}), 0.2 mL EDTA (11.1 mg mL^{-1}), 0.3 mL riboflavin (0.2 mg mL^{-1}), 0.3 mL cell-free crude extract, 0.15 mL nitro-blue tetrazolium (NBT; 1.5 mg mL^{-1}). Three sets of the above assay mixture were prepared and incubated for 10 min: the first set was illuminated with light; the second set was kept in the dark (served as blank), and the third set without cell-free extract was illuminated with light (control for 100% colour development). Absorbance was recorded at λ_{560}. One unit of SOD activity corresponds to 50% inhibition of NBT photoreduction mg^{-1} protein min^{-1}.

Assay of peroxidase (EC 1.11.1.7)

Peroxidase activity was determined by adding 0.2 mL cell-free extract to a mixture containing 2 mL distilled water, 0.3 mL phosphate buffer (0.1 M; pH 6.8), 0.2 mL of 1% H_2O_2 and 0.3 mL of 5% pyragallol (Gahagan et al., 1968). The absorbance of the mixture was recorded at λ_{420} for 5 min. Purpurogallin concentration was determined using the molar extinction coefficient of 2640 M^{-1} cm^{-1}. One unit of POD corresponds to the formation of one µmol of purpurogallin mg^{-1} protein min^{-1}.

Assay of catalase (EC 1.11.1.6)

Catalase activity was determined following Aebi (1984). The assay mixture consisted of phosphate buffer (100 mM, pH 7.0), H_2O_2 (20 mmol), and cell-free extract (0.1 mL). Decrease in H_2O_2 concentration with time was determined from a decrease in absorbance at λ_{240}. Molar extinction coefficient 43.6 M^{-1} cm^{-1} was used to determine H_2O_2 concentration. One unit of the enzyme is the amount of enzyme decomposing one µmol of H_2O_2 mg^{-1} protein min^{-1}.

Assay of glutathione peroxidase (EC 1.1.1.9)

Glutathione peroxidase activity was measured following Starlin and Gopalakrishnan (2013). The assay mixture contained 0.4 mL phosphate buffer (0.4 M; pH 7.0), 0.2 mL distilled water, 0.1 mL of 10 mM sodium azide, 0.1 mL of 2.5 mM H_2O_2, 0.2 mL of 4 mM reduced glutathione and 0.5 mL cell-free extract. After 90 seconds of incubation,

the reaction was stopped with 0.5 mL of 10% TCA, centrifuged and supernatant was mixed with 3 mL of phosphate buffer (0.1M, pH 7.8) and 1 mL of DTNB reagent (0.04% solution of 5,5'-dithiobis-(2-nitrobenzoic acid) in 1% sodium citrate). The absorbance of the solution was noted at λ_{412}. One unit of GPx is the amount of enzyme utilizing one µg glutathione mg^{-1} protein min^{-1}.

Assay of glutathione reductase (EC 1.6.4.2)

GR activity was measured following Halliwell and Foyer (1978). The assay mixture contained 2 mL phosphate buffer (0.1 M; pH 7.5), 25 µL of 10 mM NADPH, 50 µL cell-free extract and 60 µL oxidized glutathione (10 mM). Decrease in absorbance at λ_{340} was noted for 10 min. Molar extinction coefficient 6220 M^{-1} cm^{-1} was used to determine NADPH concentration. One unit of GR is the amount of the enzyme catalyzing the reduction of one µmol of NADPH mg^{-1} protein min^{-1}.

Estimation of reduced glutathione content

The amount of reduced glutathione was estimated following Ellman (1959). The reaction mixture contained 3 mL phosphate buffer (0.2 M; pH 8.0), 0.2 mL cell-free extract and 0.5 mL DTNB solution. The absorbance of the colour developed was recorded at λ_{412}.

Native polyacrylamide gel electrophoresis (PAGE) and staining of enzyme activity

Equal amounts of protein from the cell-free extract were subjected to non-denaturing, non-reducing electrophoresis (Native-PAGE) at 4°C to separate isoforms of antioxidant enzymes. Enzyme activity was visualized through biochemical staining and gels were photographed in Gel Doc (Image Quant LAS 500, GE Healthcare Bio–Science AB751 Uppsala, Sweden).

SOD, POD and CAT activities in-gel were stained following Verma and Prasad (2021) with slight modifications.

Staining for SOD activity

The gel was first incubated in the dark in 2.5 mM NBT solution for 25 min, then in phosphate buffer (50 mM; pH 7.8) containing 28 mM N,N,N',N'-tetramethylethylenediamine (TEMED) and 28 µM riboflavin. After incubation, the gel was rinsed with distilled water and exposed to fluorescent light, resulting in blue/purple colour with achromatic bands.

Staining for POD activity

The gel was placed in acetate buffer (0.2 M; pH 4.8) containing 4% benzidine in 50% methanol and 0.3% H_2O_2 until brown band(s) appeared.

Staining for CAT activity

The gel was incubated in 0.003% H_2O_2 for 10 min, washed and two separately prepared solutions (2% ferric chloride and 2% potassium ferricyanide) were poured directly onto the gel and gel was removed from staining solution after achromatic bands appeared.

Staining for GPx activity

GPx activity was stained following Lorusso et al. (2022). The gel was incubated in 2.0 mM reduced glutathione (GSH) solution for 45 min, followed by incubation for 10 min in GSH solution containing 0.003% H_2O_2. The gel was then rinsed with distilled water and stained with a freshly prepared mixture of 1% ferric chloride and 1% potassium ferricyanide until yellow bands appeared against the dark-green background.

Protein estimation

Protein content in this cell-free extracts was determined following the method of Lowry et al. (1951).

Statistical analysis

All the data shown are mean±SD of three replicates from three independent experiments (n=9). The data of physiological and biochemical parameters were analysed by applying ANOVA followed by Tukey's post hoc test using GraphPad Prism 6 version 6.02. Statistical significance differences level was set to $p<0.05$ for all data.

Results

Growth inhibition assay

The impact of different concentrations of pretilachlor (4-24 mg L^{-1}) on the growth of the organism was investigated by monitoring the increase in cell population at 24 h intervals for 144 h. The cell population of control cultures increased from 1.05 to 6.63×10^7 cells mL^{-1} within 144 h. The presence of pretilachlor (up to 16 mg L^{-1}) in the medium declined the rate of cell population increase in a concentration-dependent manner. No growth in the presence of 20 mg pretilachlor L^{-1}; and complete cell lysis occurred in the presence of 24 mg pretilachlor L^{-1} (Fig. 1). The median inhibitory concentration (IC_{50}-144 h) of pretilachlor for *Synechocystis* sp. was determined to be 12 mg L^{-1}. During the initial 48 h of exposure to all the tested concentrations of the

herbicide, the rate of cell population increase was significantly slower than in control cultures, and generation time increased with increasing herbicide concentration. After 48 h, the generation time of cultures having pretilachlor concentrations up to 16 mg L^{-1} significantly decreased (Table 1). Microscopic examination revealed that cell clumping, shrinking and yellowing started in cultures containing 10 mg pretilachlor L^{-1}, while most cells lysed in the presence of 24 mg pretilachlor L^{-1} (Fig. 2).

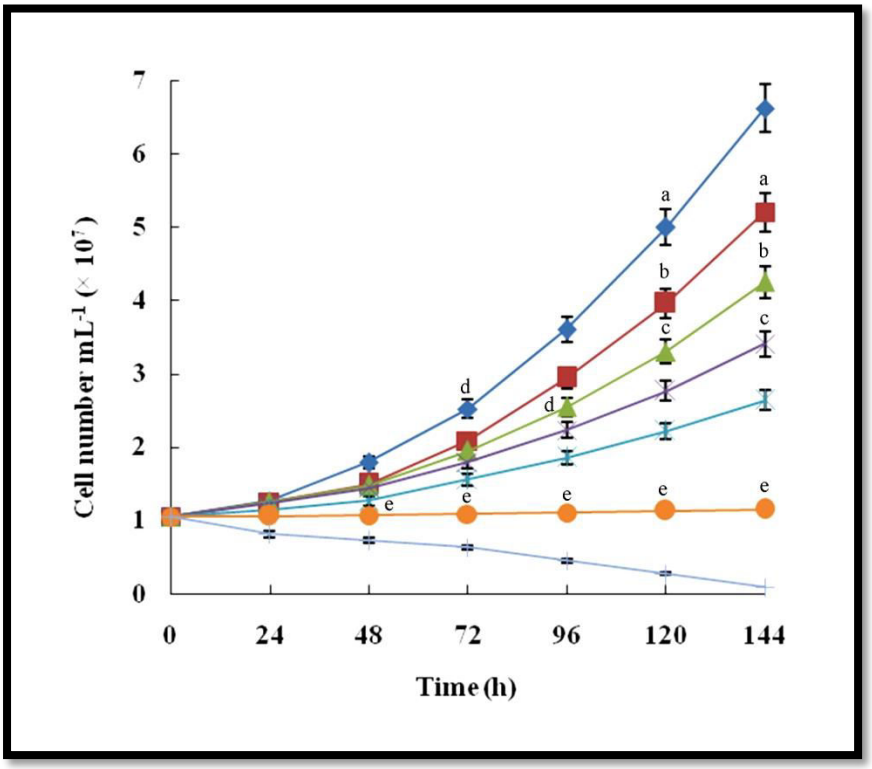

Fig. 1 Population growth curves of *Synechocystis* sp. grown in the absence and presence of pretilachlor for the determination of IC_{50}. Growth in basal medium (◆) supplemented with 4 (■), 8 (▲), 12 (×), 16 (×), 20 (●), 24 (+) mg pretilachlor L^{-1} at different times of the experiment. The results are in mean values ± SD (n=9). Data symbols, except for those marked with the same alphabet, are significantly different from one another at $p<0.05$.

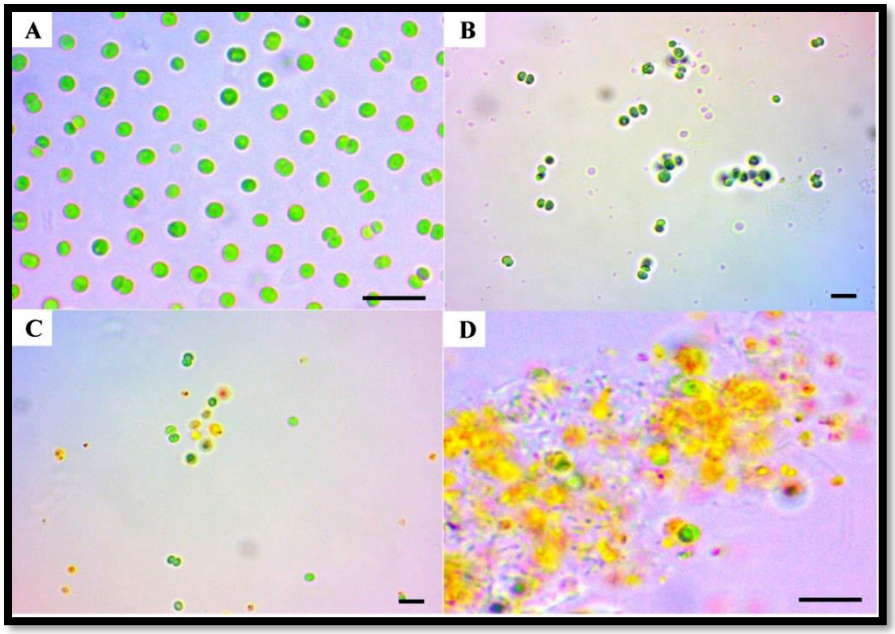

Fig. 2 Microphotographs of *Synechocystis* sp. after 48 h of exposure to pretilachlor.Control (A);10 (B); 20 (C);24 mg pretilachlor L^{-1} (D). Scale bar : 10 µm.

Table 1. Generation time of *Synechocystis* sp. cultures grown in presence of pretilachlor during the time course of the experiment.

Pretilachlor (mg L^{-1})	Generation time (h)					
	Time course (h)					
	0-24	24- 48	48-72	72-96	96-120	120-144
0	88.84±4.4	48.12^{a}±2.40	48.46^{a*}±2.42	46.20^{a*}±2.31	50.58±2.52	58.72*±2.93
4	97.60^{a*}±4.88	93.64^{a*}±4.68	49.14^{b*}±2.45	48.12^{b*}±2.40	55.88±2.79	60.78*±3.03
8	99.0^{a*}±4.95	96.25^{a}±4.81	60.26^{b}±3.01	61.87^{b}±3.09	64.16^{c}±3.20	65.37^{c}±3.26
12	108.28^{a}±5.41	106.61^{a}±5.33	73.72^{b}±3.68	77.0^{b}±3.85	77.86b±3.89	79.65^{b}±3.98
16	192.50±9.60	165.00±8.21	82.50±4.12	93.64^{a}±4.68	93.64^{a}±4.68	94.93^{a}±4.74
20	1980.00±99.00	1776.92±88.84	1004.34±50.21	962.50^{a}±48.12	924.00±46.20	949.31^{a}±47.46

All data are the mean values of three independent experiments ± SD.
Data in each row with same lower case alphabet are not significantly different from one anotherat 95% confidence level ($p < 0.05$).
Data in each column with '*' are not significanly different from one another at 95% confidence level ($p < 0.05$).

Effect on Chla and photosynthesis

Chl*a* content of the test organism decreased as a function of increasing pretilachlor concentration (Table 2). In IC_{50} dose of pretilachlor, the minimum Chl*a* content was observed (22% less than control cultures) at 48 h of exposure. After this, Chl*a* level steadily increased, being only 7% less than control cultures at 144 h. Similarly, photosynthetic activity declined by 27% at 48 h of exposure to pretilachlor but gradually recovered afterwards, being only 15% and 14% less at 96 and 144 h of exposure, respectively, compared to control cultures (Table 3).

Table 2. Chlorophyll *a* content of *Synechocystis* sp. grown in presence of pretilachlor.

Pretilachlor (mg L^{-1})	Chl a (µg mg^{-1} dry weight biomass)						
	Time (h)						
	0	24	48	72	96	120	144
0	6.89±0.34	7.42±0.37	8.01±0.40	8.30±0.41	8.45±0.42	8.56±0.42	8.61±0.43
4	6.89±0.34	7.19±0.35 (3%↓)	7.60±0.39 (5%↓)	7.96±0.39 (4%↓)	8.19±0.40 (3%↓)	8.30±0.41 (3%↓)	8.43±0.42 (2%↓)
8	6.89±0.34	7.01±0.35 (6%↓)	7.36±0.36 (8%↓)	7.80±0.39 (6%↓)	8.02±0.40 (5%↓)	8.13±0.40 (5%↓)	8.26±0.41 (4%↓)
12	6.89±0.34	6.67±0.33 (10%↓)	6.24±0.38 (22%↓)	7.13±0.35 (14%↓)	7.52±0.37 (11%↓)	7.78±0.38 (9%↓)	8.00±0.40 (7%↓)
16	6.89±0.34	5.86±0.35 (21%↓)	5.28±0.37 (34%↓)	5.89±0.29 (29%↓)	6.35±0.32 (25%↓)	6.78±0.33 (21%↓)	7.23±0.36 (16%↓)
20	6.89±0.34	5.56±0.27 (25%↓)	4.24±0.21 (47%↓)	4.75±0.23 (43%↓)	5.15±0.25 (39%↓)	5.56±0.27 (35%↓)	5.94±0.29 (31%↓)

All data are the mean values of three independent experiments ± SD.

Values in parenthesis represent decrease in comparison to value of control at the respective time of the experiment.

Table 3. Photosynthesis rate in *Synechocystis* sp. grown in basal medium without and with IC_{50} dose of pretilachlor

Pretilachlor (mg L^{-1})	Rate of Photosynthesis (µmol O_2evolved mg^{-1}Chla min^{-1})			
	Time (h)			
	0	48	96	144
Control	61.64±3.08	70.10±3.50	72.20±3.61	73.90±3.69
IC_{50}	61.64±3.08	51.20±2.56 (27% ↓)	61.40±3.07 (15% ↓)	63.70±3.18 (14% ↓)

All data are the mean values of three independent experiments ± SD.
Values in parenthesis represent decrease over values of control at respective time of the experiment.
Data of control and pretilachlor cultures at different time of experiment are significantly different from one another at 95% confidence level ($p < 0.05$).

Oxidative stress markers

Reactive oxygen species and MDA production

Maximum increase (88%) of ROS production in the organism was observed at 48 h of exposure to IC_{50} dose of pretilachlor, which later gradually decreased up to 144 h, being only 32% and 20% higher than control cultures at 120 and 144 h, respectively (Fig. 3a). Under herbicide stress, increase in cellular ROS levels was also confirmed by fluorescent microscopy (Fig. 4).

Similarly, MDA content of the organism increased up to 48 h before gradually declining. At 48 h, MDA level in pretilachlor-exposed cultures was 3.36 nmol mg^{-1} protein, a 310% increase over 0.82 nmol mg^{-1} protein in control cultures. By 144 h, MDA content of pretilachlor-exposed cultures decreased to 1.15 nmol mg^{-1} protein, being only 38% higher than control cultures (Fig. 3a).

Response of antioxidant defenses

SOD, POD, CAT, GPx activity

Consistent with growth, Chl*a,* ROS and MDA levels, SOD activity in the test organism exposed to pretilachlor increased by 100% within 48 h before gradually declining by 75% up to 144 h, compared to the control cultures (Fig. 3b). The activity of POD and CAT in control and herbicide exposed cultures was almost same, being 1.21 to 1.31 U and 1.74 to 1.8 U, respectively, throughout the experiment.

GPx activity in pretilachlor-exposed cultures peaked at 48 h, showing 189% increase compared to control cultures (Fig. 3b). Thereafter, GPx activity gradually declined, being only 29% higher than control cultures at 144 h.

To validate the biochemical activity of the above enzymes, their activity was assayed in-gel of native-PAGE. Zymogram analysis revealed two isoforms of SOD but no isoforms of POD, CAT or GPx. Consistent with biochemical observations, SOD and GPx activities of the organism peaked at 48 h of exposure to pretilachlor before declining, POD and CAT activities remained relatively constant throughout the experiment (Fig. 5).

Glutathione content and glutathione reductase

Glutathione content of the organism exposed to pretilachlor increased up to 144 h, showing 130% increase over control cultures. Glutathione reductase activity in pretilachlor-exposed test organism also increased by 196% at 48 h over control cultures, slightly declined at 72 h, remained stable until 96 h and then steadily decreased up to 144 h (Fig. 3c).

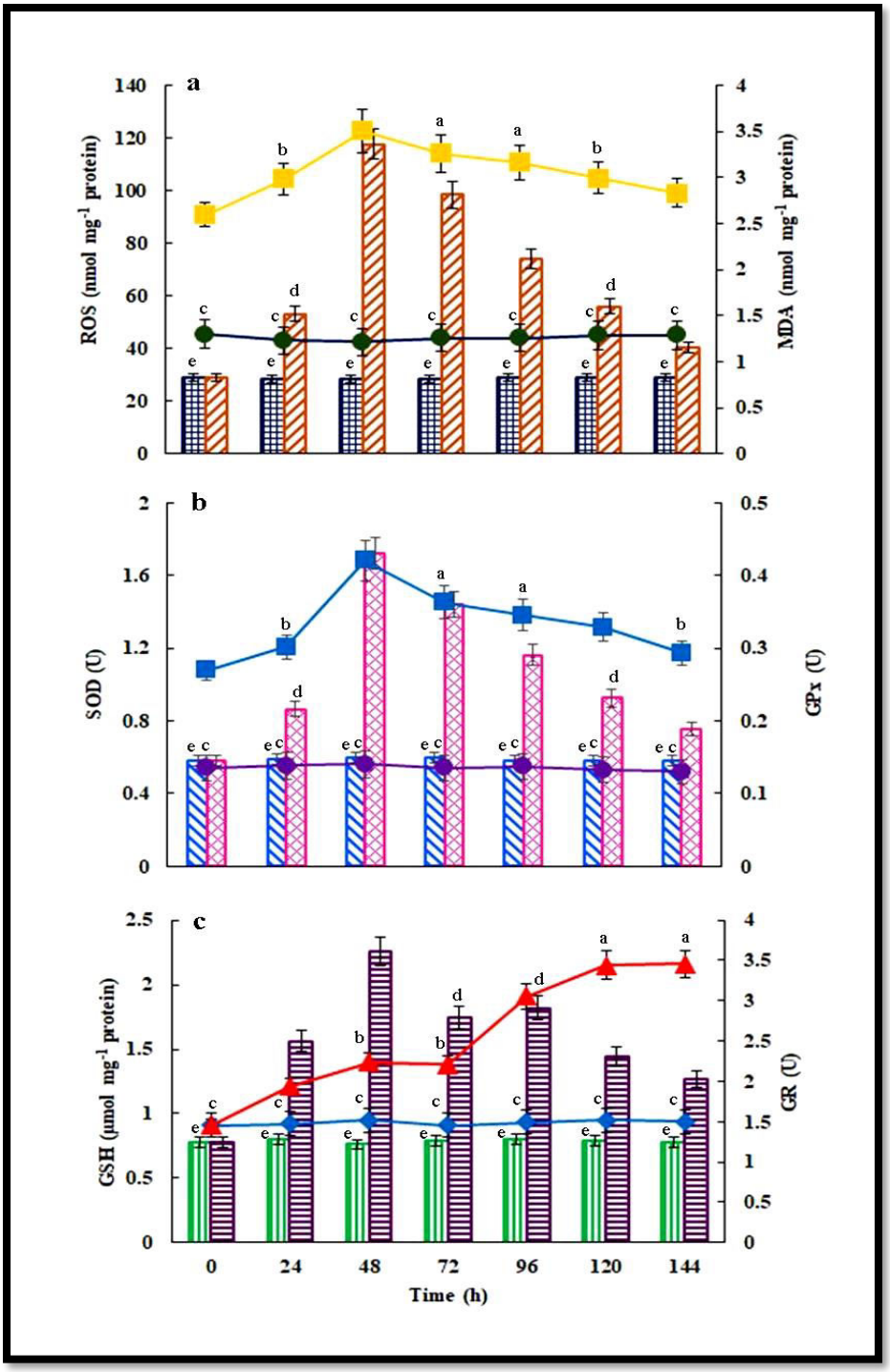

Fig. 3 Effect of IC_{50} dose of pretilachlor on oxidative stress indicators and antioxidant enzymes of *Synechocystis* sp. (a) Content of reactive oxygen species, left axis (lines with ●, ■) and malondialdehyde, right axis (bars ▦, ▨); (b) superoxide dismutase activity, left axis (lines with ●, ■) and glutathione peroxidase activity (bars: ▦, ▨); (c) content of glutathione, left axis (lines with ◆, ▲) and glutathione reductase activity, right axis (bars: ▦, ▨) in basal medium (first

symbol given in each bracket) and IC_{50} dose of pretilachlor (second symbol given in each bracket), respectively.

The results are mean values of three independent experiments ±SD (n=9). Data symbols of each parameter, except for those marked with the same alphabet, are significantly different from one another at $p<0.05$.

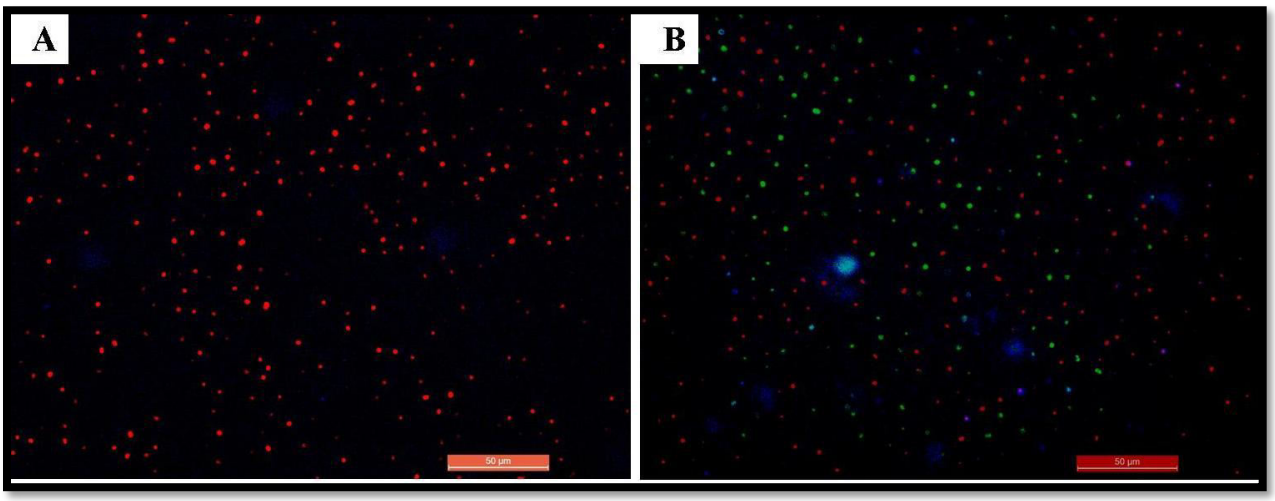

Fig. 4 Fluorescent microscope images of *Synechocystis* sp. (A) After 48 h in basal medium; (B) after 48 h exposure to IC_{50} dose of pretilachlor. Normal cells appeared red; Cells with ROS appeared green. Scale bar in each image: 50 µm.

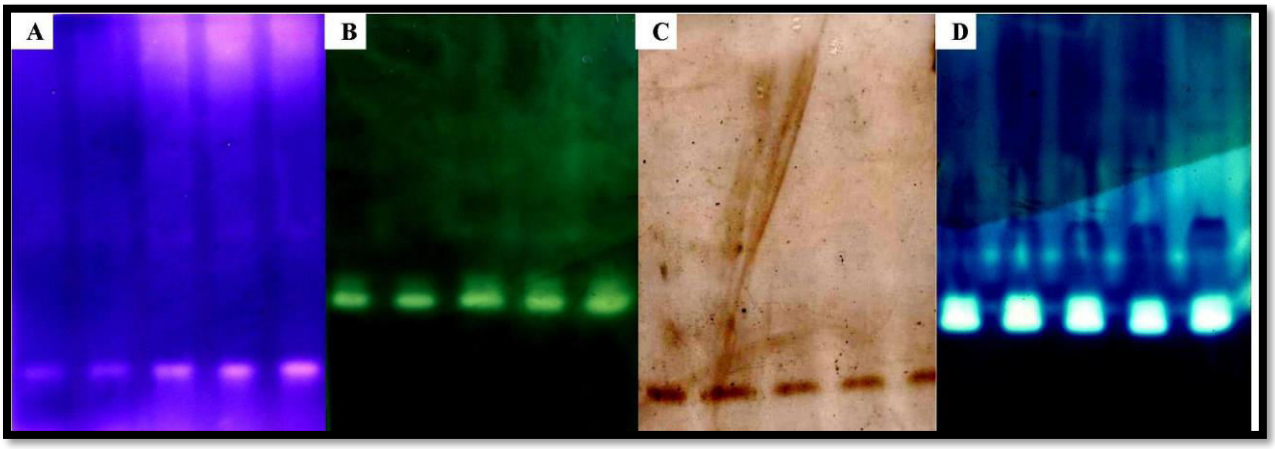

Fig. 5 Activity of SOD (A), GPx (B), POD (C), and CAT (D) of *Synechocystis* sp. on native PAGE gel after exposure to IC_{50} dose of pretilachlor. Lane 1: Control, Lane 2: 24, Lane 3: 48, Lane 4: 72, Lane 5: 96 h of exposure.

Scanning electron microscope observations

SEM images of the cultures, exposed to pretilachlor for 48 h, revealed cell clumping surrounded by a sheath. At 96 h, the sheath began to break down, and at 144 h the cells were nearly free (Fig. 6).

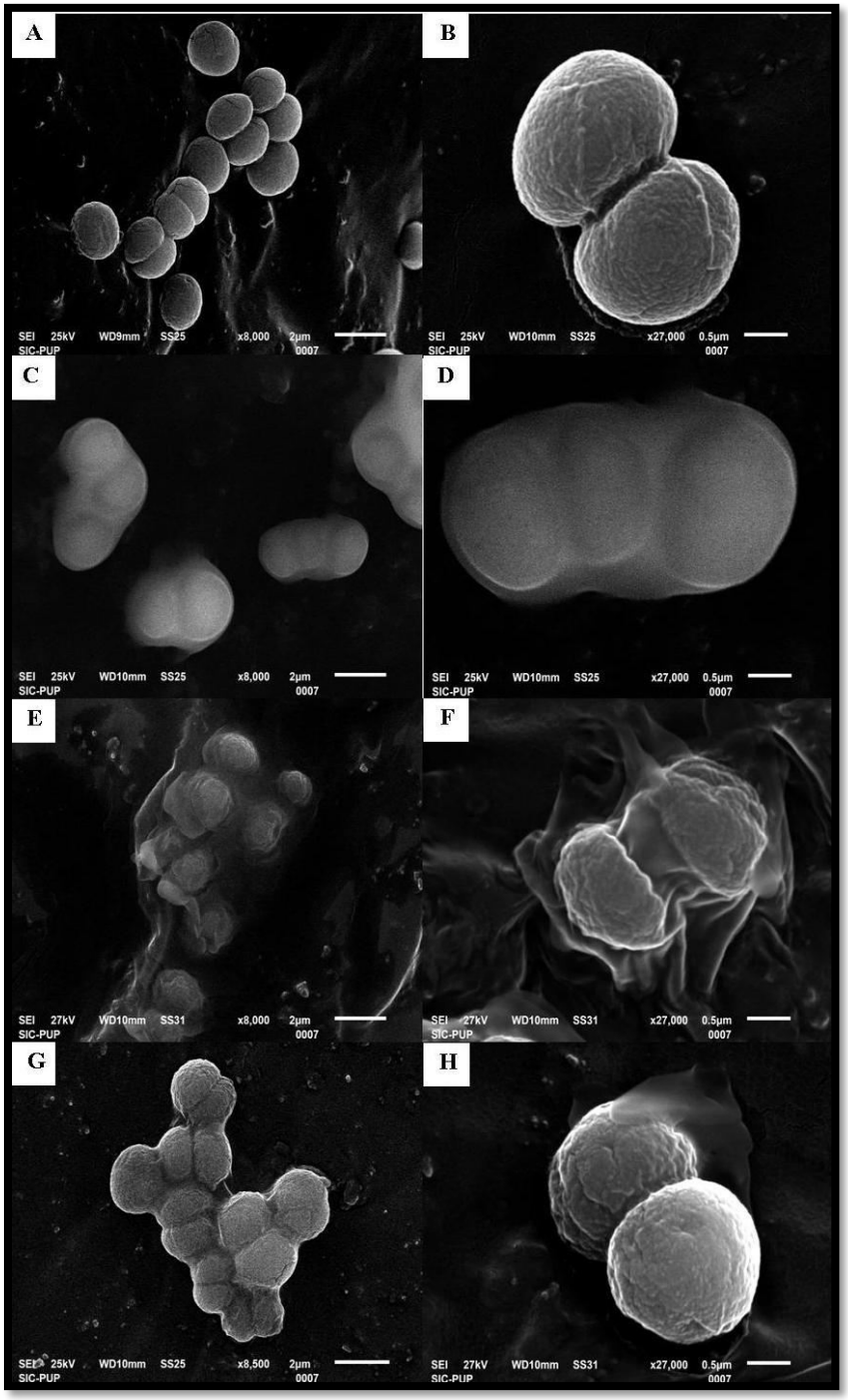

Fig. 6 SEM images of *Synechocystis* sp. cells in basal medium after 48 h (A, B); cells exposed to IC_{50} dose of pretilachlor for 48 h (C,D); 96 h(E,F); 144 h(G,H). Scale bar: A, C, E, G: 2 µm; B, D, F, H: 0.5 µm.

Staining of the cultures

To ascertain the nature of the sheath surrounding the cells under pretilachlor stress, cultures were stained for exopolysaccharides, yielding positive results (Fig. 7). Consistent with previous observations, the EPS sheath was most prominent at 48 h of exposure to pretilachlor and began to decline thereafter.

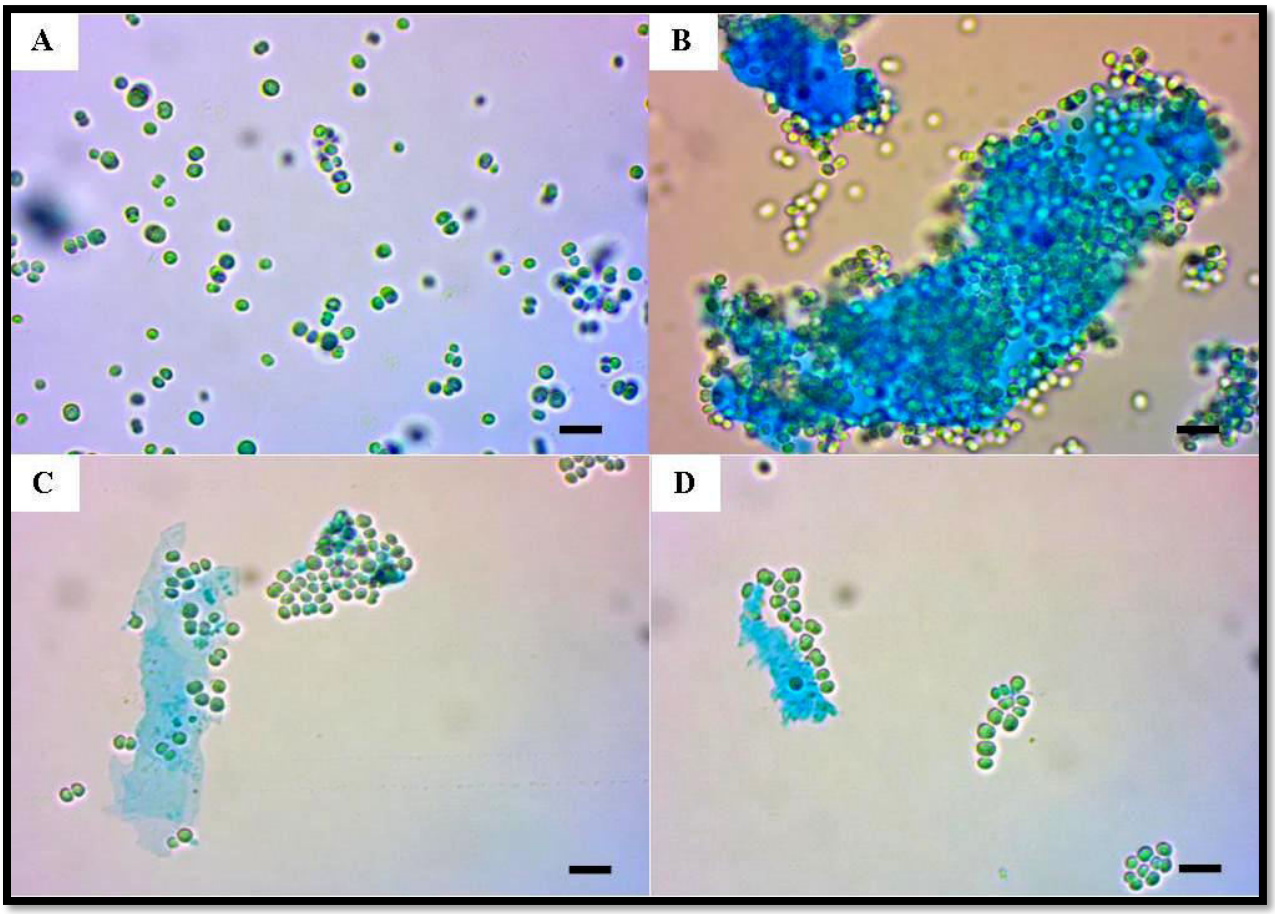

Fig. 7 Microphotographs of *Synechocystis* sp. cultures stained with alcian-blue. Cultures in basal medium at 48 h (A), supplemented with IC_{50} dose of pretilachlor after 48 (B), 96 (C) and 144 (D) h. Scale bar: 10 µm.

Discussion

For effective weed control in transplanted rice crop in India, it is recommended to apply 1.0 to 1.5 L of pretilachlor 50% EC per hectare (ppqs.gov.in). The concentration of the recommended dose of pretilachlor in field with water level 10 cm will be approximately 0.5 to 0.75 mg L^{-1}. Growth experiments during the present study revealed that IC_{50}-144 h dose of pretilachlor for *Synechocystis* sp. is 12 mg L^{-1} (Fig. 1). This indicated that the test organism exhibited strong tolerance to pretilachlor, maintaining 50% growth even at a concentration 10 times higher than

the recommended dose in rice fields. Another important observation was that the organism experienced severe herbicide toxicity during the initial 48 h, resulting in increase in generation time with an increase in herbicide concentration. After 48 h, the level of herbicide toxicity decreased, resulting in a significant decrease in generation time (Table 1) suggesting that the organism developed a mechanism to reduce the herbicide toxicity. Growth inhibition by pesticides in microalgae is because of toxic effects on pigments, chlorophyll synthesis, photosynthesis, cell membrane permeability, cell division, etc. (Singh et al., 2013).

Photosynthetic pigments affected in stress conditions, reduce photosynthesis that results indecline in the growth of the organisms. During initial 48 h exposure to pretilachlor, Chl*a* content and photosynthesis of the organism declined, followed by an increase (Table 2 and 3). Detrimental effects of pesticides on photosynthetic pigments have been observed in *Phormidium foveolarum* and *Nostoc muscorum* when treated with butachlor and monuron (Sheeba et al., 2020; Sachu et al., 2021). Not only Chl*a,* rate of photosynthesis is also negatively affected by pesticides, as these interfere with enzymes of photosynthesis and photosynthetic electron transport chain (Hamed et al., 2020). The decline in Chl*a* content and photosynthesis could have significant downstream effects on the ecological role of cyanobacteria in agroeco systems as primary producers and carbon fixers, potentially affecting soil fertility and overall ecosystem services. The test organism reduced the toxic effect of the herbicide on Chl*a* and photosynthesis, resuming growth after 48 h. Such cyanobacteria have the potential to minimize the negative impact of herbicides on their ecological functions.

Pesticide stress leads to a surge in free radicals causing damage to biomolecules such as lipids, proteins and DNA; consequently, leading to disruptions of normal cellular functions (Vanderauwera et al., 2011).

Reactive oxygen species are important stress biomarkers. During initial 48 h exposure of the test organism to pretilachlor, ROS level increased which was observed through biochemical analysis and fluorescence microscopy (Fig. 3a and Fig. 4), indicating pretilachlor caused oxidative stress. Exposure of *Nostoc muscorum* and *Phormidium foveolarum* to butachlor and UV-B irradiation individually or in combination led to an increase in ROS (Sheeba et al., 2020). The increase in ROS content during the initial hours of exposure is likely due to sudden encounter of the organism with the herbicide which led to inhibition in synthesis, or degradation of Chl*a* and disruption of photosynthetic and respiratory electron transport chains (Sachu et al., 2021). Malondialdehyde, a by-product of lipid peroxidation of the cell membrane induced by free radicals, is considered a stress indicator (Wu et al., 2016). MDA content of pretilachlor-grown cultures increased by 310% within 48 h, then started decreasing afterwards (Fig. 3a). Three key points emerge from the above discussed results: (i) exposure of the organism to varying concentrations of

herbicide led to a dose-dependent increase in ROS, along with negative impact on Chl*a*, photosynthesis and cell membrane integrity resulting in reduced or no growth (ii) the extent of ROS generation under low doses of the herbicide was not so high to cause significant stress; exposure of the organism to IC_{50} dose of the herbicide induced higher but manageable ROS levels, while higher doses induced ROS levels to such an extent that caused severe damage to the organism (iii) adverse effects of mid-level herbicide (IC_{50}) were diminished by the organism after 48 h, allowing growth to resume. Elucidating the organism's management of herbicide stress at median-level dose is significant for understanding its adaptive mechanisms.

Elevated levels of ROS in the organisms are known to increase SOD activity by its production. This facilitates dismutation of $O_2^{\bullet -}$, preventing membrane oxidation and mutilation of biomolecules (Vanderauwera et al., 2011). During initial 48 h of exposure to the herbicide, SOD activity increased by 100%, then decreased gradually up to 144 h (Fig. 3b) and the decline in SOD activity coincided with reduction in ROS levels. Studies on microalgae have reported that H_2O_2 produced by the activity of SOD is scavenged by POD and/or CAT (Wu et al., 2016; Sheeba et al., 2020). In the present study, there is no induction of POD or CAT, as their activity remained almost the same in both control and herbicide-exposed cultures. However, GPx activity in the test organism exposed to pretilachlor was maximum (189% higher than control cultures) at 48 h, which gradually decreased afterwards (Fig. 3b). GPx converts H_2O_2 to H_2O by using reduced glutathione (GSH) as an electron donor, and is thus recently considered to be involved in ROS removal in microalgae (Verma and Prasad, 2021). In-gel assay also confirmed maximum activity of SOD and GPx at 48 h of exposure of the organism to pretilachlor followed by decrease in their activity; no change in POD and CAT activity was observed (Fig. 5). The coordinated increase in GPx and SOD activities in pretilachlor-exposed test organism suggested the role of GPx in H_2O_2 detoxification. Continuous increase in glutathione content up to 144 h was intriguing as it differed from previous observations on stress biomarkers and other antioxidant enzymes (Fig. 3c). A critical evaluation of initial increase followed by decline in GPx, GR activities; and continuous increase in GSH content of the organism under pretilachlor stress is briefed as: GPx and GR activities reached their maximum (189% and 196% increase, respectively, compared to control cultures) after 48 h exposure to pretilachlor. However, the GSH content at this time was only 48% higher than control cultures. GR reduces glutathione disulphide (GSSG; oxidised glutathione) to produce GSH using NADPH (Torres et al., 2008), but the increase in GSH content did not match with increase in GR activity because GPx utilized available GSH to convert H_2O_2 to water to accelerate the glutathione redox cycle. This explains the proportional rise in the GR and GPx activities, resulting in lowered GSH levels at 48 h. After 48 h, activities of both GPx and GR gradually declined, but GPx activity decreased at a faster rate. At

144 h, the GPx and GR activities of the test organism exposed to pretilachlor were 29% and 63%, respectively, higher than their activity in control cultures. This significant difference in GPx and GR activities after 48 h resulted in sustained accumulation of GSH within the cells. GSH plays a significant role in protection against ROS by maintaining intracellular redox homeostasis (Torres et al., 2008).

Induction of high levels of antioxidant capacity in stressed microorganisms is associated with improved stress management. Most previous studies have reported elevated levels of all antioxidant enzymes and molecules in microalgae under pesticide stress (Wu et al., 2016; Hamed et al., 2020). Recent studies suggest that the antioxidant defense response in microalgae is both species-specific and pesticide-specific. In some species, SOD and POD/CAT system, while in others, GSH/GR or ascorbate-peroxidase/GR cycle is more active (Tiwari et al., 2022; Singh and Prasad, 2024). Our results indicate that *Synechocystis* sp. PUPCCC 64 exhibits a distinct reliance on GPx/GSH/GR system for detoxification of H_2O_2, differing from other microorganisms. This exclusive dependence on this system suggests a specialised evolutionary adaptation, possibly linked to oxidative stress challenges. Recently it has been reported that *Synechocystis* upregulates genes encoding GPx-like enzymes under oxidative stress conditions (Zhang et al., 2025).

SEM images revealed that 48 h exposure of the organism to pretilachlor led to cell clumping accompanied by the formation of a sheath. The sheath's extent decreased at 96 h and disappeared completely at 144 h (Fig. 6). Staining of the cultures confirmed that the sheath consisted of exopolysaccharides (Fig. 7). EPS are mainly composed of polysaccharides, nucleic acids, lipids, proteins and humic acid (Melo et al., 2022). Microbial cells excrete EPS in response to environmental conditions, in the presence of heavy metals, pesticides etc., as a stress tolerance mechanism (Melo et al., 2022). It has been suggested that EPS act as a protective layer for aggregated biomass and hinder the transport of micropollutants (Melo et al., 2022).

The results of the present study indicate that upon exposure to IC_{50} dose of the herbicide, Synechocystis sp. managed oxidative stress during the initial 48 h of exposure by activating the antioxidant defense system. However, after 48 h, the organism appeared to shift to an alternative cellular defense mechanism, reducing stress-induced ROS production.

To some extent, the organism also protected itself from herbicide toxicity by secreting EPS; otherwise, toxicity at IC_{50} dose of the herbicide would have been more severe. Based on the results of the present study, we hypothesize that the decrease in ROS levels, antioxidant enzymes activities, and EPS after 48 h is due to activation of herbicide biodegradation. Once the mechanisms of pretilachlor tolerance in *Synechocystis* sp. are fully understood, this organism could serve as a promising candidate for herbicide bioremediation in rice fields.

Conclusions

Exposure of *Synechocystis* sp. to pretilachlor triggered a significant rise in ROS production during the initial 48 h, alongwith reduced Chl*a*, photosynthesis and growth. To ensure its survival, the organism activated its antioxidant defense system and secreted EPS. Elevated ROS levels were mitigated through increased SOD activity, while H_2O_2 scavenging was managed by GPx utilizing GSH. Non-induction of POD and CAT by herbicide stress suggested a unique reliance of the organism on GPx/GSH/GR system. Additionally, EPS secretion likely slowed down the herbicides' entry into cells, enhancing tolerance. Pretilachlor tolerance in *Synechocystis* sp. followed a two-phase mechanism: the initial phase (0-48 h) involved antioxidant defense activation and EPS secretion, followed by post-48 h transition to an alternative mechanism, which is under investigation.

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Conflict Of Interest

The authors have no relevant financial or non-financial conflicts of interest to disclose.

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