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Research article

ADAPTIVE RESPONSES OF CYANOBACTERIUM *PLECTONEMA BORYANUM* TO HERBICIDE BUTACHLOR

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ABSTRACT : The Present paper deals with the herbicide *Butachlor* (5,10 ,20,40 and 80ppm)-induced changes in physiological and biochemical parameters related to photosynthesis and defense systems in paddy field cyanobacterium *Plectonema boryanum* grown under laboratory conditions. Growth and photosynthetic pigments, i.e., chlorophyll *a* and carotenoids were adversely affected by B*utachlor* treatment and the inhibition was found to be dose dependent. The toxic effect of *Butachlor* was more pronounced protein; however, a considerable reduction in chlorophyll *a,* and carotenoids was also noticed. Furthermore, *Butachlor* with increasing doses accelerated the formation of active oxygen species, i.e., O^{2-} and H^2O^2, in cells progressively. As a consequence of active oxygen species (AOS) generation in *Butachlor* -treated cells, the activity of superoxide dismutase (SOD) and peroxidase (POD) was enhanced considerably. Besides the accelerated action of enzymatic defense systems, Protein damage also showed an increasing trend with the rising concentration of *Butachlor* (5, 10, 20, 40 and 80 ppm).

Keywords: Cyanobacterium, *plectonema boryanum,* Butachlor

INTRODUCTION

The application of herbicide, a group of pesticides, in crop fields for selective control of pests in the modern age has led to serious environmental contamination resulting in greater loss of crop productivity and growth of many beneficial micro-organisms (Shetty et al., 2000). Though, the application of many herbicide are forbidden, the low cost, easy availability, lack of awareness and lax regulatory implementation have contributed to the continuous use of the herbicide in tropical and subtropical regions. The removal of these herbicide from soil and aquatic systems has become a difficult problem and as a result of this, they persist in these ecosystems for a long period of time (Das and Singh, 1977; Singh, 1973). Cyanobacteria, also known as blue-green algae, are a diverse group of gram-negative photosynthetic prokaryotes. The majority of cyanobacteria are aerobic photoautotrophs. Their life processes require only water, carbon dioxide, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism.. Many Cyanobacteria contribute greatly to the nitrogen economy of aquatic and terrestrial habitats through their ability to fix atmospheric nitrogen.

Cyanobacteria, a group of ubiquitous, photosynthetic prokaryotes which perform two key biological processes such as oxygenic photosynthesis and nitrogen fixation together in same the cells/filaments, and enrich the paddy soil particularly with nitrogen and humus contents (Watanabe and Kiyohara, 1960). During photosynthesis, cyanobacteria harvest solar energy and assimilate it into carbon compounds which provide cellular energy and a carbon skeleton for metabolic process such as nitrogen fixation in heterocystous cyanobacteria. In recent years, it has been explored whether non-heterocystous cyanobacteria which are predominantly found in paddy fields (Desikachary, 1959) may also fix atmospheric nitrogen under aerobic conditions as Ohki et al. (1992) demonstrated the potential of a non-heterocystous cyanobacterium *Trichodesmium* in nitrogen fixation. Herbicide endosulfan-induced adverse impact on photosynthesis may cause a severe effect on other related metabolic processes and over all growth performance of cyanobacteria.

A large number of pesticides are used in rice fields to protect the rice seedlings and crops, and selectively destroy the pests, but the indiscriminate use of pesticides causes great danger to the rice field cyanobacteria and other beneficial micro-organisms (Da Silva et al., 1975). Under water logged conditions as commonly observed in rice fields, pesticides may induce many cellular disorders in cyanobacteria (Greaves, 1982; Singh et al., 1986). It has been shown that insecticide, carbofuran reduced the growth of cyanobacteria *Oscillatoria* sp., and *Westiellopsis prolifica* considerably (Ravindran et al., 2000). Owing to extensive usage of endosulfan in various tropical and subtropical countries to control the insect population, it also declines the growth of microflora including cyanobacteria considerably (Satish and Tiwari, 2000; Shetty et al., 2000). Though a considerable amount of work relating to the pesticide induced inhibitory effects on growth, photosynthetic pigment contents and nitrogen fixation in cyanobacteria has been done, insecticides particularly *butachlor* induced effects on photosynthesis, AOS generation, antioxidants viz. enzymatic, non-enzymatic and lipid peroxidation in cyanobacteria in general and *Plectonema boryanum* in particular are yet to be investigated. Furthermore, another group of pesticides such as herbicides are shown to generate singlet oxygen and other active oxygen species at various sites of photosynthetic electron transport chain (Halliwell, 1987) and create oxidative stress in cells. Cellular systems scavenge these active oxygen species by invoking an increased antioxidative machinery such as enzymes superoxide dismutase, catalase and peroxidase etc., and organic chemicals like proline, ascorbate and carotenoids etc. Herbicide induced oxidative stress accelerates lipid peroxidation, thereby affecting structural integrity and permeability of cellular membranes (Halliwell, 1987). Considering the importance of cyanobacteria in rice fields, and frequent use of pesticides against pests, the authors set forth the objective of investigating the impact of herbicide *Butachlor* on growth, photosynthetic pigments ,Reactive oxygen species formation, antioxidant systems, lipid peroxidation,SDS PAGE Analysis and DNA fragmentation in a cyanobacterium *Plectonema boryanum.*

MATERIAL AND METHODS

Experimental organism:

Filamentous, non heterocystous, cyanobacterium ***Plectonema boryanum***, was used in the present study as a test organisms. *Plectonema boryanum* was obtained through Department of Biotechnology, University of Allahabad. *Plectonema boryanum* is 2-5 cm diameter; filament densely entangled, sheath distinct, only at the periphery of the thallus, cells short barrel shaped to cylindrical up to twice as long as broad.

Growth conditions: The pure culture of *Plectonema boryanum* was maintained in the culture room at 27 ± 2 °C. For regular experiments, cultures were grown in one liter N-free BG11positive medium (pH 7.5) under the light intensity of 2400 Lux and 8/16h light and dark photoperiod.

Growth medium: For the growth of *P.boryanum,* **BG -11 positive** medium was prepared in batch culture by using following composition.

Composition of BG -11 positive medium

Macronutrients	**g/L**
K_2HPO_4	0.010
$CaCl_2.7H_2O$	0.040
$MgSO_4.7H_2O$	0.025
$Na_2SiO_3.9H_2O$	0.025
Na_2CO_3	0.02
Ferric citrate	0.003
Citric acid	0.003
Micronutrient	1 ml
$NaNo_3$	1.5

Micronutrients	g/L
$MnCl_2.4H_2O$	1.81
$Na_2MoO_4.2H_2O$	0.39
$ZnSO_4.4H_2O$	0.222
$CuSO_4.5H_2O$	0.079
H_3BO_3	2.86

Sterilization of medium: All the glass wares and culture media were sterilized in an autoclave at 15 lb inch^2 pressure and at 121 ^{0}C temperature for 15 min. Before autoclaving, the desired quantities of media have been poured in to suitable sized glass wares properly plugged with cotton.

Isolation and purification: Standard microbiological techniques were used to isolate pure cultures from the contaminated stocks. The specimens were stored in screw cap bottles. A pinch of the sample was homogenized and added to 150 ml Erlenmeyer flasks containing 50 ml of sterile medium with or without combined nitrogen and incubated at room temperature under fluorescent light. Clonal and axenic populations were obtained by serial dilution.

For isolation of bacteria free cultures, few colonies from the agar slant which appeared free from bacteria were isolated and tested for bacterial contamination in the media given below.

1) Dextrose- Peptone Broth

Peptone	1.0g 100ml^{-1}
Dextrose	1.0g 100 ml^{-1}
Agar- Agar	0.8g 100 ml^{-1}

2) Caseinate – Glucose Agar

Casamino Acid	2.5g 100 ml^{-1}
Glucose	1.0g 100ml^{-1}
Agar-Agar	1.0g 100ml^{-1}

Cyanobacterial strain was inoculated into sterilized tubes containing agar slants prepared in BG11- positive medium. After 15 days of incubation in light and in darkness, cultures were examined microscopically and absence of bacteria was confirmed. Such bacteria free clones were selected and maintained on different agar slants. During the course of experimental work , cultures were tested for bacterial contamination from time to time and in case of contamination the culture were discarded and new slants from original stock was used for further study.

Incubation and maintenance of cultures

Required inoculums of cyanobacteria were transferred in fresh mineral media in a sterilized inoculation chamber. Culture were incubated and grown photoautotrophically in culture room maintained at 27 ± 1 ^{0}C, illuminated with white fluorescent tubes providing an intensity of 50 µM m^{-2}s^{-1} and a photoperiod of 8/16 h light and dark. The batch cultures were regularly shaken twice a day. Log phase cultures were used as inoculum as well as throughout experimental studies. For large scale use, cyanobacteria were also grown in a fermentor containing mineral medium.

Photosynthetic pigment extraction and estimation

Chlorophyll- a and Carotenoids

For extraction of Chl-a and carotenoids, equal volume of organism culture was centrifuged and pellets were suspended in desired volume of 80% (acetone: water, v/v). After overnight incubation at 40˚C, suspension was centrifuged and supernatant was used measuring Chl-a and carotenoids. The absorbance of pigment extracts was read for Chl-a at 663nm and carotenoids at 450 nm with a spectrophotometer. The specific coefficient as given by Myers and Kratz (1955) were used for the calculation of Chl-a and carotenoids concentrations in cultures. Quantitative estimation of these pigments in terms of g/l was done using formulae given below:

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C = D/ d α ------------------ (1)

Where α = absorption coefficient (value of α for chl-a is 82.04 and for carotenoids is 200).

D = Optical density.
d = inside path length of spectrophotometer in (cm)
C = concentration of pigment in gl^{-1}

SOD

Reagents

- 1M Na_2Co_3
- 200mM methionine
- 2.25 mM NBT
- 3mM EDTA
- 60µM riboflavin
- 0.1M phosphate buffer

Procedure

Cyanobacterialcells were harvested by centrifugation after 24 h of butachlor treatment and then homogenized at 4°C in100mM EDTA-phosphate buffer (pH 7.8) for superoxide dismutase (SOD) activity and in 100mM phosphate buffer (pH 7.8) for peroxidase activity. Supernatant obtained after centrifugation of the homogenate at 20,000_*g* for 30 min was used as a crude extract for the enzyme assay. SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium chloride (NBT) according to the method of Giannopolitis and Ries (1977) using a reaction mixture (3 ml) consisting of 1.3 mM riboflavin, 13mM methionine, 63 mM NBT, 0.05 M sodium carbonate (pH 10.2) and crude extract (600 mg protein ml_1).

Peroxidase activity

Reagents

- H_2O_2 16mM
- Pyrogallol 10mM
- Crude extract 650 mg protein ml^{-1}

Peroxidase activity in a reaction mixture (3 ml) containing 16mM H_2O_2, 10mM pyrogallol and crude extract (650 mg protein ml^{-1}) was determined spectrophotometrically according to the method of Gahagen et al. (1968) and the activity was measured as rise in optical density at 430 nm.

Determination of total peroxide and superoxide radicals

Reagents

Total peroxide

- TCA 5%,3 ml
- Ferrous ammonium sulfate 10 ml
- Potassium thiocyanate 5 ml

Superoxide

- Phosphate buffer (pH 7) 20 ml
- Hydroxylamine 10 ml
- NEDD 10 ml
- Sulphanilamide 10 ml

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Procedure

For total peroxide, test samples were homogenized in 3.5 ml of 5% TCA and after centrifugation at 10,000x*g* for 15 min, the total peroxide in the supernatant was analyzed by following the ferrithiocyanate method as described by Sagisaka (1976). The superoxide radical was measured according to the method of Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of O_2 in supernatant obtained from homogenates of 24 h treated and untreated samples with some modification as described by Jiang and Zhang (2001).

RESULTS

The cyanobacterium *Plectonema boryanum* showed inhibitory growth response against herbicide butachlor The effect of different concentrations of butachlor on photosynthetic pigments after 5 days of treatment is represented in Table 1.After 5 days of treatment decrease in the chll a and carotenoid is dose dependent.The declining trend in the pigment contents continued with the rising concentration of herbicide as 40 ppm butachlor sharply lowered chlorophyll *a* and carotenoid contents by 30and 70%, respectively. The growth of autotrophic organisms reflects the status of a key physiological process such as photosynthesis, which regulates the biomass production in autotrophs. Therefore,to understand the impact of butachlor on biomass production of cyanobacterium, photosynthesis and protective mechanisms were investigated in detail. Table 1 shows the photosynthetic pigment estimation. The generation of superoxide radical and singlet oxygen may occur at various sites of PSI and PSII in photoautotrophs, and therefore, the status of superoxide radical and hydrogen peroxide in cells treated with herbicide for 5 days of treatment was investigated. These Reactive oxygen species undergo deleterious reactions and cause oxidative stress in the cellular systems. Thus,the deleterious effects of enodosulfan on *P. boryanum* were correlated by estimating the rate of lipid peroxidation, superoxide radical and H_2O_2 production, and status of antioxidants. Results depicted in Figs(1,2,3).

Table1. Photosynthetic pigments of *Plectonema boryanum* after 5 days of Butachlor treatment

Butachlor (ppm)	(µg/ml)	
	Chlorophyll a	Carotenoids
0	3.40	1.62
5	3.11 (-8.53)	1.65 (+1.83)
10	2.53 (-25.59)	1.68 (+3.68)
20	2.39 (-29.71)	1.27 (-21.60)
40	2.36 (-30.59)	1.16 (-28.39)
80	0.76 (-77.64)	0.85 (-47.53)

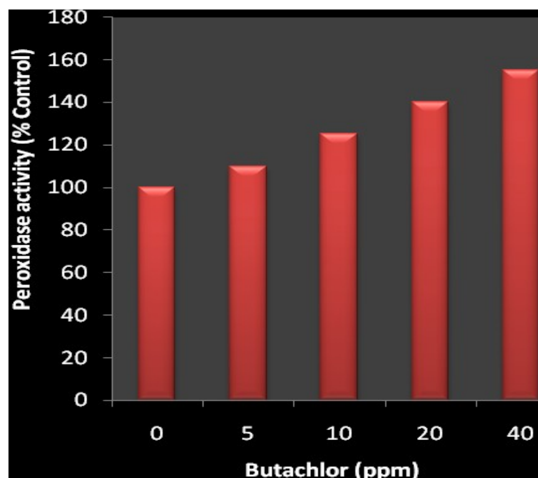

Fig 1: Effects of different concentrations of butachlor on Superoxide dismutase level after 5 days of treatment in cells of *P.boryanum*

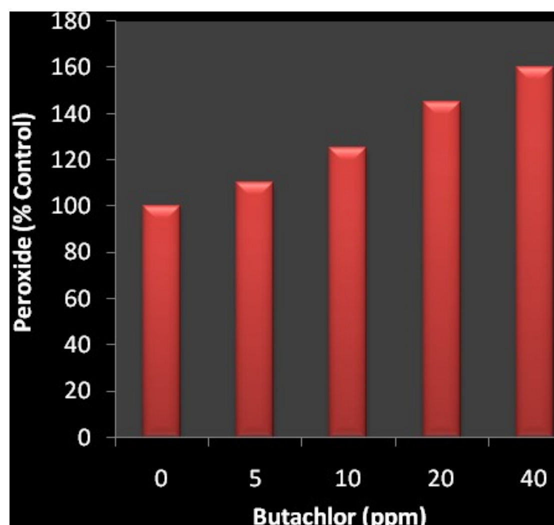

Fig 2: Effects of different concentrations of butachlor on total Peroxide level after 5 days of treatment in cells of *P.boryanum.*

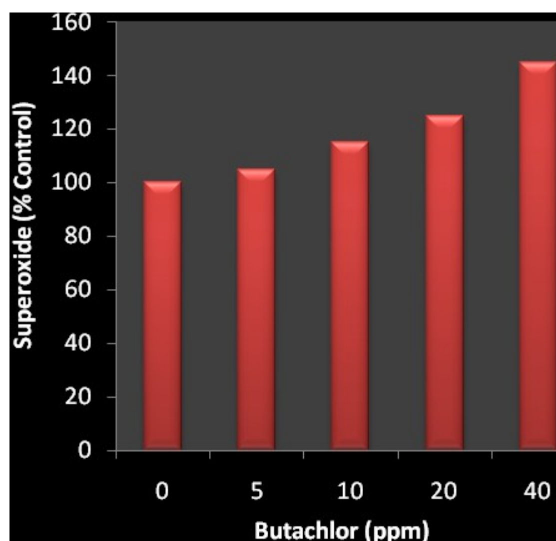

Fig 3: Effects of different concentrations of butachlor on Superoxide level after 5 days of treatment in cells of *P.boryanum*

DISCUSSION

The results of this study show butachlor induced changes in growth, photosynthetic pigment contents, photosynthesis, active oxygen species generation, lipid peroxidation and antioxidants in a paddy field cyanobacterium *Plectonema boryanum*. The inhibitory effect of butachlor on photosynthetic pigments of *P. boryanum* was found to be dose dependent and the deleterious effect was more pronounced on chlorophyll *a* followed by carotenoids. Such decrease in chlorophyll *a* and carotenoid contents may be ascribed to the inhibition of pigment synthesis directly by herbicide or accelerated degradation of pigments due to increased ROS formation at the various sites of the photosynthetic electron transport chain during stress. Butachlor-induced acceleration in the Reactive oxygen species formation in *P. boryanum* could be due to the strong inhibition of PSII and whole chain activities The stimulated generation of Reactive oxygen species caused increased peroxidation of lipid in *P. boryanum* and thus MDA production.(Fig. 7).

The enhanced level of O_2 _ and H_2O_2 following 20ppm butachlor treatment might have also caused greater damage to photosynthetic pigments (Table 1). Recent evidence has shown that ROS, especially H_2O_2 and O_2 _, are involved in cellular signaling processes as secondary messengers to induce a number of genes and enzymes such as POD and SOD (Mahalingam and Fedoroff, 2003), which invoke active oxygen species in stressed organisms. Thus, the increased level of O_2 _ and H_2O_2 triggered the activity of several antioxidant enzymes such as superoxide dismutase and peroxidase in *P. boryanum* at all the concentrations of butachlor tested. The present study demonstrates that the strong inhibitory effect on the growth of cyanobacterium *P. boryanum* could be correlated with the butachlor induced inhibition in PSII and whole photosynthetic chain activities. This inhibition might have not only increased the production of active oxygen species but also indirectly inhibited the removal of active oxygen species (H_2O_2) by reductive enzymatic reactions (glutathione reductase and dehydroascorbate reductase) due to less availability of NADPH.

CONCLUSION

In this study herbicide butachlor induced stress in cyanobacterium *Plectonema boryanum* has been seen. At high concentration there was considerable decrease in photosynthetic pigments. Furthermore, butachlor with increasing doses accelerated the formation of active oxygen species, i.e., O_2 and H_2O_2, in cells progressively, whereby an enhanced peroxidation of lipid and leakage of cell membrane were noticed. As a consequence of Reactive oxygen species (ROS) generation in butachlor -treated cells, the activity of superoxide dismutase (SOD), and peroxidase (POD) was enhanced considerably.Besides a considerable damage to the protein was observed with the rising concentration of the herbicide butachlor. Hence it can be clearly stated that butachlor has caused great damage to the cellular metabolism of a paddy field cyanobacterium *P. boryanum*, thereby affecting the paddy yield.

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