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Interaction of 2,4-D and Pencycuron with Three Different Cyanobacterial Species-*Anabaena fertilissima* Rao, *Aulosira fertilissima* Ghose and *Westiellopsis prolifica* Janet

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Abstract: Three strains of filamentous-heterocystous cyanobacteria such as *Anabaena fertilissima*, *Aulosira fertilissima* and *Westiellopsis prolifica* were screened for their photosynthetic pigments, stress metabolites and enzymatic activities in response to 2,4-dichloro phenoxy acetic acid ethyl ester and 1-(4-chlorobenzyl)-1-cyclopentyl-3-phenylurea (Pencycuron). Amongst them, *Westiellopsis prolifica* grew maximally in BG_{11} medium amended with both the pesticides concentrations. In 2,4-D ethyl ester treated cultures the phycobilin pigments showed superior plummet as compared to chlorophyll-*a* and carotenoids in all three cyanobacterial species. Phycoerythrin showed highest reduction by 86% at 60 ppm in *Anabaena fertilissima* cells grown in medium amended with Pencycuron. After 4 days of inoculation slight raise in protein content at lower concentrations of 2,4-D was recorded in three species, whereas in Pencycuron treated cultures *Anabaena fertilissima* (10% at 60 ppm) and *Aulosira fertilissima* (7% at 60ppm) showed minor lapse and *W. prolifica* was unaffected in all treated concentrations. However, carbohydrates and amino acids have shown inhibitor effect on the three species of cyanobacteria in response of the increasing concentration of both the pesticides, on the other hand, phenols exhibited the increasing trend. Activity of nitrate reductase, glutamine synthetase and succinate dehydrogenase were adversely affected in all three species when treated with the increasing concentrations of both the pesticides.

Key words: 2,4-D, Pencycuron · Pigments · Stress metabolites · Enzyme activity · Cyanobacteria

INTRODUCTION

Cyanobacteria or blue-green algae are ubiquitous group of prokaryotes that carry out oxygenic photosynthesis [1]. Their main photosynthetic pigments are chlorophyll-α and carotenoids together with phycobiliproteins. Some heterocystous genera are diazotrophic, i.e. they can use atmospheric N_2 as the sole nitrogen source and thus contributing to photodependent N_2-fixing in rice fields worldwide [2] and therefore plays a vital role in the maintenance and building up of soil fertility. Yet, the utilization of cyanobacteria as a biofertilizer in rice field requires that the strains show tolerance to a multiplicity of pesticide that is normally used. Paddy fields are unique artificial agroecosystems that are frequently disturbed by intensive agricultural practices such as flooding, drainage, ploughing and application of chemical fertilizers and pesticides (insecticides, fungicides and herbicides) [3]. Thus, under the water-logged conditions of rice fields, both nitrogen-fixing cyanobacteria and rice field herbicides are found to interact resulting in the destruction of cyanobacteria [4].

2,4-D (2,4-Dichlorophenoxy acetic acid ethyl ester) is an active ingredient in most of the commercial herbicides and belongs to chlorophenoxy group. It is a selective and contact herbicide used to control broadleaved and grassy weeds in post-emergence rice. 2,4-D inhibits photosynthetic electron transport chain and also reduces heterocyst frequency [5]. Kobbia and Sharouny [6] employed cyanobacteria-*Nostoc muscorum, Tolypothrix lanata* and *Aulosira laxa* to assess different responses against 2,4-D herbicide at all concentrations.

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Table 1: Physico-chemical properties of 2,4-D ethyl ester and Pencycuron

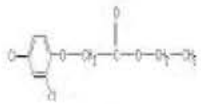
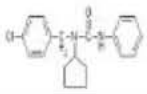
Pesticides	Empirical Formula	Chemical name and number (Chemical Abstract Service)	Chemical structure	Molecular weight (g.mol-1)	Melting point(°C)
2,4-D ethyl ester(herbicide)	$C_{10}H_{10}O_3Cl_2$	Ethyl Ester of 2,4-Dichloro Phenoxy Acetic Acid CAS no.94-76-6		249	136°C to 140°C
Pencycuron (fungicide)	$C_{19}H_{21}ClN_2O$	N-[(4-chlorophenyl)methyl]-N-cyclopentyl-N '-phenylureaCAS no.6 6063-05-6		328.8	129.5°C

Table 2: Differential susceptibilities of three heterocystous filamentous cyanobacteria, Anabaena fertilissima, Aulosira fertilissima and Westiellopsis prolifica to two pesticides

	Concentration (ppm)		
Pesticides	Anabaena fertilissima	Aulosira fertilissima	Westiellopsis prolifica
2,4-D ethyl ester	15	20	30
	30	40	60
	60	80	120
Pencycuron	15	15	50
	30	30	100
	60	60	200

Rana and Nirmal Kumar [7] have also made some observations of the effect of the herbicide N-(4-isopropylphenyl)-N, N-dimethyl urea on the aquatic organisms including cyanobacteria.

Pencycuron (1-(4-chlorobenzyl)-1-cyclopentyl-3-phenylurea) is the active ingredient of the commercial fungicide Monceron 250 SC. Pencycuron is a relatively new non-systemic protective fungicide for controlling sheath blight (*Rhizoctonia solani*) of rice [8], is expected to be used widely in agricultural production particularly in Asia. However, information on the dissipation pattern of Pencycuron on cyanobacteria is lacking. The details of chemical names, structures and other properties of both herbicide and fungicide are detailed (Table 1).

Every pesticide having specific effect on each organism, therefore, the aim of this work was to establish the differential and toxicity effects of the selected rice field pesticides on photosynthetic pigmentation and metabolites content of three species of rice field cyanobacteria and also explore the effects of both pesticides on enzyme activities as important physiological parameters of pesticide toxicity evaluation.

MATERIALS AND METHODS

Cyanobacterial Cultures: The axenic cultures of nitrogen-fixing cyanobacteria, viz., *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica* were obtained from the National Facility for Blue Green Algal Collections, IARI, New Delhi. The cyanobacteria were grown under controlled illumination of 40 $\mu Em^{-2}s^{-1}$ at 27±1°C in a nitrogen-free BG_{11} liquid medium at pH 7.0±0.2 under aerobic and static conditions. All inoculations were carried out under aseptic conditions and the cultures were periodically checked for any contamination. Only axenic cultures were used for experimental studies.

Pesticides: The pesticides chosen for the study were 2,4-D (38% EC 2,4-D ethyl ester) and Monceren (22.9% SC Pencycuron) obtained from Northern minerals limited, Haryana and Bayer CropScience limited, Mumbai respectively. Three concentrations for each pesticide were selected for the present investigation to analyze the response in *Anabaena fertilissima, Aulosira fertilissima* and *W. prolifica* (Table 2) on determining LC_{50}. Stock solution of both the pesticide were prepared in sterilized double-distilled water and added aseptically to the culture medium to the final concentrations indicated for each treatment.

Diagnostic Methods: Samples were taken after every four days up to sixteen days for the determination of pigments, metabolites and enzyme activity. Analytical grade (Merck Ltd and Himedia Ltd, India) chemicals were used throughout the study. Each experiment was conducted in replicates of three and their ±SD values were calculated.

The pigments included were chlorophyll-α [9], carotenoids [10] and phycobilin pigments [11]. The changes in metabolites content like total carbohydrates [12], proteins [13], phenols [14] and amino

acids [15] were measured. Nitrate Reductase (NR) activity in vivo was estimated by the method of Sempruch *et al.* [16] while Glutamine Synthetase (GS) enzyme was extracted in Tris HCl buffer (pH 7.5) and estimated by slight modification of the method described by Pamiljans *et al.* [17]. The estimation of in vivo succinate dehydrogenase (SDH) activity was measured by the method of Kun and Abood [18].

RESULTS

Effect of 2,4-D Ethyl Ester: The highest chlorophyll-*a* content was recorded in the untreated cells and the maximum reduction in chlorophyll-α content was registered in *Anabaena fertilissima* (86% at 60 ppm), followed by *Aulosira fertilissima* (77% at 80 ppm) and the lowest in *W. prolifica* (60% at 120 ppm) by the end of 16th day of growth (Fig.1,3 and 5). Carotenoid content in all three selected strains was affected, in a time-dose response manner, in cultures treated with 2, 4-D. At the end of the experiment after 16 days, carotenoids in *Anabaena fertilissima* were depleted by 80% at 60 ppm (Fig. 1). However, as compared to *Anabaena fertilissima* less reduction of carotenoids was observed in *W. prolifica* by 64% (at 120 ppm) followed by *Aulosira fertilissima* where the values were reduced by 72% relative to control (Fig. 3 and 5). In 2,4-D treated cultures the phycobilin pigments were more adversely affected than chlorophyll-α and carotenoids in all three cyanobacteria (Fig. 1, 3 and 5).

The release of carbohydrates was found to be higher in untreated cells in all tested periodic intervals in three species. *W. prolifica* was found to be more resistant as compared to other two strains. In *Anabaena fertilissima* 81% decrease in carbohydrate content was observed at 60 ppm, whereas in *Aulosira fertilissima* and *W. prolifica* it was 70% at 80 ppm and 65% at 120 ppm after 16 days of incubation (Fig.7, 9 and 11). 2,4-D, an inhibitor of protein synthesis showed a more marked suppression of total protein content than of amino acids. However, slight increase at lower concentrations of three species was recorded after 4 days of inoculation, which was succeeded by a period of depression (Fig. 7, 9 and 11). The release of phenols was slightly but significantly stimulated after 4-days of exposure and was higher in all treated cultures as compared to untreated cultures (control) in all the test species (Fig. 7, 9 and 11).

2,4-D caused a progressive decrease in two most important nitrogen assimilating enzymes i.e. NR and GS activity when supplemented with its lethal concentration. 2,4-D treatment for 16 days resulted in 78% retardation of NR activity in *Anabaena fertilissima* at 60 ppm, 73% at 80 ppm in *Aulosira fertilissima* and 62% at 120 ppm in *W. prolifica,* whereas GS was suppressed by 97% at 60 ppm, 76% at 80 ppm and 69% at 120 ppm (Fig. 13, 15 and 16). After 16 days of growth SDH activity was reduced to about 89% of the control in *Anabaena fertilissima* as compared to *Aulosira fertilissima* (83% at 80 ppm) and *W. prolifica* (73% at 120 ppm) (Fig. 13, 15 and 16).

Effect of Pencycuron: Minimum growth in terms of chlorophyll-*a* and carotenoid contents was observed in cells subjected to 60 ppm Pencycuron in *Anabaena fertilissima.* After 16 days of fungicide exposure chlorophyll-*a* content was decreased by 70% at 60 ppm in *Anabaena fertilissima*, 64% at 60 ppm in *Aulosira fertilissima* and 50% at 200 ppm in *W. prolifica,* whereas carotenoid was diminished by 65% at 60 ppm in *Anabaena fertilissima,* 50% at 60 ppm *Aulosira fertilissima* and 47% at 200 ppm in *W. prolifica* (Fig. 2, 4 and 6). Reduced levels of phycobilin pigments were also recorded in cells grown in medium amended with Pencycuron. Among phycobilin pigments phycoerythrin showed highest reduction by 86% at 60 ppm in *Anabaena fertilissima* after 16 days of incubation (Fig. 2, 4 and 6).

In all three cultures synthesis of carbohydrate and aminoacid declined in response to the stress,when supplemented with different doses of Pencycuron, however, the synthesis of phenol was greater in cells grown in higher concentrations. After 4 days of inoculation the release of protein was slightly decreased at highest concentration in *Anabaena fertilissima* (10% at 60 ppm) and *Aulosira fertilissima* (7% at 60 ppm), but in *W. prolifica* it was unaffected in all treated concentrations. Parallel experiments after 16 days showed significant reduction of protein in all three strains at their respective concentrations (Fig. 8, 10 and 12).

NR activity was also affected by the different concentrations of Pencycuron and was reduced in *Anabaena fertilissima* (60% at 60 ppm) (Fig. 14) where reduction in GS activity by 55% was observed after 16 days of inoculation (Fig. 14). The suppression of succinate dehydrogenase activity after 16 days treatment by Pencycuron was 58% at 60 ppm in *Anabaena fertilissima* followed by *Aulosira fertilissima* (45% at 60 ppm) and *W. prolifica* (35% at 200 ppm) (Fig. 14,16 and 18).

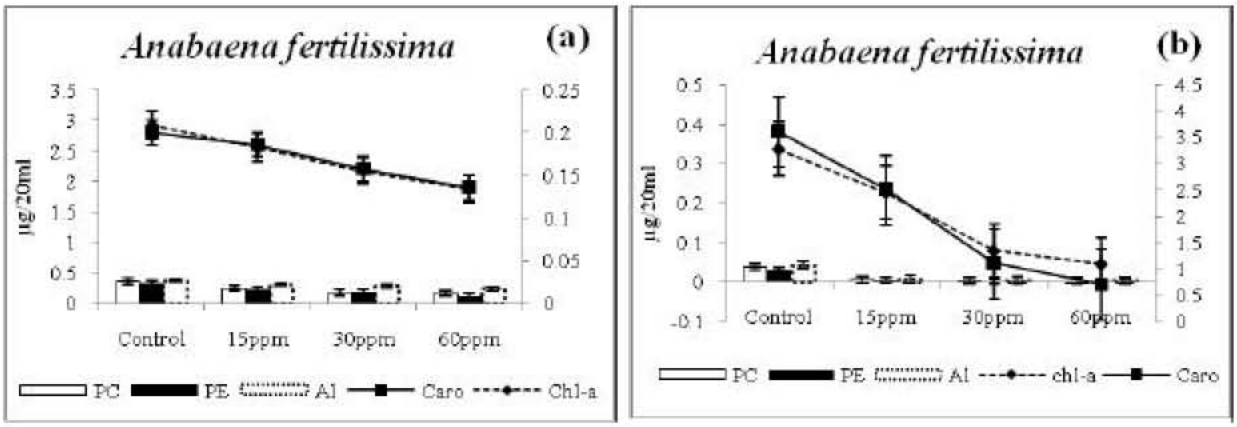


Fig. 1: Shows the pigment concentration of *Anabaena fertilissima* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester [PC=phycocyanin, PE=phycoerythrin, Al=allophycocyanin, Chl-a =chlorophyll-*a*, and Caro= carotenoids]

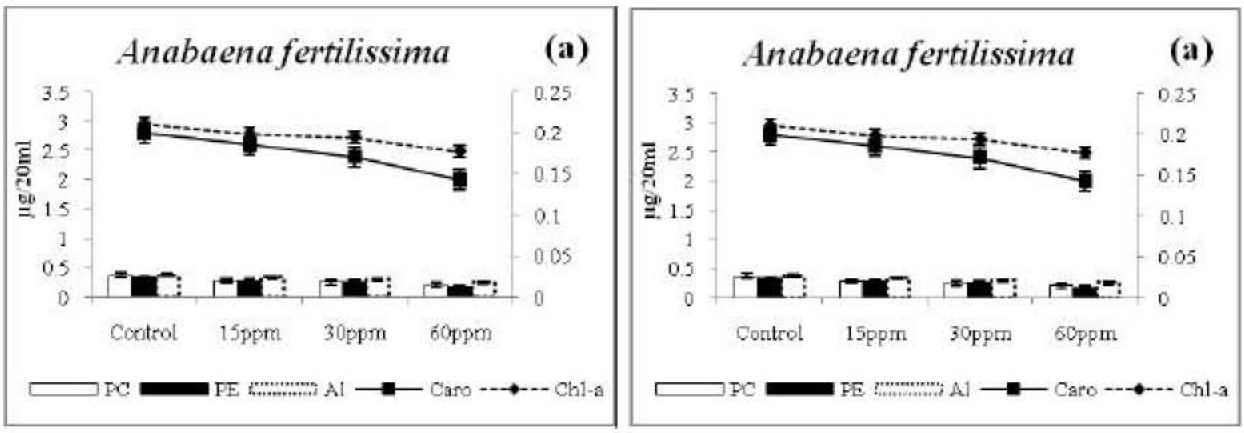


Fig. 2: Shows the pigment concentration of *Anabaena fertilissima* after 4 days (a) and 16 days (b) treatment of Pencycuron

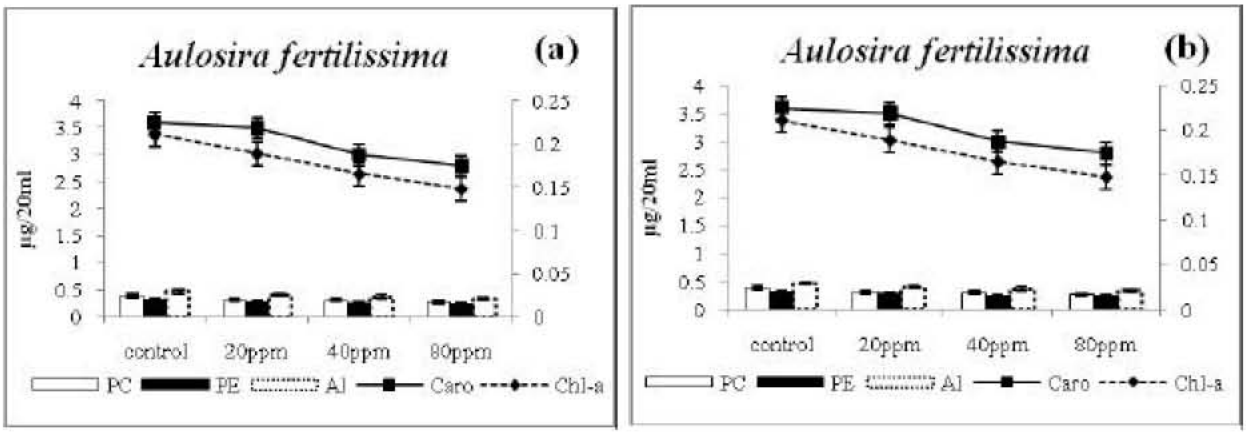


Fig. 3: Shows the pigment concentration of *Aulosira fertilissima* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester

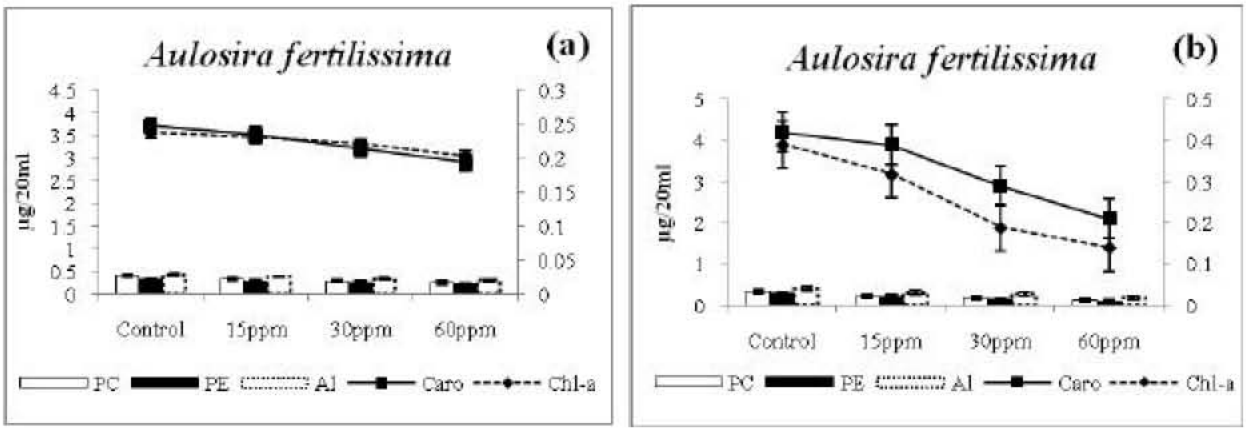


Fig. 4: Shows the pigment concentration of *Aulosira fertilissima* after 4 days (a) and 16 days (b) treatment of Pencycuron

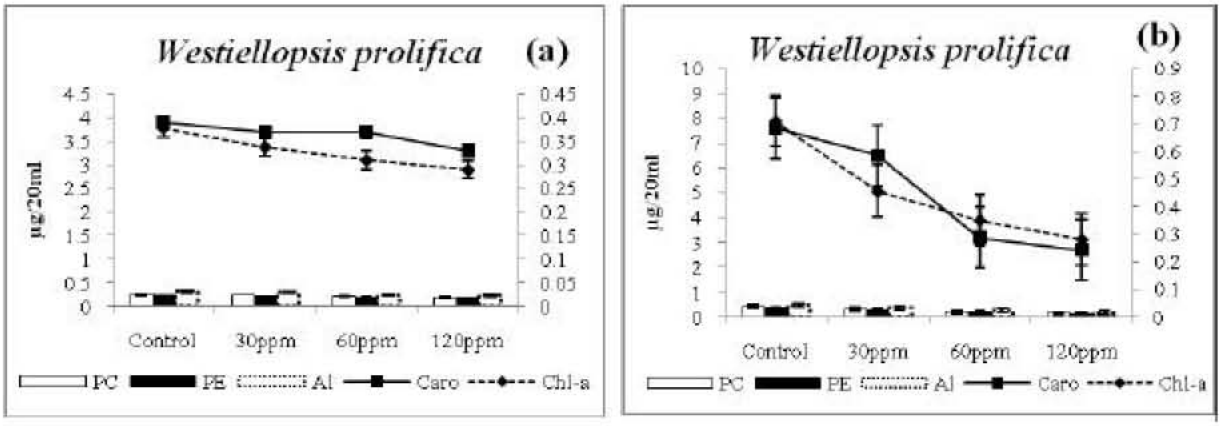


Fig. 5: Shows the pigment concentration of *Westiellopsis prolifica* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester

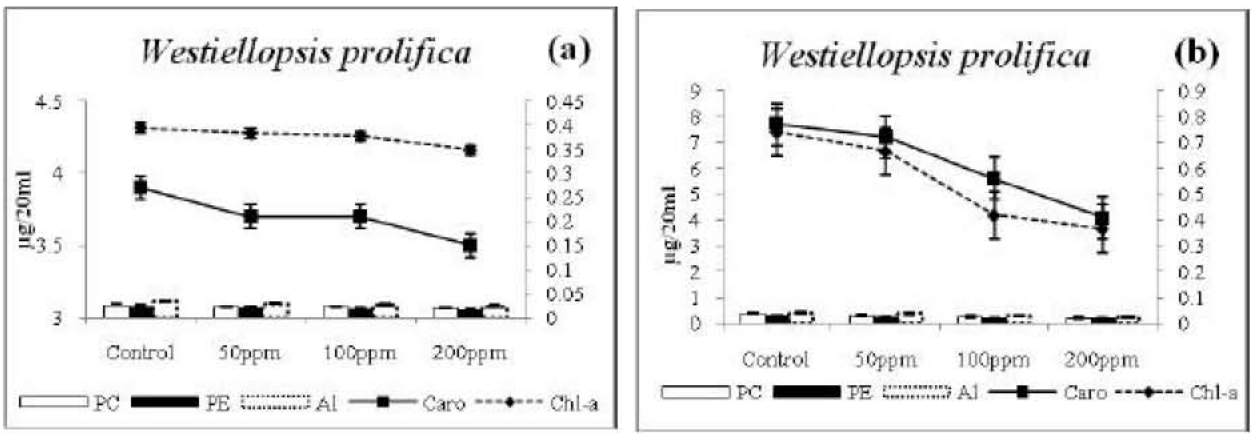


Fig. 6: Shows the pigment concentration of *Westiellopsis prolifica* after 4 days (a) and 16 days (b) treatment of Pencycuron

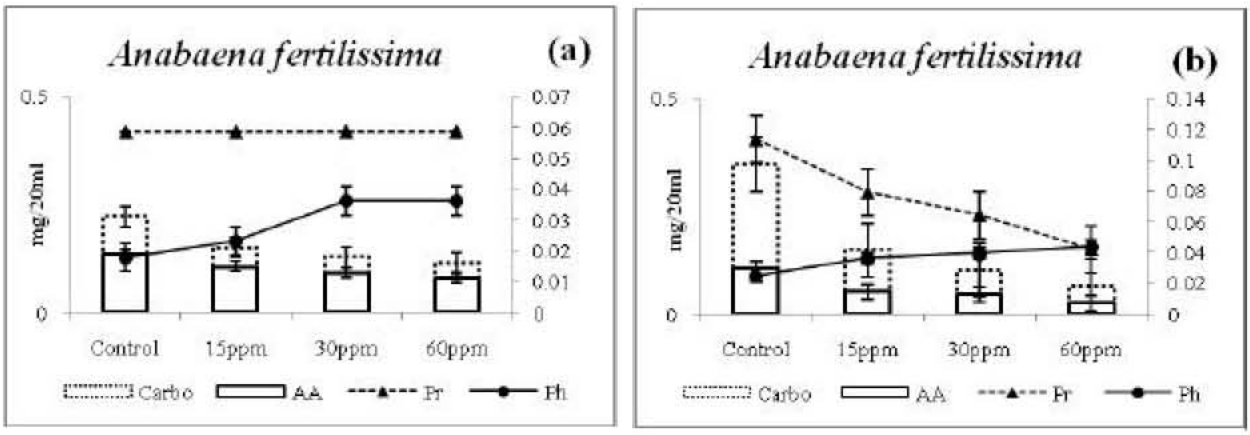


Fig. 7: Shows the metabolite concentration of *Anabaena fertilissima* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester [Carbo= carbohydrates, AA= amino acid, Pr= protein, and Ph= phenol]

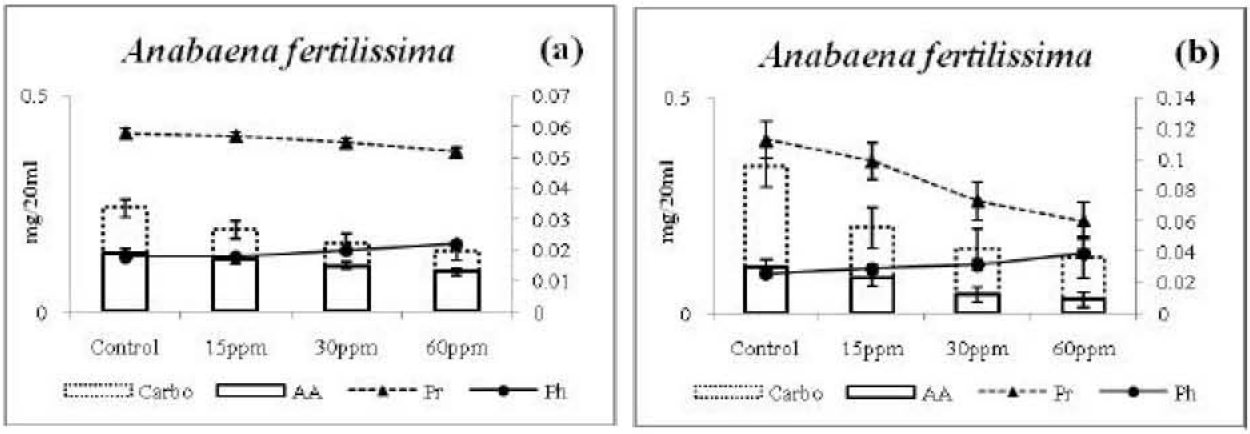


Fig. 8: Shows the metabolite concentration of *Anabaena fertilissima* after 4 days (a) and 16 days (b) treatment of Pencycuron

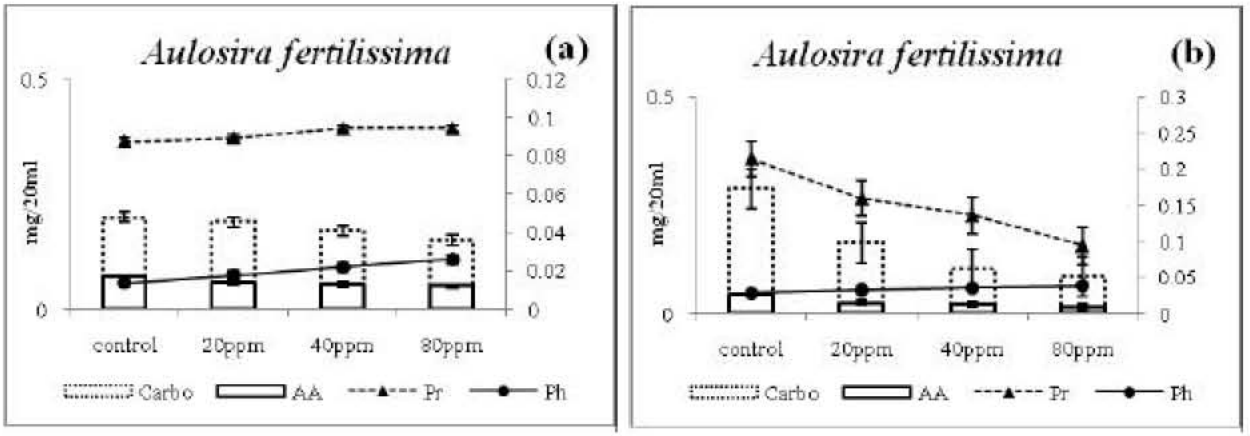


Fig. 9: Shows the metabolite concentration of *Aulosira fertilissima* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester

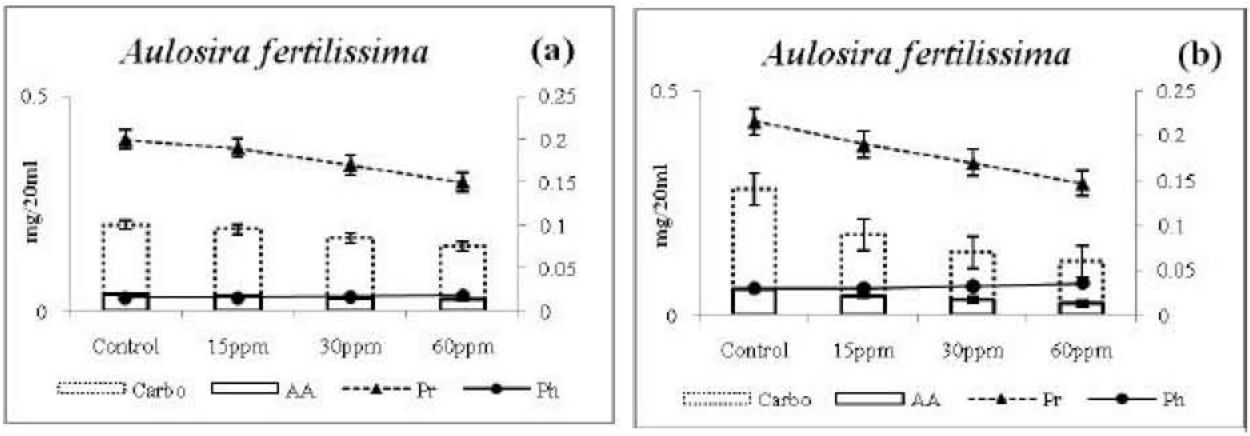


Fig. 10: Shows the metabolite concentration of *Aulosira fertilissima* after 4 days (a) and 16 days (b) treatment of Pencycuron

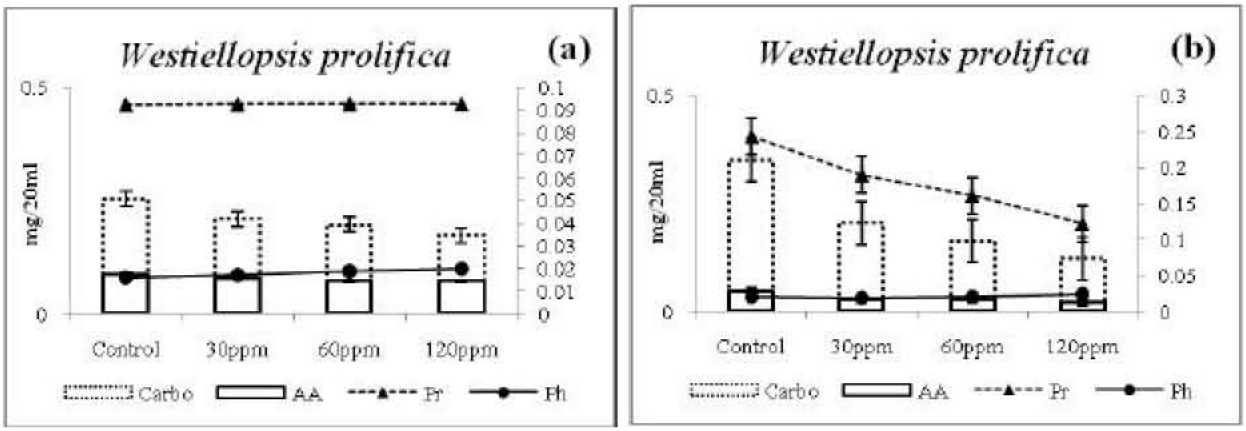


Fig. 11: Shows the metabolite concentration of *Westiellopsis prolifica* after 4 days (a) and 16days (b) treatment of 2,4-D Ethyl ester

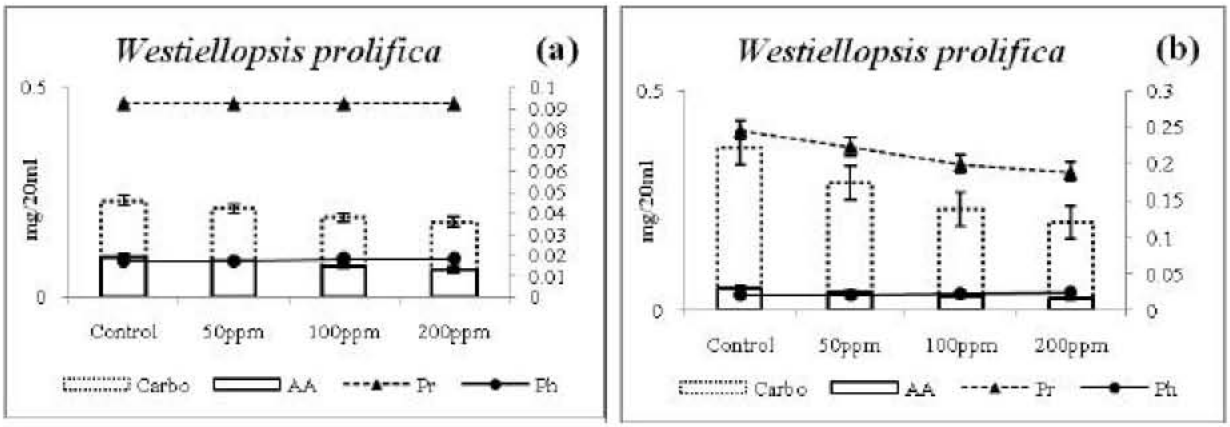


Fig. 12: Shows the metabolite concentration of *Westiellopsis prolifica* after 4 days (a) and 16 days (b) treatment of Pencycuron

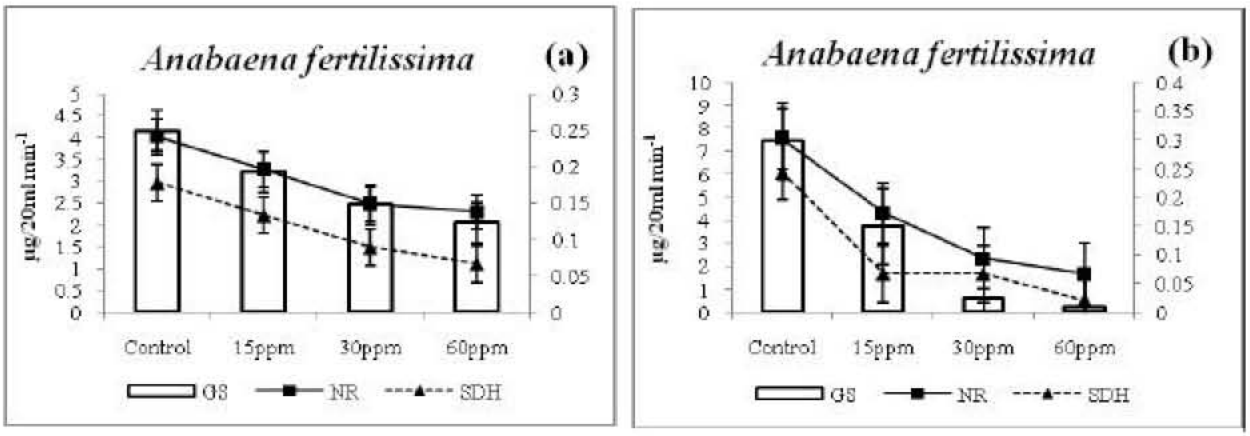


Fig. 13: Shows the enzymatic activities of *Anabaena fertilissima* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester [NR= nitrate reductase, GS= glutamine synthetase, SDH= succinate dehydrogenase]

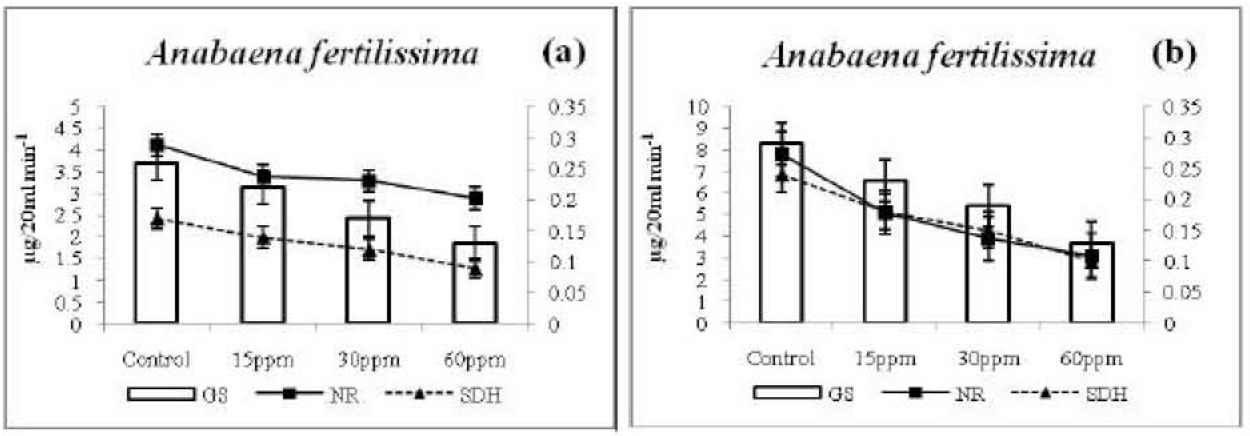


Fig. 14: Shows the enzymatic activities of *Anabaena fertilissima* after 4 days (a) and 16 days (b) treatment of Pencycuron

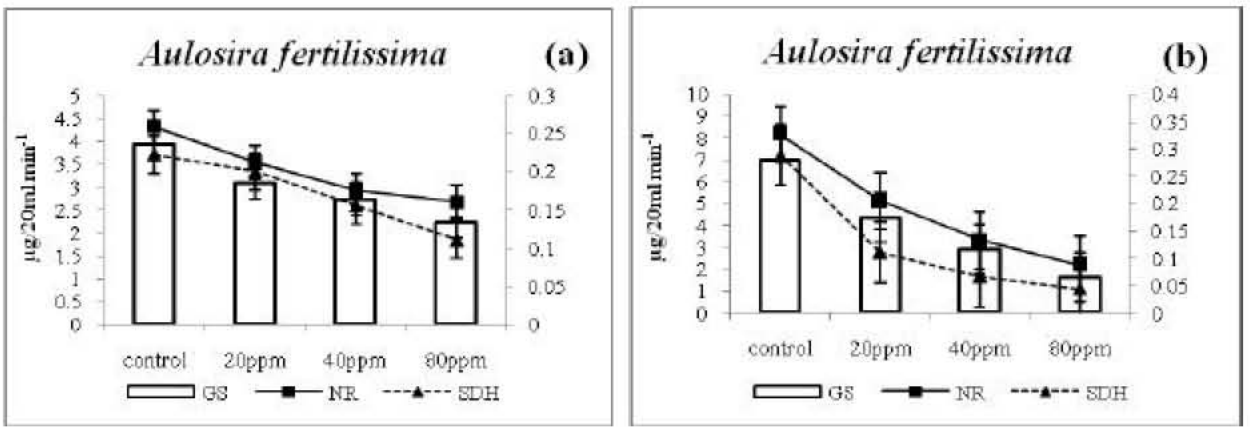


Fig. 15: Shows the enzymatic activities of *Aulosira fertilissima* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester

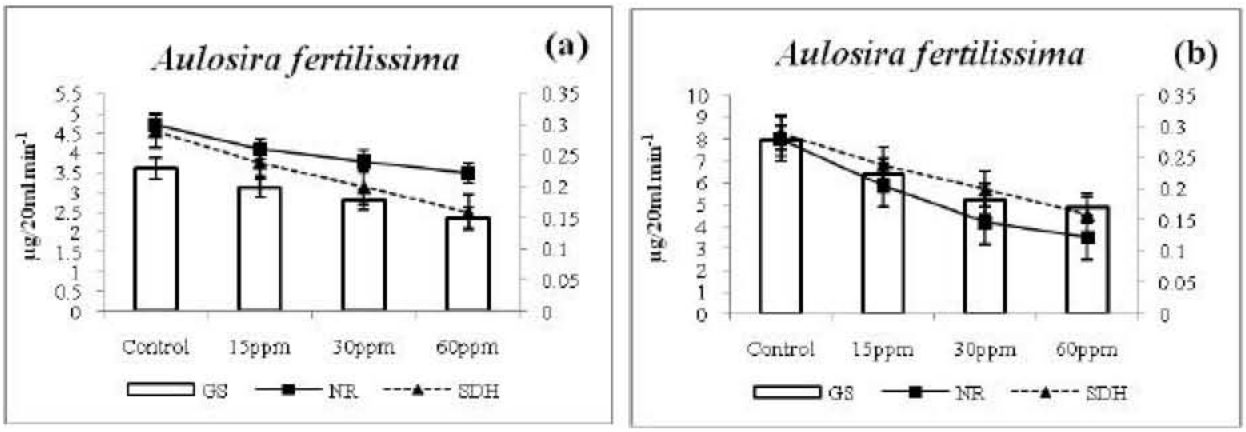


Fig. 16: Shows the enzymatic activities of *Aulosira fertilissima* after 4 days (a) and 16 days (b) treatment of Pencycuron

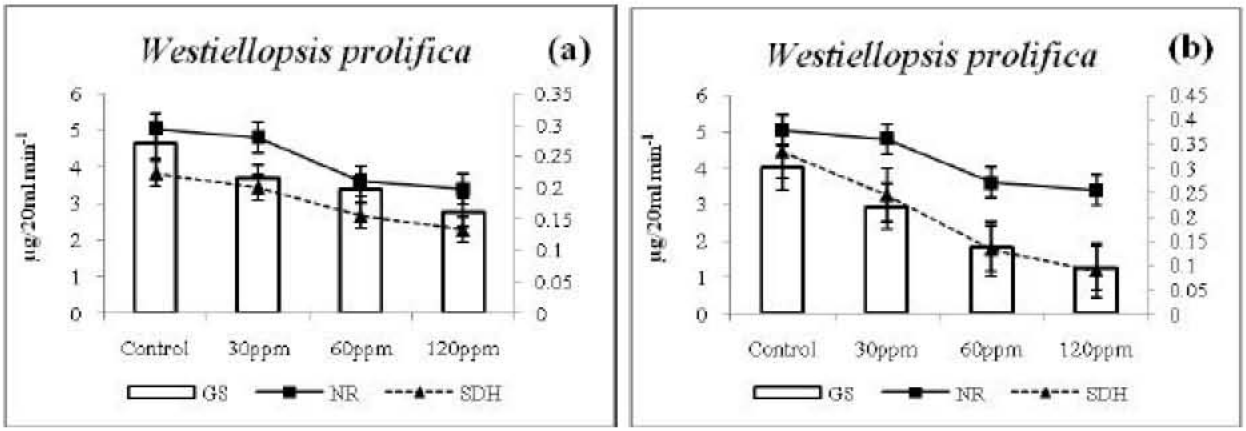


Fig. 17: Shows the enzymatic activities of *Westiellopsis prolifica* after 4 days (a) and 16 days (b) treatment of 2,4-D Ethyl ester

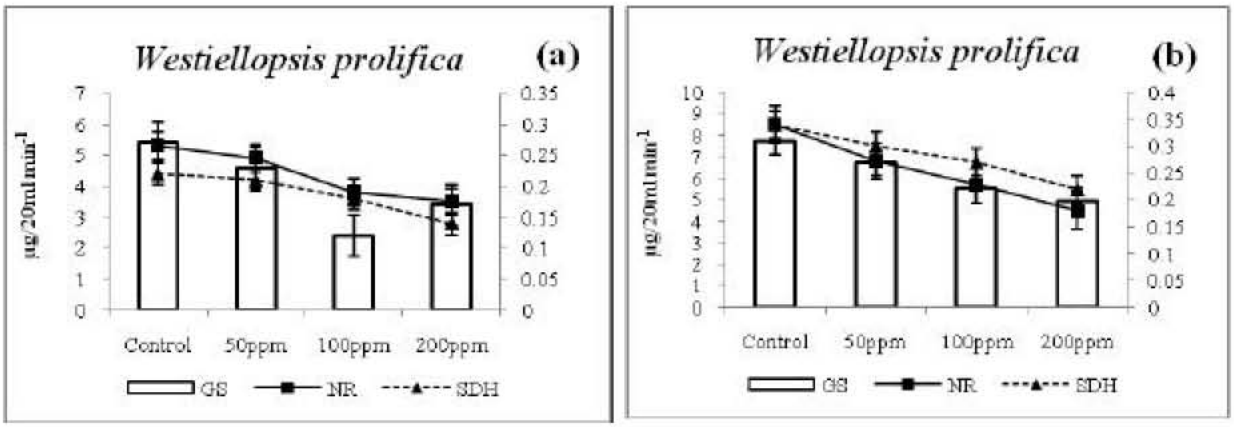


Fig. 18: Shows the enzymatic activities of *Westiellopsis prolifica* after 4 days (a) and 16d ays (b) treatment of Pencycuron

DISCUSSION

The effect of pesticides on cyanobacterial populations has been considered to be inhibitory at high doses [19]. Data obtained in the present investigation revealed that all three strains were more susceptible to 2,4-D ethyl ester than to Pencycuron in most of the parameters studied. In present study, the effect of both pesticides has been considered to be inhibitory at higher doses. Growth in terms of chlorophyll-*a* was greatest in the untreated cells, which might be due to inhibition of chlorophyll synthesis in pesticide treated cells. However, growth rate was less than 50% in the maximum concentrations of pesticides. Inhibition of chlorophyll synthesis by pesticides in *Anabaena* sp. has been reported by Chinnaswamy and Patel [20] and Nirmal Kumar [21].

Both the tested pesticide affected total carotenoids of *Anabaena fertilissima, Aulosira fertilissima* and *W. prolifica*. The content of these accessory pigments was decreased after 16 days, mainly with the highest pesticide concentration. Galhano *et al.,* [3] verified adverse effect of Molinate on carotenoids. The concentration dependent reduction in phycobilin pigment content may be due to inhibition caused by 2,4-D and Pencycuron. The remarkable depletion of phycobilin pigment content with progress of time in cultures treated with pesticide was probably due to a disturbance in lipid bilayer integrity at thylakoid membranes [3]. A decline in phycobilin pigment content due to adaptation to high levels of thiobencarb had also been reported in *N. sphaeroides* cells [22].

Literatures are scanty on the effect of pesticides, particularly of fungicide, on carbohydrate metabolism in cyanobacteria. Pesticides may generally decrease or increase the sugar content of treated organisms depending on type of the organism, age, treated part, duration of contact time and type of chemical used [23]. The data herein obtained further reveal that total carbohydrate content of all three strains decreased significantly when cultures were exposed to the highest pesticide concentration. These observations are in agreement with the findings of Kobbia *et al.,* [24] for simazine. Based on the inhibitory effect and growth arrest, the release of certain products, like amino acid and proteins were also affected at the earlier stage itself, which is in agreement with a study of Standyk *et al.,* [25]. The release of phenols in the presence of different concentration of phenylurea fungicide was higher than untreated cultures which could be due to accumulation of phenolic compounds of the fungicide. Nirmal Kumar and Rita Kumar [26] also substantiated the findings that phenols could act as protectants by the organisms under stress or drought conditions.

The results revealed that there is inhibition in activity of NR which is also in confirmation with the results of Mallick and Ria [27]. Moreover, GS activity was found to decline in response to all treatment of both pesticides. The suppression of GS activity in herbicide-treated culture of *Anabaena fertilissima*, *Aulosira fertilissima* and *W. prolifica* may be related to the suppressed NR activity under similar conditions. In *Gloeocaspa* sp., GS was reported to be more active under the nitrogen-fixing conditions [28] and the activation of GS gene (gln A) requires the nitrogen fixing conditions [29]. The decrease of SDH activity observed with both pesticides after 16 days of exposure might due to pesticide toxicity. Similarly, inhibition of SDH due to fungitoxicity toward *Rhizoctonia solani* (Kuhn) was reported by Phillips and Rejda-Heath [30].

In conclusion, it appears that all three strains of cyanobacteria in general and *A. fertilissima* in particular, do not resist to very high concentrations of 2,4-D (herbicide) and Pencycuron (fungicide). However, the effect of pesticide on the populations of nitrogen-fixing cyanobacteria in rice fields also depends on other pesticide concentrations and the water regimes of flooding or non-flooding associated with paddy rice fields [31]. Consequently, for a more thorough understanding of the environmental impact of pesticide on natural nitrogen-fixing cyanobacteria, more detailed field studies are needed. Nonetheless, avoiding the use of extremely toxic herbicide 2,4-D ethyl ester in rice fields, as identified in this study, is still advisable until a better understanding is available.

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