RESEARCH PAPER:

Chronic toxicity of chlorophenoxy herbicide on growth, metabolites and enzymatic activities of *Anabaena fertilissima* Rao

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SUMMARY

Study was carried out to investigate the chronic response of Cyanobacterium, *Anabaena fertilissima* to chlorophenoxy herbicide 2,4-Dichlorophynoxyacetic Acid (2,4-D) ethyl ester at different concentrations 15, 30 and 60ppm. The influence of 2,4-D on growth (pigments), release of metabolites such as carbohydrates, protein, amino acid, phenols and nitrate reductase and glutamine synthetase activities was analyzed. The test concentrations caused a concentration dependent decrease in pigments. Depletion in carbohydrate and protein content was registered with rise in herbicide concentrations. However, phenols were found to rise with increased herbicide concentrations but amino acids were reported to decline. The inhibition of nitrate reductase and glutamine synthetase activity was also concentration dependent and showed more sensitivity for substituted phenoxy herbicide. This study therefore suggests that decrease in metabolite content and enzyme activity can be used as a signal of herbicide toxicity in Cyanobacteria

Key words : *Anabaena fertilissima*, Chronic response, Glutamine synthetase, Growth, Inhibition, Metabolites,

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Herbicides are produced to kill or injure plants and therefore affect various mechanisms associated with photosynthesis, respiration, growth, cell and nucleus division or synthesis of proteins, carotenoids or lipids (Ecobichon, 1991). 2,4-D is the most widely used and extensively studied herbicide (Marie *et al.*, 2001) and is generally used to control excess growth of weeds in aquatic habitat (Das and Singh, 1977). The application of herbicides in crop fields for selective control of herbs in the modern age has led to serious environmental contamination resulting in greater loss of crop productivity and growth of many beneficial micro-organisms like Cyanobacteria (Shetty *et al.*, 2000). For better exploitation of Cyanobacteria as biofertiliser, it is indispensable to select tolerant strains along with understanding of their tolerance (Kumar *et al.*, 2008).

Kobbia and El-Sharouny (2007) employed Cyanobacteria- *Nostoc muscorum*, *Tolypothrix lanata* and *Aulosira laxa* to assess different responses against 2,4-D herbicide at all concentrations. Rana and Nirmal Kumar (1995) have also made some observations of the effect of the herbicide N-(4-isopropylphenyl)-N, N-dimethyl urea on the aquatic organisms. Perhaps, it is evident that many pesticides at the recommended field application have none or accelerating effect on growth of Cyanobacteria but may affect various physiological processes like nitrogenase activity, photosynthesis, carbon fixation and enzymes of assimilatory nitrate reduction and ammonia assimilation in cyanobacteria (Nagpal and Goyal, 1992). In this perception, an investigation was carried out to elucidate the effect of commercial chlorophenoxy herbicide, 2,4 dichlorophenoxyacetic acid (2,4-D) ethyl ester at different doses like 15, 30 and 60 ppm in response to metabolites like proteins, carbohydrates, amino acids, phenolic compounds and enzymes nitrate reductase and glutamine synthetase for every four days up to sixteen days.

MATERIALS AND METHODS

Pure cultures of *Anabaena fertilissima*, a heterocystous form was obtained from culture collection of NFF blue-green algae, IARI, New Delhi, and grown photoautotrophically in BG11 medium under controlled illumination of 800 lux light for 14:10 hours photo and dark period per day at 25 ± 2^{0}C. *A. fertilissima* was maintained in nitrogen free BG11 medium and was subjected to different concentrations of chlorophenoxy herbicide 2, 4-D response. To analyze the effect of different concentrations of herbicide on growth, the experimental medium was prepared with 15, 30 and 60 ppm of 2,4-D (finalized the dose on the basis of LC50 values tested for different concentrations). Stock solutions of test herbicide were prepared in sterilized double-distilled and added to the culture medium to obtain their respective concentrations. Exponentially growing 2ml homogenous culture was inoculated and made upto 20 ml with and without herbicide (as control) constant.

Samples were taken after every four days up to sixteen days for the determination of pigments, metabolites, and enzyme activity. The pigments included were chlorophyll *a* (Jeffrey and Humphrey, 1975), carotenoids (Parsons andmStrickland, 1963) and phycobilin pigments (Bennet and Bogorad, 1973). The changes in metabolites content like total carbohydrates (Hedge and Hofreitte, 1991), proteins (Lowry *et al*., 1951), phenols (Malick and Singh, 1980) and amino acids (Lee and Takahashi, 1966) of *A. fertilissima* exposed to 2,4-D were measured. Nitrate reductase activity *in vivo* was estimated by the method of Sempruch *et al*. (2008) while Glutamine synthetase enzyme was extracted in Tris HCl buffer (pH 7.5) and estimated by slight modification of the method described by Yuan *et al*. (2001).

RESULTS AND DISCUSSION

The results obtained from the present investigation are summarized below :

Growth and pigments:

Growth in terms of Chlorophyll-*a* of the *A. fertilissima* was adversely affected by 2,4-D (2,4 dichlorophenoxy acetic acid) at higher concentrations. The minimum growth of 0.13 $\mu g\ ml^{-20}$ was recorded at 60 ppm followed by 0.21 $\mu g\ ml^{-20}$ at 30 ppm and 0.43 $\mu g\ ml^{-20}$ at 15 ppm after interval of 16 days. However, *A. fertilissima* showed maximum growth of 0.55 $\mu g\ ml^{-20}$ on the 16[th] day in the control cells. Similarly reduction in growth pattern was reported by Jianrong (2005) in *Nostoc sphaeroides* when treated with herbicide thiobencarb. Kapoor and Sharma (1980) exposed cultures of the nitrogen-fixing, filamentous Cyanobacterium *Anabaena doliolum* to 2,4-D ethyl ester (as 'Weedone 48' concentrate) at concentrations of 36, 108, 180, 252 and 324 mg/litre.

Effect of the herbicide 2,4-D on various pigments of *A.fertilissima* is represented in Fig. 1 (a) to (e). It was observed that chlorophyll *a* content decreased with increased concentrations as the time progressed; a total decrease of 21%, 62%, and 76% was observed at 15, 30, and 60 ppm, respectively. After 16 days of treatment a low concentration (15 ppm) of herbicide reduced

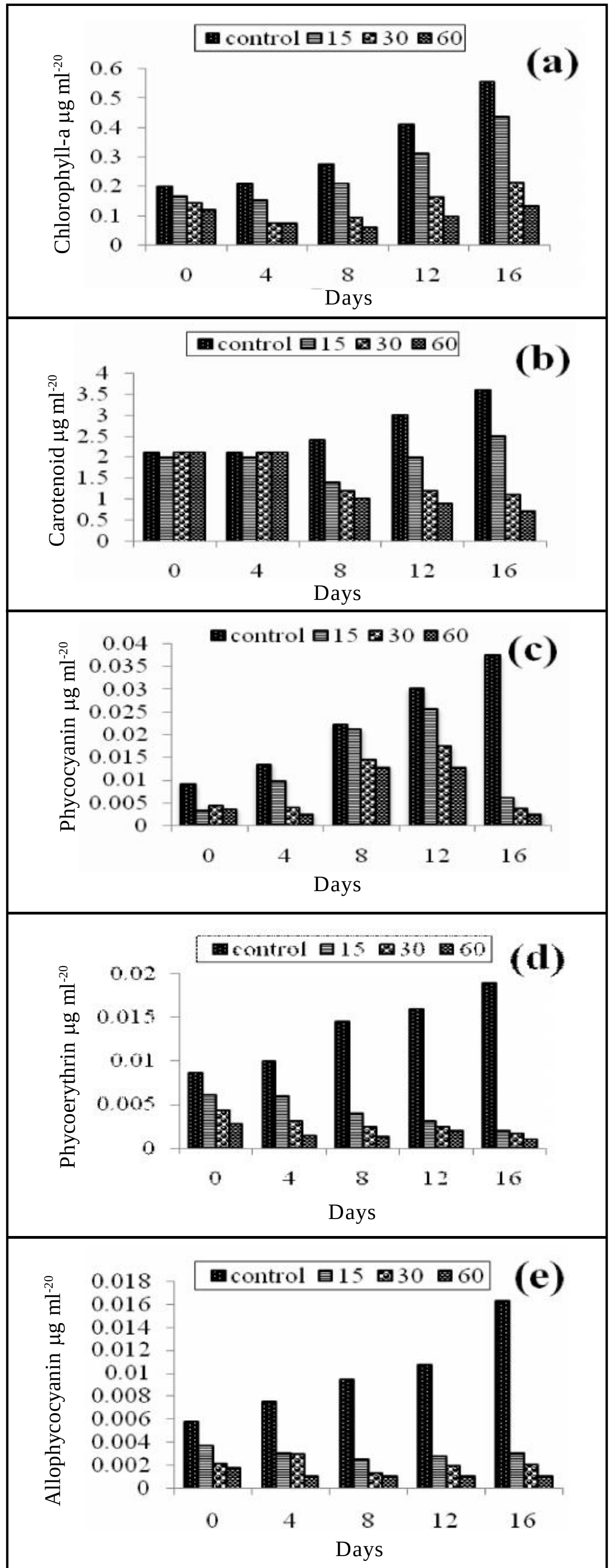

Fig. 1 (a – e) : Effect of different concentrations of 2,4-D on different pigments of *A. fertilissima*

chlorophyll-*a*, carotenoid, phycocyanin, phycoerythrin and allophycocyanin contents by 21%, 30%, 83%, 81% and 89%, respectively. The declining trend in the pigment content continued with the rising concentration of herbicide at 60 ppm of 2,4-D sharply lowered chlorophyll *a*, carotenoid, phycocyanin, phycoerythrin, and allophycocyanin contents by 76%, 80%, 93%, 95% and 93 %, respectively after 16 days. The test herbicide dose caused a concentration-dependent decrease in pigment contents. The inhibition of pigments followed the trend: carotenoids > chlorophyll > allophycocyanin > phycoerythrin > phycocyanin. Laval- Martin *et al*. (1977) found a significant loss of chlorophyll and disorganization of chloroplasts in *Euglena gracilis* by diuron. Probably this could be the reason in the present study that the herbicide disintegrate and destroy the chlorophyll molecule of the test Cyanobacteria.

Phycocyanins are major reserves for nitrogen (Cohen-Bazire and Bryant, 1982) in Cyanobacteria. The level of phycocyanin declined markedly with increasing concentrations of 2,4-D. Such a decline in phycocyanin level might be attributed to its possible degradation, thus leading to nitrogen starvation (Allen and Smith, 1969). Decline in pigment contents may be due to lysis of the cell-wall and disruption of the thylakoid membrane as known for *A. flos-aquae* (Rai *et al*., 1989). Phycocyanin, phycoerythrin, and allophycocyanin located in phycobilisomes are the main accessory pigments in cyanobacteria (Jianrong, 2005). It is quite clear from our observations that the test herbicide 2,4-D affected the synthesis of phycocyanin, phycoerythrin, and allophycocyanin pigments in *A.fertilissima*. Thus, the study supports the earlier findings of Jianrong (2005) where significant decrease in phycoerythrin, phycocyanin and allophycocyanin content was noticed in *Nostoc sphaeroides* as a result of thiobencarb toxicity.

Carbohydrates:

There was a decrease in carbohydrate content when the cultures were treated with 15, 30 and 60 ppm of 2,4-D. The highest amount of carbohydrate content was found in untreated cultures (0.34 mg ml^{-20}) followed by 15 ppm (0.30 mg ml^{-20}) and 30 ppm (0.25 mg ml^{-20}) of 2,4-D treatment, whereas, lowest amount of carbohydrate content (0.06 mg ml^{-20}) was observed at 60ppm (Fig. 2a). The gradual fall of carbohydrate concentration was registered by 13%, 27% and 81% at 15, 30 and 60ppm, respectively after duration of 16 days. The higher concentrations of 2,4-D retarded the carbohydrate contents of *A. fertilissima* could be due to conversion of sugars into other metabolites which corroborated the findings of Chai and Chung (1975) that interaction of 2,4-D suppressed the carbohydrate content of *Chlorella ellipsoidea*.

Proteins:

The protein content increased in all the treatments as time progressed but the values were lower when compared with control (Fig. 2b). The reduction was observed in protein content after 16 days treatment by 14%, 46% and 57% in 15, 30 and 60 ppm, respectively. The protein content of treated *A. fertilissima* depleted with the progress of time, could be possibly due to inhibition of enzymes and structural protein essential for the growth of Cyanobacteria. Battah *et al*. (2001) recorded reduction in protein content in *A. variabilis* when treated with a dose of 3 mg L^{-1} thiobencarb.

Amino acids:

It was observed that the amino acid content decreased with increased concentrations of herbicide. As the time progressed, a total decrease of 39%, 44%, and 72% in 15, 30 and 60 ppm, respectively was recorded after 16 days. At the end of 16 days treatment, the untreated culture showed higher amino acid content (0.02mg ml^{-20}) than that of the treated cultures (Fig. 2c). Like proteins, amino acid concentration was sharply reduced with increasing concentrations of 2,4-D. Similar observations were recorded by Nirmal Kumar *et al.* (2008), on treatment of *Tolypothrix tenuis* with fertilizer industrial effluents.

Phenols:

An increase in the phenol content of *A. fertilissima* was noticed in applied treatments when compared to control (Fig. 2d). Highest amount of phenols was recorded in 60 ppm (0.04 mg ml^{-20}) after 16 days treatment, while the lowest was observed in control (0.03 mg ml^{-20}). Phenols are important aromatic metabolites formed during stress conditions which trigger the various biochemical processes of the organisms. Initially, phenol content was noticed to increase at higher concentration as compared to control could be due to the chemical nature and composition of treated phenoxy herbicide. Mann *et al*. (1965) and Nirmal kumar and Rita Kumar (2002) also substantiate the findings that phenols could be act as protectants by the organisms under stress or drought conditions.

Nitrate reductase:

The nitrate reductase activity in cultures treated with 2,4-D was substantially inhibited and the degree of

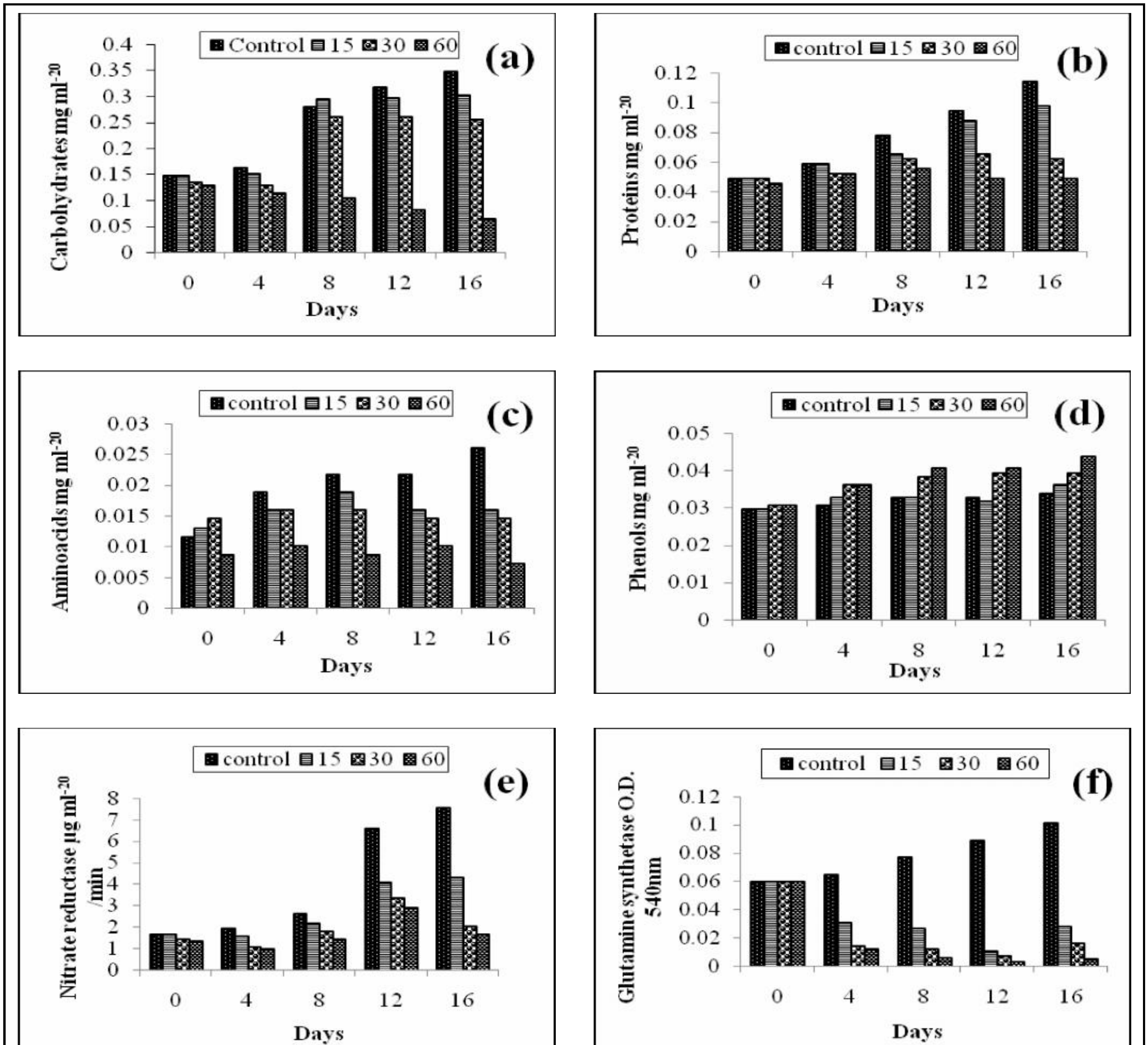

Fig. 2 (a – f) : Effect of different concentrations of 2,4-D on various metabolites and enzymes activity of *A. fertilissima*

inhibition increased with increase in concentration and time (Fig. 2e). The 2,4-D toxicity on nitrate reductase was observed maximum at 60 ppm followed by 30 and 15 ppm. The retardation of nitrate reductase activity was 43%, 73%, and 78% at 15, 30, and 60 ppm concentration of 2,4-D, respectively after 16 days treatment. Experiments on NR activity revealed that different concentrations of 2,4-D inhibited the synthesis of NR. The displacement of an essential metal ion forming the central and functional part of the enzyme protein may be one of the reasons for the inhibition of nitrate reductase and secondly, interference with sulphydryl (-SH) groups which often determine the secondary and tertiary structure of proteins (Awasthi, 2005).

Glutamine synthetase:

Inhibition of glutamine synthetase activity was also concentration-dependent (Fig. 2f) with maximum toxicity of 2,4-D recorded at 60 ppm (95% reduction) followed by 30 ppm (84%) and 15 ppm (72%). Like other parameters, glutamine synthetase also displayed highest sensitivity for 2,4-D at 60 ppm. The effect of 2,4-D on *A. fertilissima* exhibited reduction in glutamine synthetase with progress of time and an increase in concentrations. The inhibition of glutamine synthetase can be attributed either to exhaustion of energy-yielding substrates or direct inactivation of enzyme complexes (Rai *et al.*, 1990).

Conclusion:

From the present investigation, it is clearly evident that the growth and metabolic activities of *A. fertilissima* is adversely affected by the herbicide 2,4-D ethyl ester, commonly used in agriculture. Based on the inhibitory effect and growth arrest, the release of certain products, like carbohydrates, proteins, amino acids and phenols were also affected at 15, 30 and 60 ppm of 2,4-D, even at an earlier stage of treatment. Experiments on enzyme activity revealed that 2,4-D inhibits the synthesis of nitrate reductase and glutamine synthetase. On the basis of the present observations, it can be stated that the application of this herbicide to rice fields should be considered because of its toxicity to the heterocystous filamentous Cyanobacteria.

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