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Study of herbicidal effect 0f 2,4-D on growth and cellular metabolites in cyanobacterium *Synechococcus aeruginosus* Nägeli from rice fields

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ABSTRACT

The effect of the herbicide 2,4-D on growth and cellular metabolites in blue-green alga *Synechococcus aeruginosus* was determined. The herbicide is the mostly used in rice-fields of Andhra Pradesh, India. The algal growth and cellular metabolites synthesis was stimulated by the presence at low concentrations of 200 µg/ml of 2,4-D. The growth of the algae was gradually decreased with the increased concentrations of herbicide at 500, 800 and 1000 µg/ml within the medium, whereas the presence of 1100 µg/ml 2,4-D has completely inhibited algal growth and cellular metabolites synthesis. The above observation has indicated that, 2,4-D at lower doses acts as growth hormone/auxin and promotes the luxuriant growth of the algae, while the higher doses of the same herbicide shows the detrimental effect on growth. The present study indicated that as the use of lower concentrations of the herbicide 2,4-D has significantly increased the growth rate and is same as imperative in the changes observed in the biochemical metabolites.

INTRODUCTION

Indiscriminate employment of pesticides on crops to control pests cause environmental hazards and imbalance of microorganisms in aquatic and terrestrial ecosystems comprising of inhabitated biological components viz. algae, fungi, protozoa and bacteria. Water and soils of rice-fields would very often be heavily contaminated with pesticides by one or another means of agricultural practices (Venkataraman, 1975). Pesticides, in general, may be inhibitory, stimulatory or neutral depending on the nature of the chemical, its concentration and the algal strain (Roger & Kulasooriya, 1980; Reddy et al,1999). The pesticides are selective in their action against these pests or the microorganisms inhabitating in the soil or water and thereby change the population of microorganisms, their growth and survival either blocking the pertinent metabolic pathways or utilized as co-metabolites. The survival of the organisms depends upon the concentration of the pesticide and their persistence besides the physiological state of the microorganisms.

The rice-fields of coastal Andhra Pradesh are sometimes heavily dominated with weeds viz. *Lippia nodiflora, Merremie* Hallier F. at times, which finally cause reduced rice yield (Tadulingam and Venkatanarayana, 1955). 2, 4-Dichlorophenoxyacetic acid (2,4-D) referred to as synthetic auxin, is a common selective systemic herbicide generally used in rice- fields and cereals to arrest a number of broad leaf weeds (Brian,1964). It has been known to affect non- target beneficial organisms (Godsby et al., 1997) primarily diazotrophic cyanobacteria. The herbicidal activity is mediated by an auxin – like capacity to alter the normal protein synthesis and cell division (Stevens and Breckenridge, 2001). While at low concentrations 2,4-D acts as an auxin analogue promoting plant growth, at high concentrations it is lethal and used as a herbicide against broad leafed plants (Mullison, 1987).

Cyanobacteria also known as blue-green algae, are a diverse group of gram negative photosynthetic prokaryotes, which perform two biological processes such as oxygenic photosynthesis and nitrogen fixation together in the same cells or filaments and enrich the paddy soil with nitrogen and humus. The role of blue-green algae in the nitrogen economy of paddy fields of tropical countries like India and other far east countries (Watanabe et al., 1951) has been well documented. The results obtained at field level in rice crop are evident from the fact that the nitrogen fixation and its transfer is carried out by blue-green algae. They can be incorporated into soil as organic matter and also as a source of enzymes as they produce acid and alkaline phosphatases that are active in solution or in the periplasmic space of the cell wall. Both biomass and extra cellular Polymeric Substances (EPS) incorporated into soil, induced a growth promotion of other microorganisms and increased the activity of soil enzymes that participate in the liberation of nutrients required by plants (Caire et al., 1997).

In view of the beneficial functions of blue-green algae in agriculture, despite the indiscriminate use of the herbicides by the farmers to eradicate pests and weeds growing in the fields, it has been necessitated to undertake studies of herbicides on the growth, survival and biochemical changes of these algae. The influence of 2, 4-D on growth and nitrogen fixation (Singh, 1974) of blue-green algae has been reported earlier. In the present study unicellular, non-heterocystous, microalgae *Synechococcus aeruginosus,* found abundantly in rice- fields have been selected to investigate the effect of 2,4-D. The *in vitro* study focus to get an insight of the dose effect on the process of growth and metabolisms in *Synechococcus aeruginosus* .

MATERIAL AND METHODS

Synechococcus aeruginosus was isolated from the rice-fields of Andhra Pradesh to study the toxic effects of 2, 4-D on the growth and biochemical changes. The isolated samples were cultured in Chu 10 basal medium and the log phase cultures were used as inoculum in the subsequent experiment. The experiments were performed in liquid basal media as well as on agar media and cultures were incubated under continuous illumination with fluorescent tubes (1400 lux) at room temperature (27+-2 C). The herbicide stock solution was added to the media to give a final concentration of 200, 500, 800,1000 & 1100 µg/ml. Activity of 2, 4-D as a hormone and or herbicide is investigated in terms of growth and biochemical metabolite levels present in the culture subjected to 2, 4-D stress.

The growth pattern was studied in terms of a growth curve on the agar plates supplemented with varied doses of 2,4-D. Cellular metabolites were measured in liquid medium. The growth was measured in terms of optical density of acetone soluble pigments at 660nm, total protein and also algal cell counting with Neubauer's haemocytometer for a period of 30 days. The sensitivity of *Synechococcus aeruginosus* was tested on cellular metabolites in basal liquid medium containing different concentrations of 2, 4-D and the amount of active metabolites viz. Chlorophyll-a, carbohydrates, proteins, RNA, DNA, C-phycocyanin, allophycocyanin, alpha-amylase and acid phosphotase activity were estimated after 15 days.

Active cellular metabolites were extracted by standard biochemical procedures and estimated by respective assay methods. Acetone soluble pigments were extracted from equal volumes of aliquots by suspending the pellets in 80% of acetone. Following the formula of Mac Lachlan and Zalik (1963) as mentioned by Holden (1976) estimation of Chlorophyll-a was done. Water soluble pigments C-phycocyanin and allophycocyanin were extracted in phosphate buffer and estimated by the method of Siegelman and Kycie (1978). Protein estimation was done by Lowry et al., (1951) method. The anthrone reagent method (Spiro, 1966) was used for carbohydrates estimation. Nucleic acids were extracted using Herbert et al., (1971) method. RNA was estimated by orcinol reagent as mentioned by Elliot and Wald (1974) While DNA estimation was done in terms of phosphate by the method described by Fiske and Subba Row (1925). Apha amylase as well as acid phosphotase enzyme activity was estimated by Oser (1965) and Bernfeld (1955) & Patni and Aaronson (1978) methods. The spectrophotometric method was used for colorometric estimation of respective components in each method.

RESULTS

Effects of increasing 2,4-D concentrations on cyanobacterial growth:

The results recorded in the present investigation showed that the growth pattern of algae in control as well as 2,4-D treated cultures (200, 500, 800, 1000 and 1100 µg per ml 2, 4-D) showed a lag phase followed by the exponential phase up to 18th day and declined thereafter as shown in Fig 1. Both control and 2, 4-D treated cultures showed triphasic growth except at 1000 µg per ml concentration. The growth of the algae was gradually inhibited by the increased concentrations of the 2,4-D in the medium. The lower

concentration of 200 µg 2, 4-D per ml in basal medium promoted the growth of the algae without lag phase when compared to the control, implying its role as a hormone. While 500 µg per ml concentration proves to be a growth retardant when compared to 200 µg per ml dose cultures and the exponential phase in the culture was continued up to 20th day and later became static, it means that at 500 µg per ml effect of 2, 4-D as a herbicide begins. The herbicide effect is very prominent at 800 µg per ml dose. The growth of the algae was gradually inhibited by the increased concentrations of 2, 4-D in the medium until 1000 µg per ml. At 1100 µg per ml 2, 4-D concentration, the growth of the alga is completely inhibited.

Effects on cellular metabolites:

In the long term experiments, after fifteen days of incubation period, the sensitivity of algae was tested in liquid medium to find out the adverse effects of various concentrations of 2, 4-D (200, 500, 800 and 1000 µg per ml) on cellular metabolites of *Synechococcus aeruginosus* i.e. chlorophyll-a, carbohydrates, proteins, RNA, DNA, C-phycocyanin, allophycocyanin, activity of α-amylase and acid phosphatase . Results of experiments revealed that, as compared to control, the cellular metabolites were proportionately decreased with the increase of concentrations of 2, 4-D, except at 200 µg per ml, which was proved as growth promoter(Table 1). The concentration at 1000 µg 2, 4-D per ml caused effective decline in the quantity of chlorophyll-a, carbohydrates, proteins, RNA, DNA, C-phycocyanin, allophycocyanin. Activity of α -amylase and acid - phosphatase showed a significant decline when compared to control. Among all cellular metabolites, the levels of chlorophyll-a, proteins, RNA and C-phycocyanin appeared to be remarkably affected at 1000 µg 2, 4-D per ml. A drastic reduction in nucleic acid implies that, 2,4-D has an impact on transcription and simultaneously effects the genome of algae. Rise in 2, 4-D concentration i.e. at 500 , 800 and 1000 µg per ml, results in a progressive decline in the quantities of all the metabolites. The amount of DNA reduction is in accordance with the amount of RNA reduction and the same is reflected in growth that finally leads to death of the algal cells. In our experiments , we found that the effect of 2,4-D at lower doses i.e.200 µg per ml acts as auxin and promoted the growth both on agar medium and liquid medium. However, higher doses decreases the growth and quantities of the cellular metabolites. The toxic tolerance level of the algae (1000 µg per ml) was not changed both on agar and liquid medium .

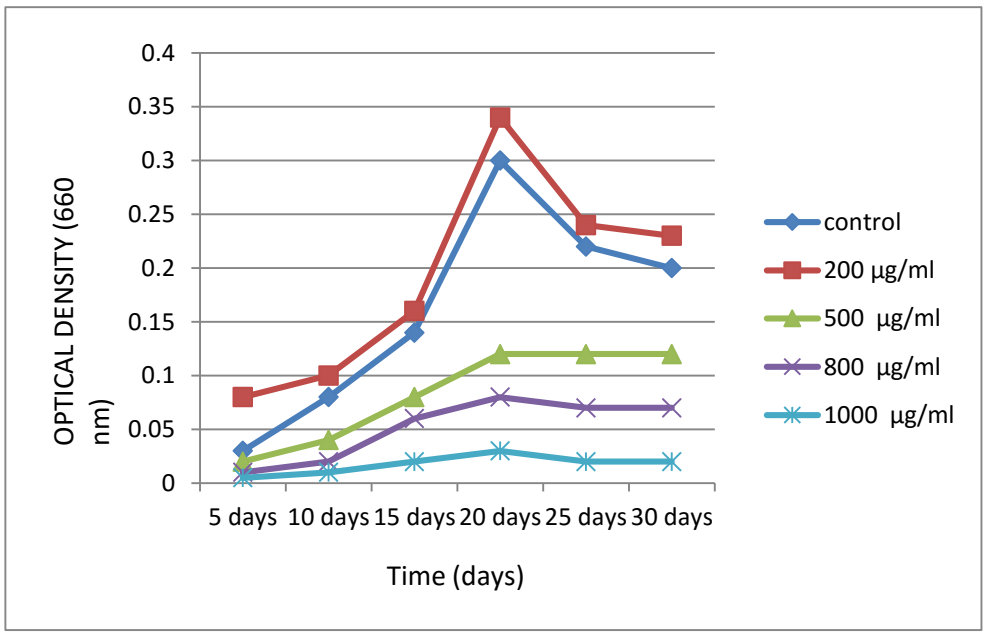

Fig 1: Time – course Growth of *Synechococcus aeruginosus* in basal medium supplemented with graded concentration of 2,4- D

Table – 1: Long-term effect of 2, 4-D on cellular metabolites in *Synechococcus aeruginosus*

Concentrations	Chloro phyll-a (mg/g f.w.	Carbo-hydrates (mg/100 mg f.w.)	Proteins (mg/100 mg. f.w.)	RNA (µg/100 mg f.w.)	DNA (µg phosphate/ 100 mg.f.w.)	C-phyco-cyanin (mg/ml)	Allo- Phyco-cyanin (mg/ml)	α-amylase (µg/ml maltose released in 15 min./100 µg protein)	Acid phosphatase (µg/ml PnP released in 30 min./100 µg protein)
Basal medium (control)	0.4218	0.963	0.085	125.7	29.30	0.27	0.0042	79.14	11.01
Basal medium + 200 µg/ml 2, 4-D	0.5380	1.320	0.093	91.0	22.86	0.22	0.0035	75.19	7.34
	(+21.59)	**(+27.04)**	**(+8.60)**	**(-27.60)**	**(-21.97)**	**(-18.51)**	**(16.66)**	(-4.99)	(-33.33)
Basal medium + 500 µg/ml 2, 4-D	0.1173	0.735	0.009	63.60	12.81	0.13	0.0027	50.00	4.28
Basal medium + 800 µg/ml 2, 4-D	0.0516	0.556	0.004	34.5	10.92	0.004	0.0038	32.29	3.57
Basal medium + 1000 µg/ml 2, 4-D	0.0139	0.445	0.002	10.1	8.75	0.001	0.0018	22.29	1.75
	(-96.70)	**(-53.79)**	**(-97.64)**	**(-91.96)**	**(-70.13)**	**(-99.62)**	**(-57.14)**	**(-71.83)**	**(-84.10)**

f.w. = fresh weight; PnP = P-nitrophenol; 2, 4-D = 2, Dichlorophenoxyacetic acid; Parentheses show the percentage of promotion (+) or inhibition (-) over control (Basal medium)

DISCUSSION

Among the pesticides, herbicides are the most widely used class of pesticides in agriculture (Zimdahl, 2002) and have been found to have varied effects in various organisms (Metting, 1981). Studies on deleterious effects of herbicide on growth and metabolism of cyanobacteria are very few (Chinnaswamy and Patel, 1984) . Understanding the mode of action of herbicides has been an important tool in research to improve application methods in various agricultural practices, handle weed resistance problems and explore toxicological properties. For identifying the mechanism of action of herbicide, there was a need to investigate and identify the sites, where the herbicides exert their action either at cellular level or molecular level. 2,4-D has been designated as a powerful herbicide of hormonal type , which is generally used in rice- fields and cereals to arrest a number of broad leaf weeds (Brians, 1964), has been known to effect non- target beneficial organism (Godsby et al., 1997) primarily diazotrophic cyanobacteria. In the present study *Synechococcus aeruginosus,* a microalgal species found abundantly in rice- fields have been selected to investigate the effect of 2,4-D. The *in vitro* study of the dose effect in the present investigation gives an insight into the changes in the process of growth and metabolism in *Synechococcus aeruginosus .*

Auxin promotes the growth by DNA synthesis and cell division as suggested by Skoog, 1954, Schroeder et al. 1967). Based on the results, lower concentrations of 2, 4-D i.e. 200 µg/ml as shown in Fig 1 stimulates the growth by enhancing the chlorophyll-a, protein, carbohydrates as compared to control and functions as auxins (Dodge, 1975). At this dose, herbicide 2,4-D attaches to cell membrane and alters the nutrient flow to direct enzyme stimulation which leads to synthesis of photosynthetic pigments (table- 1) (Rivera and Penner, 1979).

A receptor for auxin was identified as the F-box protein TIR1 (transport inhibitor response 1) is a component of cellular protein complex (SCFTIR1) (Tan et al., 2007) which recognizes synthetic auxin analogues such as 1-naphthalene acetic acid (1-NAA) and 2,4-dichlorophenoxiacetic acid (2,4-D). With the help of an amide bond, amino acids swiftly conjugate with 2, 4-D (Sidler,1994) and the resultant conjugates functions as auxins as shown in Fig. 1 (Feung et al.,1974). Pathways of protein turn over and activation of various amino acid synthetic pathways, as a part of auxin function is evident (Istvan Jablonkai,2011).

Higher concentration of 2,4-D i.e 500,.800 µg per ml were proved as the inhibitory range to *Synechococcus aeruginosus*. While the concentration of 1000 µg per ml 2,4-D , proves to be lethal dose and toxic to *Synechococcus aeruginosus* cells, resulting in decline in growth (Klotz and Duysen, 1972). The inhibition in growth (Fig 1) might be attributed to toxic effect of 2,4-D. The toxic action on growth and photosynthesis is evident in various studies (Leganes and Femandez-Valiente, 1992, k. Bhagya Lakshmi Jyothi,2013). As suggested by Moreland (1967) at higher concentrations ,2,4-D effected not only the cell wall but also at multiple sites inside the cell. As soon as the bio-concentration of pesticides builds up inside the plant cell or an organism as reported in *Scenedesmus qaudricaude* by Valentine and Bingham(1974) develop additional sites of pesticides which may be prone to involve and affect the various metabolic pathways and finally cause the damage to the cell and organism's death as evidenced from the literature as well as confirmed by our experimental results.

CONCLUSION

Synechococcus aeruginosus have varying tolerance potential to various doses of 2,4-Dstresses. During present investigation we have attempted to investigate the growth pattern changes and biochemical metabolites in this unicellular cyanobacteria. . We found that though the stresses caused lethal effects but cyanobacteria survived in that stressful conditions. As indicated from the obtained results that both, absorption spectra or pigments concentration, can also be used for a quantification of viable cells. Hyperaccumulation and tolerance are genetically inherited traits; this in turn would provide some valuable insight into the role of cyanobacteria in the microbial community as well as their potential impact on biofertilizer role in herbicide contaminated soil. Also, these strains can be used as axenic culture to biodetoxify the contaminated soil and at the same time provide natural fertilizer role. This study if extended by evaluating biotic and abiotic factors stress, would in future serve as an ecological tool to manage chemicals used in modern agriculture with least impact on environment.

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