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Biological Effects of Rice-Field Herbicide Stam F-34 on the Nitrogen-Fixing Cyanobacterium *Anabaena doliolum*

SURENDRA SINGH
Department of Biochemistry, North-Eastern Hill University, Shillong 793 014

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The herbicide, stam F-34 inhibited the growth of the cyanobacterium *Anabaena doliolum* in a concentration dependent manner and 30 μm ml^{-1} was found to be lethal. The toxicity of the herbicide was reversed by the addition of glucose, sodium acetate, and amino acids (arginine, aspartic acid, serine, threonine, and glutamine), even when herbicide was present at lethal dose. The herbicide also inhibited heterocyst differentiation and nitrogen-fixation. Since, toxicity of the herbicide could be reversed by inorganic nitrogen sources (nitrate and ammonium), it is suggested that nitrogen-fixation is sensitive to herbicide.

Key Words : *Anabaena doliolum*; Cyanobacterium; Herbicide; Stam F-34

Introduction

Cyanobacteria are N_2-fixing oxygenic phototrophs with potential as source of nitrogenous biofertilizers independent of fossil fuels. Recently, attention has been paid to their role in this respect, and to strain selection to facilitate enhanced agricultural productivity (Venkataraman 1975). The capacities to fix nitrogen in the presence of combined nitrogen, to resist herbicide and to tolerate salinity changes and dessication are of particular importance. The present day rice cultivation technology requires a high input of synthetic fertilizers besides the accumulating load of pesticides and herbicides in the field (French & Gay 1963). The man made pesticides and herbicides pose the threat of their side effects on the non-target organisms and such effects have not yet been fully understood. Investigations have revealed that stam F-34 may be very useful in increasing the rice yield by selective elimination of weeds from the rice fields (French & Gay 1963, Subrahmanyam et al. 1965, Kobayashi et al. 1968, Park & Park 1971). Since, nitrogen-fixing cyanobacteria are an invariable component of the rice fields and considered best from the environmental contaminations, therefore, they should be thoroughly screened for deleterious effects of herbicides.

The paper reports effects of stam F-34, a commonly used herbicide on the growth, heterocyst differentiation and nitrogen fixation in the cyanobacterium *Anabaena doliolum*.

Materials and Methods

Anabaena doliolum Bharadwaja, a heterocystous nitrogen-fixing cyanobacterium was axenically grown in modified Chu No. 10 medium (Safferman & Morris 1964) at $24 \pm 1°C$ and illuminated with day-light fluorescent tubes (photon flux 50 $\mu E.\ m^{-2}.\ s^{-1}$ on the surface of the vessels) for 14 hr/day. The nitrogen free medium was obtained by replacing calcium nitrate with calcium chloride (0.007 M). For obtaining different inorganic

nitrogen supplemented media, nitrate as calcium nitrate (1mM) and ammonium as ammonium chloride (0.05mM) were used. Glucose and sodium acetate (500 μg ml,$^{-1}$ each) and amino acids (arginine, aspartic acid, serine, threonine and glutamine) (25μg ml^{-1} each) were supplemented in the medium after filter sterlization. Growth of the cyanobacterium was estimated by following changes in chlorophyll content per ml and expressed in terms of specific growth rate (*K*). Specific growth rate (*K*) was computed by the method of Guillard (1973). Chlorophyll *a* content was measured by using the method of Mackinney (1941).

Cyanobacterium grown in ammonium medium, where the heterocyst frequency was less than 0.01%, was used for heterocyst differentiation studies. Heterocyst differentiation was initiated by transferring the washed cyanobacterial suspension into basal medium. The number of heterocysts was estimated microscopically following incubation for 96 hr and their frequency (average atleast 30 independent random samples) has been expressed as number of heterocysts per 100 vegetative cells. Nitrogenase activity was estimated using acetylene reduction assay (Stewart et al. 1967). The assays were performed in 13 ml serum vials under an atmosphere of 10% (v/v) acetylene. The reaction mixture contained 5 ml of cyanobacterial suspension and graded concentrations of stam F-34 (0.5 – 30μg ml^{-1}). The vials were illuminated on shaker at 25°C and a photon fluence rate of 50 μE. m^{-2}. s^{-1} Gas samples (1 ml) in triplicate were collected after 1 hr and the amount of ethylene produced was analysed in a Gas Chromatograph (Model Tracor 540).

Propanil, the trade name of commonly used herbicide stam F-34 (3,4-dichloropropionanilide, 35% E.C.) was a product of Bharat Pulverising Mills Ltd., Bombay. Different concentrations (0.5-30 μg ml^{-1}) were prepared by diluting the filter sterilized stock in sterile medium and adding to the culture after inoculation with the cyanobacterium.

Results

Figure 1 shows the effects of inorganic nitrogen sources on the toxicity of the herbicide stam F-34. Growth was expressed in terms of specific growth rate (*K*). Considering the specific growth rate in the presence and absence of herbicide, it was evident that the herbicide affected the growth of *A. doliolum* in a concentration-dependent manner. Higher concentration of stam F-34 completely arrested growth and 30 μg. ml^{-1} proved to be lethal. Since, cultures treated with herbicide exhibited symptoms of nitrogen-starvation (in terms of reduction in protein contents, data not shown), it was of interest to investigate whether combined nitrogen sources protected the cyanobacterium from herbicide toxicity or not. Nitrogen in the form of nitrate readily suppressed the toxicity of stam F-34 and showed more protection than N_2 and ammonia-containing media. Ammonia-containing medium showed an enhanced algicidal effect even at 20 μg. ml^{-1} (figure 1).

Figure 2 shows the toxic effects of stam F-34 on growth of *A. doliolum* in relation to carbon metabolism. The cultures devoid of herbicide grew well with glucose and sodium acetate and did not show any lag.

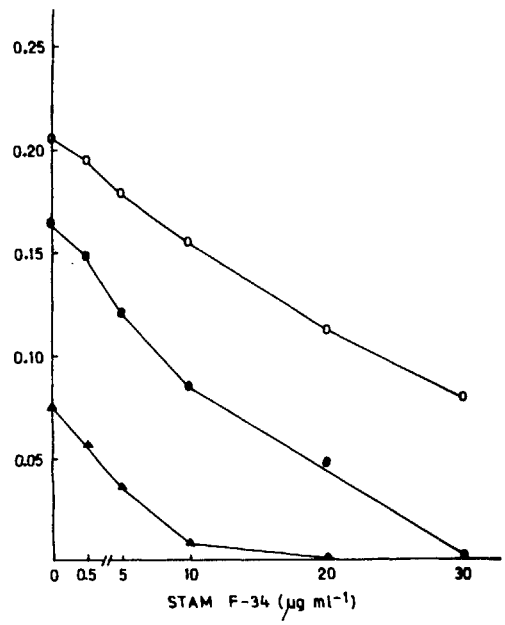

Figure 1 Effect of different concentrations of stam f-34 on growth (in terms of specific growth rate, K) of *Anabaena doliolum* in media supplemented with N_2 (●), NO_3^- (○), NH_4^+ (▲)

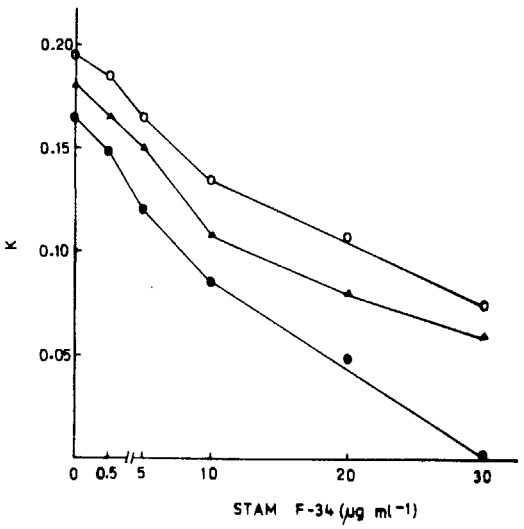

Figure 2 Effect of different concentrations of stam f-34 in the absence (●) and presence of glucose (O) and sodium acetate (▲) on growth (in terms of specific growth rate, K) of *Anabaena doliolum*

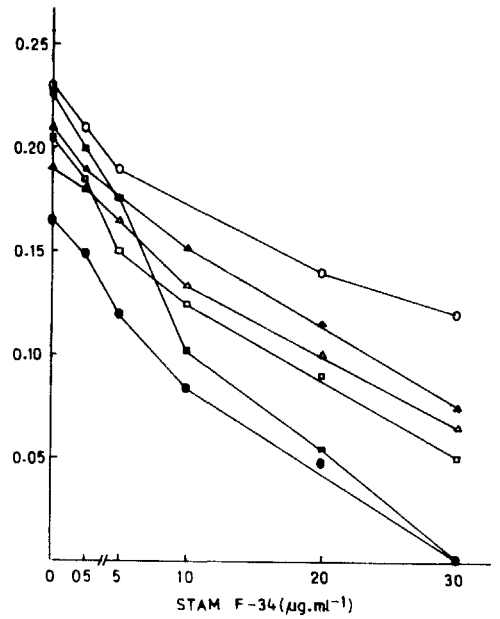

Figure 3 Effect of different concentrations of stam f-34 on growth (in terms of specific growth rate, K) of *Anabaena doliolum* in N_2 (●), and amino acids; arginine (O), aspartic acid (▲), serine (△), threonine (□) and glutamine (■).

Simultanious addition of glucose and sodium acetate alongwith various concentrations of the herbicide protected the cyanobacterium from herbicide toxicity. Both the carbon sources revered the toxicity of herbicide at algicidal dose but no recovery was obtained at higher concentration of stam F-34 (50 $\mu g\ ml^{-1}$, data not shown). Glucose as well as sodium acetate caused a significant reduction in the inhibitory effect of herbicide, therefore, it seems that stam F-34 inhibits the growth of the cyanobacterium by inhibiting the photosynthetic CO_2 assimilation.

Figure 3 shows the role of various amino acids, arginine, aspartic acid, serine, threonine and glutamine in relation to the stam F-34 toxicity to the cyanobacterium in nitrogen-fixing growth conditions. Cultures supplemented with herbicide and amino acids did not show any lag (figure 3). All the amino acids except glutamine overcome the herbicide toxicity even at algicidal doses. Arginine, which serves as a nitrogen reserve in cyanobacteria, showed maximum protection against stam F-34 toxicity and was followed by aspartic acid, serine, threonine and glutamine (figure 3).

Figure 4 shows the stam F-34 induced inhibition of heterocyst differentiation and nitrogenase activity level in the cyanobacterism *A. doliolum*. Heterocyst differentation and nitrogenase activity attained their maximum values of 4.9% and 4.5 nmol $C_2H_4\ \mu g^{-1}$ chl *a* hr^{-1}, respectively when the cells were incubated in the medium devoid of stam F-34. Stam F-34 inhibited the heterocyst differentiation and nitrogenase activity in a concentration dependent manner. At 0.5 $\mu g\ ml^{-1}$ concentration, heterocyst differentiation and nitrogenase activity showed slight inhibition, whereas at higher concentration (30 $\mu g\ ml^{-1}$), heterocyst differentiation and nitrogenase activity both were completely inhibited (figure 4). Microscopic observation revealed that in such cultures detachment of heterocysts from the vegetative cells was a common feature, consequently giving rise to short filaments. Thus, it is quite likely that detach-

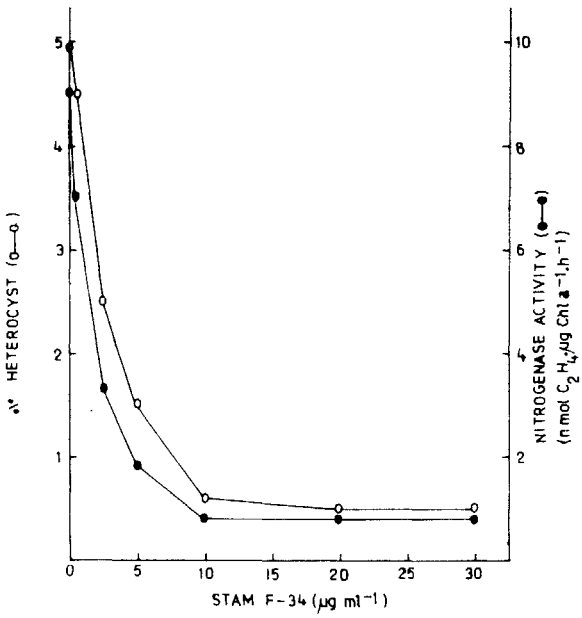

Figure 4 Effect of different concentrations of stam f-34 on heterocyst frequency (○) and nitrogenase activity (●) in *Anabaena doliolum*

ment of differentiated heterocysts could give rise to lowering of heterocyst frequency.

Discussion

The herbicide stam F-34 is structurally releated to DCMU (3 (3,4-dichlorophenyl) 1,1-dimethyl ura). It selectively iradicates weeds by metabolic conversion into DLC (3,4-dichloroacetanilide) by oxidative phosphorylation and subsequent hydrolysis is DCA (3,4-dichloroaniline) and lactic acid (Yin et al. 1968). Growth of the cyanobacterium was inhibited at a very low concentration of the herbicide (0.5 µg ml^{-1}) which is quite low as usually employed in rice fields. Thus, it is quite likely that the accumulating load of herbicide might cause more damage to the cyanobacteria multiplying in the rice fields. A combination of inorganic nitrogen sources (nitrate and ammonium) proved to be more supportive for cyanobacterial growth than elemental nitrogen (N_2). Nitrate reversed the herbicide induced growth inhibition of the cyanobacterium even at the lethal concentration of 30 µg ml^{-1}. This indicates a higher sensitivity of the nitrogen-fixing ability of the cyanobacterium to herbicide stress than metabolic assimilation of combined nitrogen sources. The data obtained with ammonia as nitrogen source showed that it supported the slower growth than other nitrogen sources. Allen and Arnon (1955) reported that ammonium nitrogen being energetically the most favourable nitrogen source often supported poorer growth. The same cyanobacterium *A. doliolum*, had, however, previously been examined by Singh and Srivastava (1968), and the present result on ammonia, supporting poorer growth than nitrate, agree with their observations. Similar results were also observed with *Phormidium persicinum* (Pinter & Provasoli 1958) and with *Calothrix scopulorum* (Stewart 1964). The slow growth rate and poor growth on ammonium has been attributed in part to a rapid selective accumulation of ammonia within the cell and the concomitant drop in the *p*H of the external environment (Fogg et al. 1973). The results with ammonia were in agreement with NaCl. induced DDT toxicity to cyanobacteria (Batterton et al. 1972). An alternative explanation may also be given as the formation of a complex between stam F-34 and ammonium nitrogen which releases more toxic substances on hydrolysis.

The inorganic nitrogen assimilation and inorganic carbon assimilation are reductive processes. The reversal of herbicide toxicity of cyanobacterial growth in the presence of glucose or acetate may be attributed to their action both as carbon source and a reductant source. Photosynthetic assimilation of CO_2 is a reductive process occuring on the basis of photochemically generated reductant during oxygenic photosynthesis. A substance which interferes with the above requirements will inhibit the photosynthetic cyanobacterial growth either by reducing photolysis of water or interferring with the level of electron transport (Moreland 1980). Van Rensen (1982) also showed that more than 50% of all herbicides used, interact with the process of photosynthesis. Most of them are inhibitors of photosystem II dependent electron flow.

The amino acids, arginine, aspartic acid, serine, and threenine protected the cyanobacterium from herbicide toxicity even at algicidal concentration. Glutamine was

the only amino acid which failed to reduce the harbicide toxicity at the concentration of 30μg ml^{-1}. Arginine protected the cyanobacterium from herbicide toxicity maximally since, it serves as a nitrogen reserve in cyanobacteria (Simon 1971). Thus, the herbicide toxicity produced as a result of nitrogen starvation in cyanobacterium may be overcome by exogenous supplementation of arginine. These results suggest that amino acids play a dual role, either by reducing herbicide toxicity by forming biologically unavailable chelators or by altering the transport of resulting herbicide-amino acid complexes. The latter possibility has also been suggested for 2,4-D uptake in higher plants and in cyanobacteria (Andreae & Good 1957 Feung et al. 1973, 1974). An alternative explanation may also be given that stam F-34 was too antagonistic to amino acids or acted as an organic carbon source since, amino acids are usually a poor source of nitrogen for the growth of cyanobacteria (Neilson & Doudoroff 1973).

The herbicide, stam f-34 inhibited the heterocyst differentiation and nitrogenase activity even at a low concentration (0.5μg ml^{-1}). Since, at higher concentrations (30μg ml^{-1}) it inhibited the heterocyst differentiation and nitrogenase activity completely, it is suggested that at physiological level, the herbicide affects nitrogen fixation in cyanobacteria. The heterocysts are regarded as the nitrogen-fixing factories in cyanobacteria. The conversion of N_2 to NH_3 takes place via the enzyme nitrogenase which requires energy for its functioning. The energy (ATP) required for nitrogenase is met by photophosphorylation (Rai & Singh 1982). Such observations suggest that nitrogen fixation and carbon assimilation in cyanobacteria are quite sensitive to stam F-34 mainly because of herbicide induced depletion of cellular ATP and reductant pool and likewise, nitrogenase is also known to be dependent on ATP, reductant and carbon skeleton derived through photosynthesis. This conclusion is further supported by the present observations that photosynthetic CO_2 assimilation inhibited in the presence of herbicide is reproducibly reversed by the exogenous supplementation of inorganic carbon sources (glucose and sodium acetate) Heterocysts are differentiated cells physiologically unable to assimilate CO_2 because of the absence of ribulose 1,5-biphosphate corboxylase/oxygenase and depend upon vegetative cells for import of photosynthetically produced carbohydrate which provides the reductant and other necessary requirements for effective nitrogen fixation (Wolk 1968, Winkenbach & Wolk 1973). Thus, overall it is suggested that herbicide mainly inhibits the nitrogen fixation by inhibiting CO_2 assimilation, photosynthetic electron flow, ATP and reductant supply to heterocysts in cyanobacteria

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