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Response of multiple herbicide resistant strain of diazotrophic cyanobacterium, *Anabaena variabilis*, exposed to atrazine and DCMU

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Effect of two photosynthetic inhibitor herbicides, atrazine (both purified and formulated) and [3-(3,4-dichlorophenyl)-1,1-dimethyl urea] (DCMU), on the growth, macromolecular contents, heterocyst frequency, photosynthetic O_2 evolution and dark O_2 uptake of wild type and multiple herbicide resistant (MHR) strain of diazotrophic cyanobacterium *A. variabilis* was studied. Cyanobacterial strains showed gradual inhibition in growth with increasing dosage of herbicides. Both wild type and MHR strain tolerated < 6.0 mg L^{-1} of atrazine (purified), < 2.0 mg L^{-1} of atrazine (formulated) and < 0.4 mg L^{-1} of DCMU indicating similar level of herbicide tolerance. Atrazine (pure) (8.0 mg L^{-1}) and 4.0 mg L^{-1} of atrazine (formulated) were growth inhibitory concentrations (lethal) for both wild type and MHR strain indicating formulated atrazine was more toxic than the purified form. Comparatively lower concentrations of DCMU were found to be lethal for wild type and MHR strain, respectively. Thus, between the two herbicides tested DCMU was more growth toxic than atrazine. At sublethal dosages of herbicides, photosynthetic O_2 evolution showed highest inhibition followed by chlorophyll *a*, phycobhiliproteins and heterocyst differentiation as compared to carotenoid, protein and respiratory O_2 uptake.

Keywords: Atrazine, Cyanobacteria, DCMU, Herbicides, Mutants, Photosynthesis

In modern agriculture weed control by herbicides is a common practice to increase in crop productivity[1]. Continuous applications of herbicides are not only detrimental to undesired weeds, but also to the beneficial microbial flora of soil including cyanobacteria[2-6]. Cyanobacteria or blue green algae are a remarkable group of gram negative, oxygenic, photosynthetic, prokaryotes that grow and multiply at the simple expense of water, light and air[7]. Cyanobacteria have been shown to be agriculturally important as biofertilizer, particularly in tropical rice field soils, because of the capacity of some of these to synthesize organic substances and also to fix atmospheric nitrogen[8-10]. The similarity of cyanobacteria to the higher plants and eukaryotic algae in terms of photoautotrophic mode of nutrition invites similar growth inhibitory actions of photosynthetic inhibitor herbicides that are among the most widespread herbicides used[11]. Successful exploitation of diazotrophic cyanobacteria as biofertilizer requires them to have capability to either tolerate or resist toxic actions of various rice field herbicides. Much attention has not been paid in developing resistant strains of diazotrophic cyanobacteria capable of sustaining the detrimental impacts of herbicides [12-14].

Efforts were made to isolate a spontaneous mutant of nitrogen fixing cyanobacterium *Anabaena variabilis* a local rice field isolate[15], exhibiting resistance to four commonly used rice field herbicides viz. arozin, alachlor, butachlor and 2,4-D; the mutant was designated as *Av* (MHR)[16]. In the present study investigations have been made to examine the biological responses under graded concentration of two photosynthetic inhibitor, rice field herbicides atrazine (both purified and formulated) and 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) with an aim to further incorporate atrazine and DCMU resistant traits. Both wild type and mutant strain showed similar level of tolerance towards these herbicides. DCMU was found to be more growth toxic as compared to Atrazine. Thus, the possibility of constructing multiple herbicide resistant strain of diazotrophic cyanobacterium tolerating six common rice field herbicides would serve as gene bank for further biotechnological studies and would prove beneficial in rice agriculture.

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Materials and Methods

Organism and growth conditions—The axenic clonal culture of wild type N_2-fixing cyanobacterium *Anabaena variabilis*, a rice field isolate[15], its spontaneous mutant [*Av* (MHR)] exhibiting resistance to the lethal dosages of arozin, alachlor, butachlor and 2,4-D[5,16] was cultivated in BG_{11} medium[17] devoid of any combined nitrogen source (N_2-medium). Culture was incubated in an air-conditioned culture room maintained at 25°±1°C fitted with cool day fluorescent light. Photon flux density of light on the surface of the vessel was 45 $\mu Em^{-2} s^{-1}$ for 18 h/day.

Herbicides—Atrazine (purified): (2 chloro-4 ethylamino) –6 (isopropylamino) –5- triazine; 95%) purity was obtained from Rallis India Bombay, India (designated as At-P). Atrazine (formulated): 50 EC was obtained from Northern Minerals limited, Daultabad Road, Gurgaon, Haryanna, India (designated as At-F). DCMU [3-(3,4 – dichloro phenyl)-1, 1- dimethyl urea] of 98% purity was obtained from ICN pharmaceuticals, Inc. life Sciences group Plainview, N.Y.

Determination of growth, macromolecular contents, photosynthesis and heterocyst frequency of wild type and multiple herbicide resistant mutant of A. variabilis under herbicide stress—Impact of increasing concentrations (0-100 mg L^{-1}) of herbicides atrazine and DCMU on the growth and survival of diazotrophic cyanobacterial isolates was determined in N_2-medium by monitoring variations in the concentrations of chlorophyll *a* pigments[18] at regular intervals of 24 h as a parameter of growth. For all the experiments, exponentially growing cells (6 day old) were harvested by centrifugation (5000 × *g*, 5 min), washed thrice with sterilized double distilled water and dispensed equally in assay flasks. Cultures were incubated under photo-autotrophic growth conditions as described above. The cultures without addition of herbicides were taken as control.

The 50% inhibitory growth concentrations for the strains were determined by monitoring survivability of cells on plates containing graded concentrations of herbicides. The percentage survival of the strains was calculated by the following method:

$$\frac{\text{Number of colonies on the herbicide treated plates}}{\text{Number of colonies on the untreated control plates}} \times 100$$

The concentration of the herbicide at which 50% of colonies survived as compared to untreated control culture was termed as IGC_{50} and the concentration at which no colonies survived (complete lysis) were considered the lethal concentration. The experiment was repeated thrice and every time five sets of plates were taken for each herbicide concentration. The data were analyzed by using a statistical computer program by determining means and standard errors.

Macromolecular contents i.e. protein[19], carotenoids[20] and phycobiliprotein[21] was determined at regular intervals of 48 h of diazotrophic growth.

Heterocyst frequency of exponentially grown diazotrophic culture of both MHR and wild type strain was determined microscopically and is expressed in percentage as total number of heterocysts occurring/ 100 vegetative cells.

The photosynthetic O_2 evolution and dark O_2 uptake of both wild type and mutant strain was measured with Clark type Oxygen electrode (Hansatech, U.K.) as per Rao *et al.*[22].

Statistical analysis—The results are expressed as mean ± SE.

Results

Effect of graded concentration (0 -100 mg L^{-1}) of herbicides (Atrazine and DCMU) on growth and survival of wild type and multiple herbicide resistant mutant of *A. variabilis* was monitored by estimating chlorophyll *a* content up to 12 days at an interval of 24 h. The initial chlorophyll *a* concentration ($\mu g ml^{-1}$) recorded 0.19 and 0.17 in wild type and *Av* (MHR) was increased to 2.5 and 2.9, respectively at 10th day of growth in untreated control cultures whereas the cultures treated with graded concentration of herbicide could not record any substantial increase in chlorophyll *a* content except in cultures treated with 1.0 and 2.0 mg L^{-1} of pure atrazine, 0.5 mg L^{-1} of formulated atrazine and 0.05-0.2 mg L^{-1} of DCMU during their growth. On the basis of survival potentiality of wild type and MHR

Table 1—Lethal, sub-lethal and IGC_{50} values (mg L^{-1}) of herbicides for wild type and MHR strain of *A. variabilis.*
[Values are mean ± SE of 3 independent experiments]

Herbicides	Lethal		Sub-lethal		IGC_{50}	
	Wild type	MHR	Wild type	MHR	Wild type	MHR
Atrazine (P)	8.0±0.25	8.0±0.35	6.0±0.22	6.0±0.46	1.9±0.33	1.8±0.24
Atrazine (F)	4.0±0.31	4.0±0.18	2.0±0.19	2.0±0.45	1.7±0.24	1.4±0.19
DCMU	0.6±0.15	0.5±0.23	0.5±0.45	0.4±0.17	0.08±0.20	0.03±0.36

under graded herbicide concentrations, the lethal, sublethal and IGC_{50} concentrations of herbicides were determined (Table 1).

Complete lysis of the cultures occurred at 8 mg L^{-1} of purified atrazine; 4 mg L^{-1} of formulated atrazine in wild type and MHR; 0.6 mg L^{-1} (Wild type), 0.5 mg L^{-1} (MHR) of DCMU between 6-10 days of incubation. Maximum inhibition in chlorophyll *a* content of 74 - 80% in wild type and 68 - 77% of control in MHR was observed at sublethal dosages of atrazine pure, formulated and DCMU treated cells at 8^{th} day of growth (Fig. 1 a). As compared to untreated control cultures, at sublethal dosages of herbicides PC was reduced to 73-79%, PE to 72-79%, protein to 45-54%, carotenoid to 46-58%, heterocysts to 74-78% (Fig. 1 b) in wild type and in MHR strain PC was reduced to 61-67%, PE to 66-71%, protein to 47- 52%, carotenoid to 40-47%, heterocysts to 73% (Fig. 1 b) at 8^{th} day of growth. Photosynthetic O_2 evolution was suppressed to 83-87% in wild type and 80-85% in MHR (Fig. 1 c) whereas 65-74% suppression in wild type and 60-69% in MHR strain was observed in dark O_2 uptake (Fig. 1 d) at sublethal dosages as compared to untreated control cultures at 8^{th} day of growth.

Discussion

The present results indicate gradual but substantial inhibition in growth with increasing concentration of herbicides. Both parent and mutant tolerated atrazine (purified) upto 4 mg L^{-1} and atrazine (formulated) upto 1 mg L^{-1} and complete lysis was observed in 8 and 4 mg L^{-1} indicating atrazine (formulated) to be more toxic as compared to the purified form. Almost similar growth inhibitory concentration (Atrazine formulated) of 2 mg L^{-1} was reported in *Anabaena flosaquae*[23] 5 mg L^{-1} in *Nostoc* sp.[24]. However, higher level of tolerance up to 10 mg L^{-1} was observed in *Nostoc muscorum* and *Anabaena variabilis*[25]. On the contrary, a cyanobacterial strain SG2 tolerated markedly higher concentration of atrazine (purified) i.e. upto 1000 mg L^{-1} and the herbicide only marginally affected photosynthetic ability (10% of control) and had no effect on growth rate. However, 89% inhibition in O_2 evolution was exhibited by *Synechocystis* sp. Strain 6803 at 1000 mg L^{-1} of atrazine[26]. This is in contrast to results of Narusaka *et al.*[27] using several herbicide resistant mutants *of Synechocystis* sp. Strain PCC 6803, which grew slower under photosynthetic growth conditions and evolved 70% less O_2 than control strain grown under herbicide free conditions.

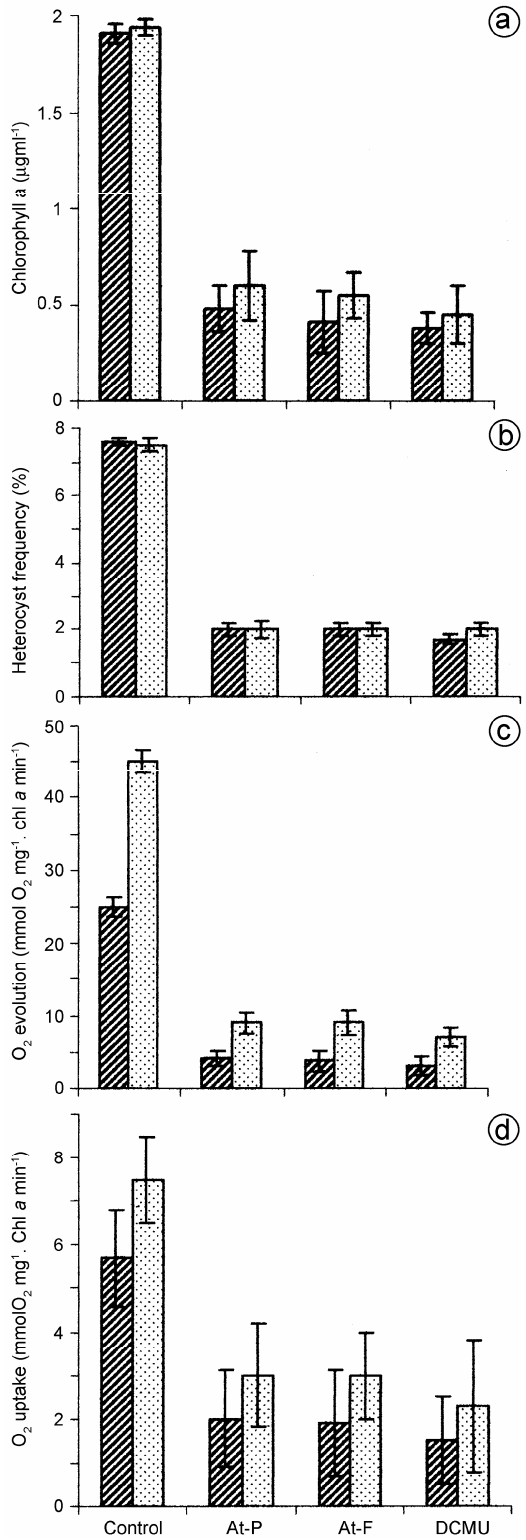

Fig. 1— Effect of sublethal dosages of herbicides on (a) chlorophyll *a* content, (b) heterocyst frequency, (c) photosynthetic O_2 evolution and (d) dark O_2 uptake of wild type (stripped) and MHR (dotted) strain of *A. variabilis*. [Values are mean ± SE of 3 independent experiments].

In the present study DCMU was found to be more growth toxic as compared to atrazine to both parent and mutant strain. Concentration of 0.6 and 0.5 mg L^{-1} was growth inhibitory to parent and MHR strain. Almost similar growth inhibitory concentration of 0.2 mg L^{-1} was reported in *Nostoc muscorum*[13]. However, a cyanobacterial strain SG2 tolerated DCMU upto 15 mg L^{-1} but oxygen evolution was inhibited 75% at 5 mg L^{-1} as reported by Sajjaphan *et al.*[26].

Herbicide treated wild type and MHR showed higher inhibition (as compared to untreated control) in photosynthetic O_2 evolution (83-87% in wild type and 80-85% in MHR) followed by chlorophyll *a* content (74-80 % in wild type and 68-77 % in MHR). The water soluble pigments phycocyanin and phycoerythrin followed almost similar trend of inhibition as chlorophyll *a.* Similarly heterocysts the site of nitrogen fixation showed marked suppression (74-78% inhibition in parent and 73% inhibition in mutant) under herbicide stress. The results further indicated that as compared to chlorophyll *a* and phycobiliproteins, carotenoid (46-58% in wild type and 40-47% in MHR) and protein content (45-54% in wild type and 47-52% in MHR) were less severely inhibited. Atrazine and DCMU are Triazine and phenylurea herbicides, which have been shown to inhibit photosystem II (PSII) in many plant species, algae and cyanobacteria. The structure and function of PSII in the cyanobacteria are similar to those found in higher plants[28], and PSII dependent electron transport in the cyanobacteria is similarly inhibited by these herbicides[29]. The photosynthetic apparatus in cyanobacteria consists of thylakoid membrane bound protein assemblies composed of a number of polypeptide components, including the 32-kDa D_1 polypeptide[30] encoded by the *psb A* gene. The D_1 protein is a subunit of the PSII core complex[31], and this protein also known as the Q_B binding protein, is the main target for phenylurea, triazine and phenolic group herbicides[32,33]. These herbicides compete with plastoquinone for binding and inhibit photosynthesis by blocking electron transfer from quinone acceptors QA to QB[34,35]. This inhibition is caused due to the primary effect of these herbicides at the photosynthetic level, which then leads to several secondary effects. The depletion of pigments seems to result from photoxidation induced by the inability of chlorophyll to dissipate its absorbed excitation energy due to inhibition of electron transport. This inhibition limits the availability of NADPH[36]. Since energy and reducing potential provided by NADPH play a vital role in many biosynthetic pathways, the changes in other parameters are also expected.

Heterocyst development depends upon the algal growth, herbicide concentrations inhibiting growth; also inhibit heterocyst formation[37]. The results further indicated similar level of tolerance in the parent and MHR strain to the growth toxic effects of the two herbicides tested. The tolerance level of both parent and MHR strain was more in case of atrazine (< 6.0 mg L^{-1} purified and < 2.0 mg L^{-1} formulated) than DCMU (< 0.4 mg L^{-1}). Though both are photosynthetic inhibitor herbicides results from the analysis of D_1 mutants of several cyanobacteria and *Chlamydomonas* strains indicated that there is not a direct correspondence between atrazine resistance and resistance to DCMU. Moreover, this relationship differs considerably among mutations in the *psb A* genes from different species of cyanoabcteria [32,38,39,33].

The results of the previous studies made with the survival potentialities of parent *A. variabilis* under graded concentration of Arozin, Alachlor, Butachlor and 2,4-D indicated that the isolate tolerated upto 15 mg L^{-1} of the herbicides and complete lysis was observed at 25 mg L^{-1}. Thus this indicates that as compared to Arozin, Alachlor, Butachlor and 2,4-D the two photosynthetic inhibitor herbicides tested were more growth toxic. This variation in the relative sensitivity of the strain to the growth toxic effects of the herbicides tested under laboratory conditions seems to result from interactions between mode of herbicidal actions with morphological, physiological, biochemical and genetic properties of cyanobacteria. Herbicides have differential effects on various metabolic processes and the sensitivity of the strain varies depending upon the species, kind of herbicides and chemical formulations[40-42]. Thus, further incorporating resistant markers of these two herbicides by spontaneous mutational techniques can increase the herbicide tolerance potentiality of the strain. Therefore, construction of such novel multiple herbicide resistant strain with herbicide resistant metabolic activities are bound to serve as a potent biofertilizer inoculant in rice-agriculture applied with common rice field herbicides.

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