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Biochemical Effects of Carbaryl on Nitrogen Assimilating Enzymes of Cyanobacteria *Nostoc muscorum*

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The blue green algae (cyanobacteria) is implicated in the soil enrichment due to nitrogen-fixing ability (Swaminathan 1982). Although these nitrogen fixing cyanobacteria are found in all environmental situations, their luxuriant growth is most common in water logged paddy fields (Singh *et al* 1986). *Nostoc muscorum*, one of the most promising prokaryotes, contributes nitrogen 15-49 kg/ha through nitrogen fixation (Venkataraman 1981; Tandon *et al* 1988). After the restricted use of organochloro pesticides, carbamate pesticides are extensively used in modern agriculture due to their low persistence and high effectiveness (Horvath 1986). Since soil is the ultimate sink of pesticides in agricultural fields, they are bound to interact with these nontarget soil microflora. The aim of the present investigation is to study the effect of carbamate insecticide carbaryl on growth, nitrogen assimilating enzymes like nitrogenase, nitrate reductase (NR), glutamine synthetase (GS) and photosynthesis and respiration of cyanobacteria *Nostoc muscorum*.

MATERIALS AND METHODS

Nostoc muscorum strain ISU (formerly *Anabaena* ATCC 27893, a generous gift from Dr. D.N. Tiwari, Banaras Hindu University, India) was used in this study. The insecticide carbaryl (99.9%) was supplied by Union Carbide, USA. ADP, bovine serum albumin (BSA), γ-glutamyl hydroxamate, Tris, methylviologen were purchased from Sigma Chemical Company, USA. Toluene was purchased from E. Merck. All other reagents used in the experiments were of analytical grade. Organisms were grown in combined nitrogen free CHU-10 medium (Saffermann and Morris 1964) under fluorescent light, intensity 2500 lux, with a photoperiod 14 hr/d, in a growth chamber at 26 ± 2°C under controlled 90% humidity. On the sixth day of growth (at exponential phage) carbaryl was added at 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L and 75 mg/L. The growth of cyanobacteria was measured by counting the cell number using haemocytometer.

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Activities of nitrogenase and nitrate reductase and respiration and photosynthetic pigment chlorophyll *a* content were measured after 96 hr of carbaryl exposure. For nitrate reductase assay cultures were grown in CHU-10 medium supplemented with 1 mM $Ca(NO_3)_2$ or $NaNO_3$.

Chlorophyll *a* was extracted and measured as described by Bhunia *et al* (1991) and quantified using extinction coefficient of 82.04. Respiratory O_2 uptake by intact cells was measured in a Gilson oxygraph equipped with a clark type oxygen electrode. Dense treated culture, 0.5 mL was placed in the reaction chamber and rate of O_2 uptake was then measured in the dark. Temperature was the same as the original growth condition. Nitrogenase activity was measured using acetylene reduction technique according to the method of Stratton *et al* (1979). Treated culture, 3 mL, were sealed in a 9 mL container bottle with rubber cork and 10% volume of air was replaced with acetylene. Then it was incubated for 2 hr under identical growth conditions as described earlier. Ethylene production was measured by injecting gas samples (1 mL) from the sealed bottle into a Gas Chromatograph (Model PYE Unicam GC 104, U.K.) equipped with a porapak N column. For gas chromatographic analysis the conditions were as follows : column temperature 110°C, detector temperature 150°C, injector temperature 150°C, nitrogen gas (carrier gas) flow rate 30 mL/ min.

Nitrate reductase activity was assayed *in situ* as described by Herrero *et al* (1981). Treated cyanobacterial cells were harvested by centrifugation at 5000 x g for 15 min and cell pellet was washed with Tris-HCl buffer (pH 7.5) followed by the addition of 10% toluene and shaken vigorously for 3 min. A known volume of this preparation was immediately added to a reaction mixture for nitrate reductase assay. The reaction mixture in a final volume of 1 mL contained 100 µmol $NaHCO_3$-Na_2CO_3 buffer pH 10.5, 20 µmol KNO_3, 4 µmol methylviologen. The reaction mixture including the cyanobacterial sample was incubated for 5 min at 30°C. The reaction was stopped by the addition of 10 mL of 1 M Zn-acetate and nitrite was thus produced was determined by following the azo-coupling method of Snell and Snell (1949). Glutamine synthetase activity was measured by estimating γ-glutamyl hydroxamate produced during the reaction following the method of Sampaio *et al* (1979). The cyanobacterial cells were centrifuged at 5000 x g for 5 min and the cell pellet was washed with buffer A (50 mM Tris-HCl buffer, pH 7.5) and buffer B (buffer A supplemented with 5 mM $MgCl_2$, 10 mM Na-glutamate, 5 mM mercaptoethanol and 1 mM EDTA). The cyanobacterial cells were disrupted by morter-pestle in buffer B and centrifuged at 35000 x g for 30 min. Supernatant was the source of enzyme. The final volume 1 mL of reaction mixture contained 40 mM Tris-HCl buffer, pH 7.0, 3 mM $MnCl_2$, 20 mM K-arsenate, 0.4 mM ADP (Na-salt), 60 mM hydroxylamine hydrochloride ($NH_2OH.HCl$) and 30 mM glutamine. The reaction is allowed to proceed for 15 min at

30°C in the dark. Finally reaction was stopped by adding 0.2 mL of a stop mixture (4.0 mL of 10% $FeCl_3$ + 1.0 mL of 24% TCA + 0.5 mL of 6 (N) HCl + 6.5 mL of distilled water). The readings were taken at 540 nm against a blank which contained all the components except ADP and $NH_2OH.HCl$ with Hitachi U2000 (Japan) spectrophotometer. Protein was estimated by the method of Lowry *et al* (1951) using BSA as standard.

RESULTS AND DISCUSSION

The effect of carbaryl on the growth of *Nostoc muscorum* cells is shown in Figure 1. It is evident from the figure 1, that growth retardation of the cyanobacteria occurred in a dose dependent manner of carbaryl. At 10 and 25 mg/L carbaryl the growth of the cyanobacteria were significantly decreased but at 50 mg/L severe reduction in growth was observed. Lethal dose of carbaryl was found to be 75 mg/L. The growth inhibition by 50 mg/L carbaryl concentration seems to be biphasic; following 4 d of treatment the growth inhibition by carbaryl appeared to be partially reversed. This may be due to the partial degradation of carbaryl to non-toxic metabolites. Chlorophyll *a* content of carbaryl treated cyanobacterial cells were reduced in a dose dependent manner as shown in Table 1. Conversely respiratory O_2 uptake was enhanced in the same trend (shown in Table 1). Significant enhancement of respiration rate was found at 10 mg/L and above concentrations of carbaryl exposure. At high concentration of carbaryl, low chlorophyll *a* content indicated that photosynthetic activity of *N. muscorum* cells were impaired during carbaryl treatment. The stimulation of respiratory O_2 uptake suggests respiratory electron transport chain is not inhibited by this pesticide. The overall increase in respiration rate may be due to triggering a series of mechanisms which the microorganisms are believed to posses to counter the effects of the chemical. These mechanisms may include uptake, accumulation, biodegradation and transport of chemical out of the cells (Davis and Smith 1978). Moreover any possible suppressive activity on the photosynthetic ATP generation might compel the organism to rely more on endogenous carbon reserves (polyglucose and poly-alpha-hydroxy butyrate) and oxidative phosphorylation to meet the extraenergy demands under the stress condition. The low photosynthetic pigment content as observed in these studies may result from photooxidation arising from inability of chlorophyll *a* to dissipate its absorbed excitation energy when electron transport is inhibited. Inhibition of electron transport limits the availability of NADPH and chemical energy as ATP (Moreland 1980). The nitrogenase activity was reduced significantly in presence of carbaryl except at 5 mg/L (as shown in Table 2). The inhibition of nitrogenase activity by the pesticide treatment may be explained by the low photosynthetic pigment content and reduced photosynthetic activity of *Nostoc muscorum* cells under such experimental condition. In heterocystous cyanobacteria the photosynthetically fixed carbon which is essential for nitrogen fixation is supplied to the heterocyst

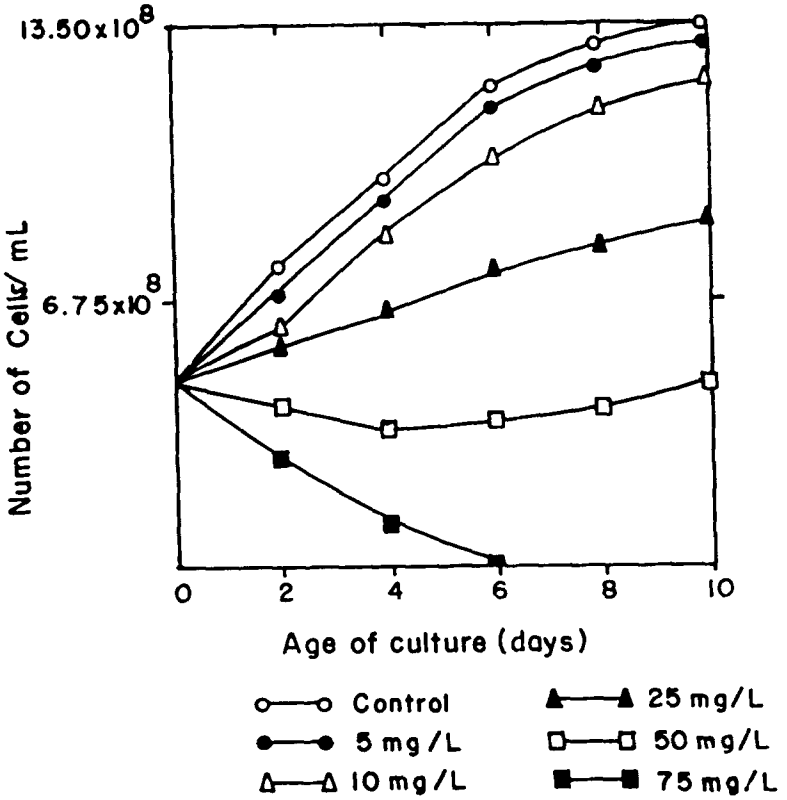

Figure 1. Growth patterns of *Nostoc muscorum* cells in presence of different concentrations of carbaryl.
Each point is the mean of four sets of experiments.

Table 1. Effect of carbaryl on chlorophyll *a* content and respiratory O_2 uptake in *Nostoc muscorum* after 96 hr of treatment.

	Control	Carbaryl mg/L 5	10	25	50
Chlorophyll *a* content (µg/mL culture)	6.10 ± 0.18	5.90 ± 0.16	4.08 ± 0.13**	2.75 ± 0.08**	1.70 ± 0.05**
Respiratory O_2 uptake[a]	162.12 ± 6.4	168.52 ± 6.5	180.35 ± 6.9*	190.39 ± 7.2**	216.42 ± 8.1**

a = nmole/min/mg protein
The values are means ± SD four sets of experiments; *$p<0.01$, **$p<0.001$ when compared with control.

Table 2. Activities of nitrogenase, nitrate reductase and glutamine synthetase after 96 hr of carbaryl treatment.

	Control	Carbaryl (mg/L) 5	10	25	50
Nitrogenase (n mole C_2H_4/hr/mg protein)	57.10 ± 1.26	55.10 ± 1.25	40.10 ± 1.14*	29.00 ± 0.70*	16.90 ± 0.39*
Nitrate reductase (n mole NO_2/min/mg protein)	419.35 ± 12.22	400.00 ± 11.90	280.20 ± 8.66*	235.20 ± 7.40*	190.25 ± 5.30*
Glutamine synthetase (n mole product^x/ min/mg protein)	1680.31 ± 33.10	1630.09 ± 33.00	1155.44 ± 23.90*	900.48 ± 19.60*	795.64 ± 16.39*

x = γ-glutamyl hydroxamate.
The values are means ± SD four sets of experiments; *$p<0.001$.

by the vegetative cells (Stewart 1980). Haselkorn (1978) reported that chemical energy ATP and reducing agent NADPH, required for nitrogenase activity are mainly derived from photosynthetic reaction. Photosynthesis as measured by photosynthetic O_2 evolution of *Nostoc muscorum* cells was impaired by carbaryl treatment was reported earlier from our laboratory (Bhunia *et al* 1993). The observed diminution of nitrogenase activity in presence of different concentration of carbaryl was due to the primary effects at the photosynthetic level. Both nitrate reductase and glutamine synthetase activities were reduced significantly at 10 mg/L and above concentrations of carbaryl exposure, as shown in Table 2. NR and GS activity were not reduced significantly at 5 mg/L concentration of carbaryl. The enzyme NR is membrane bound and its activity depends on reduced ferredoxin produced during oxygenic photosynthesis (Flores *et al* 1983). The progressive decrease in NR activity may be due to the different levels of interference by this pesticide with their photosynthetic mechanisms. The GS activity suppression in carbaryl treated *N. muscorum* cells may be related to the suppressed nitrogenase and NR activities under similar conditions. GS is most active under nitrogen fixing conditions in *Gloeocapsa* sp. (Thomas *et al* 1982). Moreover the activation GS gene (gln A) requires the nitrogen fixing conditions (Tumer *et al* 1983). Therefore suppression of nitrogenase and NR activity may be responsible for low GS activity of *Nostoc muscorum* cells under the carbaryl exposure.

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