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Growth, Chlorophyll *a* Content, Nitrogen-Fixing Ability, and Certain Metabolic Activities of *Nostoc muscorum*: Effect of Methylparathion and Benthiocarb

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Among the most ubiquitous nitrogen-fixing blue-green algae in Indian soil, *Nostoc muscorum* is one of the most promising biological systems for dinitrogen fixation (Tandon *et al.* 1988). Although they are found in all environmental situations, their luxuriant growth is most common in water-logged paddy fields (Singh *et al.* 1986). Under the submerged rice fields, this system contributes nitrogen from 15 to 49 kg/ha (Venkataraman 1981) through nitrogen fixation. Due to low persistance and high effectiveness, organophosphate and carbamate pesticides are extensively used in modern agriculture (Fest 1977). However, application of such pesticides for plant protection and in increasing plant productivity also bring about adverse effects on the algal population affecting soil fertility (Tandon *et al.* 1988). Although many reports are available on the toxicities of pesticides on blue-green algae particularly on their growth, survival, and photosynthesis (Muralikrishna and Venkateswarlu 1984; Singh and Tiwari 1988), their effects on metabolic activities including nitrogen fixation, nuclease activities and macromolecular profiles in algae have been little investigated. The present communication reports the studies on the effects of organophosphate insecticide, methylparathion, and carbamate herbicide, benthiocarb, on the growth, chlorophyll a content, nitrogen fixation, nucleases and protease activities of filamentous nitrogen-fixing cyanobacteria *Nostoc muscorum*.

MATERIALS AND METHODS

Pure culture of *Nostoc muscorum* ISU (formerly known as *Anabaena* ATCC 27893), used in this study, was a gift from Dr. D.N. Tiwari, Algal Research Laboratory, Banaras Hindu University, India. Insecticide methylparathion (84% purity) and herbicide benthiocarb (90% purity) were supplied by Pesticide India, India. DNA, RNA and Bovine serum albumin (BSA) were purchased from Sigma Chemical Company, USA. All other reagents used in the studies were of analytical grade.

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The cultures were grown in combined nitrogen free (conveniently called N_2 medium) CHU-10 medium (Saffermann and Morris 1964) and temperature maintained in an air conditioned growth chamber at 26 ± 2°C under the fluorescent light illumination of intensity nearly 2500 lux for 14 hr/day under controlled humidity. The organisms were grown in several replicates. On the 6th day (exponential phase) methylparathion and benthiocarb were added separately at desired concentrations (5, 10 and 20 mg/L for methylparathion and 2, 4 and 6 mg/L for benthiocarb). The growth was measured turbidometrically at 650 nm at 48-hr interval using spectrophotometer (Hitachi, Japan, U2000) for 10 d. The cell numbers were counted with a haemocytometer and the number of cells/mL were plotted from the standard curve (O.D. vs cell density). Contents of DNA, RNA and protein of the cells, and enzymes DNase, RNase, protease and nitrogenase from the subcellular fractions of the cells, and pigment chlorophyll a content of the cells was measured after 96-hr of pesticide treatment. Cyanobacterial cells were harvested by centrifugation of culture medium at 5000 x g for 5 min and washed with distilled water. DNA was measured by the diphenylamine method using calf thymus DNA as standard and RNA by the orcinol reagent method using yeast RNA as standard (Herbert *et al.* 1971). Protein was estimated by Folin phenol reagent method (Lowry *et al.* 1951) using Bovine serum albumin (BSA) as standard. For the extraction of RNase, cells were homogenised in 50 mM Tris-HCl buffer (pH 7.5), containing 10 mM mercaptoethanol and centrifuged at 20,000 x g for 30 min. The spectrophotometric (Hitachi, Japan, U2000) assay of RNase was made at 260 nm using yeast RNA as substrate according to the method of Srivastava (1968). DNase was extracted from cells by grinding cells in 50 mM sucrose-citrate buffer (pH 6.0) and the homogenate was centrifuged at 20,000 x g for 30 min. The supernatant was used for the assay of DNase spectrophotometrically at 260 nm using calf thymus DNA as substrate (Worthington enzyme manual 1972). For the extraction of protease, cells were disrupted in 20 mM sodium phosphate buffer (pH 7.2), containing 10 mM mercaptoethanol and was centrifuged at 20,000 x g for 20 min. From the supernatant protease activity was determined spectrophotometrically at 280 nm using Bovine serum albumin as substrate, as described by Basha and Cherry (1978).

Chlorophyll a was extracted in acetone (80%, v/v) at 4°C for 12-hr and estimated by measuring absorbance at 665 nm with spectrophotometer (Hitachi, Japan, U2000) and quantified by using extinction coefficient of 82.04 (Mayers and Kratz 1955).

Nitrogenase activity was determined using an acetylene reduction technique as described by 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 10-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, U.K., GC-104) 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. 100 nM ethylene was identified as 99.9 nM in this Gas Chromatography.

RESULTS AND DISCUSSION

The growth of methylparathion and benthiocarb treated cultures is shown in Figure 1. At 2 and 4 mg/L benthiocarb the growth of the algae were significantly decreased but at 6 mg/L severe reduction in growth occurred. Benthiocarb, 8 mg/L, was found to be lethal. No significant recovery from growth inhibition was observed up to 10 d of growth studies. In case of methylparathion treated cultures no significant growth inhibition occurred at 5 mg/L, but concentrations above 10 mg/L significantly lowered the growth. The lethal dose of methylparathion was observed to be 35 mg/L.

Benthiocarb reduced photosynthetic pigment chlorophyll a content about 97% of the algae *Nostoc muscorum* and pigment content reduced by 56% and 74% at 2 and 4 mg/L benthiocarb treated culture respectively. Pandey (1985) reported that propanil, an organochlorine herbicide induced inhibition of chlorophyll a synthesis in cyanobacteria. Methylparathion at lower concentration, i.e., at 5 mg/L did not inhibit the growth significantly but at 10 and 20 mg/L the same inhibition was about 36% and 65%, respectively. Methylparathion, an organophosphorous insecticide, inhibits photosynthetic pigment chlorophyll a in algae as was observed earlier (Bose and Subbaraj 1984). The observation that the photosynthetic pigment content chlorophyll a inhibition in cyanobacteria indicates impairment of photosynthesis by both benthiocarb and methylparathion, the effect being more predominant with benthiocarb treatment. At 2 mg/L of benthiocarb treatment nitrogenase activity was decreased by 66% after 48-hr and the decrease was marked at concentrations of 4 and 6 mg/L. Unlike benthiocarb, at lower concentration (5 mg/L), however, methylparathion does not significantly change the nitrogenase activity. But at 10 and 20 mg/L nitrogenase activity was decreased by 55.6% and 71%, respectively. The inhibition of nitrogenase activity of cyanobacteria by the application of pesticides was also observed by other workers (Tandon *et al.* 1988; Singh *et al.* 1988). This inhibition may be explained by the low photosynthetic pigment content of the cells under such experimental conditions. The low 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). Haselkorn (1978) reported that ATP and reducing agent required for nitrogenase activity are derived mainly from photosynthetic reaction. The observed diminution of nitrogenase activity in presence of benthiocarb and methylparathion in *Nostoc moscorum* is therefore due to their

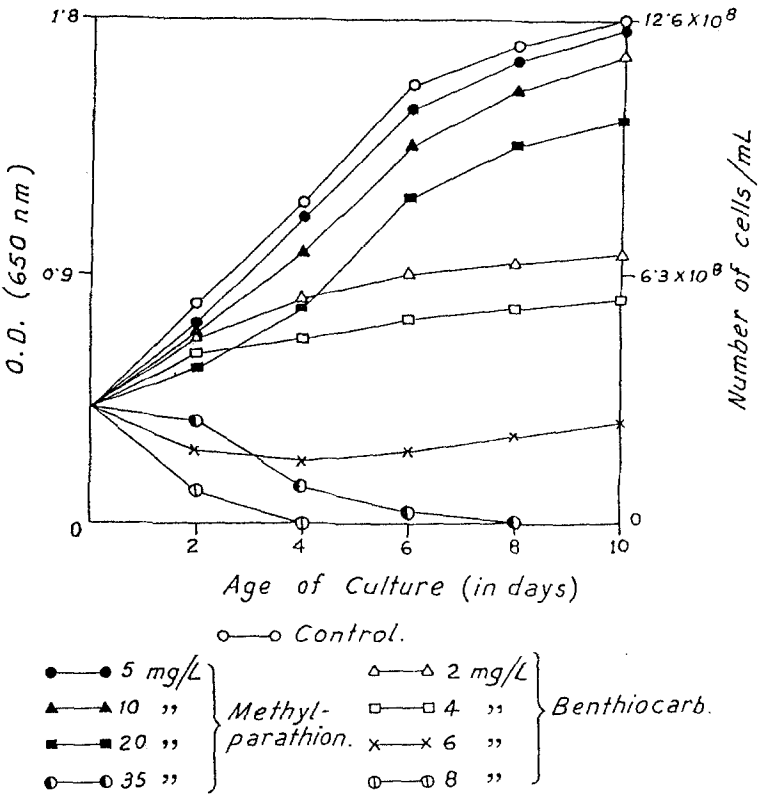

Figure 1. Growth patterns measured as cell density of *Nostoc muscorum* in presence of different doses of Benthiocarb and Methylparathion

primary effects at the photosynthetic level. Nuclease activity, i.e., DNase and RNase activity induced by the application of benthiocarb and methylparathion application, is presented in Table 2. With the increase in the activity of DNase and RNase a simultaneous decrease in the respective nucleic acid levels were observed. Protease activity in case of methylparathion treated cell was not significantly changed at 5 and 10 mg/L, but at 20 mg/L it was increased by 25% with respect to control. Benthiocarb treated algae show higher protease activity in dose dependent manner even at low concentration. Pandey (1985) observed that ricefield herbicide propanil rapidly decreased the DNA, RNA, and protein content of cyanobacteria *Nostoc calcicola*. The stimulation of nuclease activities with concomitant decrease in total DNA and RNA content was also observed in germinating seeds *Vigna sinensis* by organophosphate insecticide malathion (Chakravorty *et al.* 1981) and by carbamate group insecticide carbaryl (Sengupta *et al.* 1986). Low protein

Table 1. Effect of benthiocarb and methylparathion on chlorophyll a content and acetylene reduction (nitrogenase activity) in *Nostoc muscorum* (after 96-hr of treatment).

	Control	Benthiocarb (mg/L)			Methylparathion (mg/L)		
		2	4	6	5	10	20
Chlorophyll a content (µg/mL culture)	6.40±0.06	2.80±0.03*	1.66±0.02*	0.15±0.01*	6.00±0.02*	4.10±0.01*	2.20±0.03*
Nitrogenase activity (n mole C_2H_4 produced/hr/ mg protein)	68.50±1.35	22.80±0.70*	10.40±0.30*	1.76±0.54*	65.80±1.10	30.40±0.81*	20.00±0.32*

The values are the means ± SD of four sets of experiments.

*differ significantly $p<0.001$ when compared with control.

Table 2. Effects of various concentrations of benthiocarb and methylparathion on contents of nucleic acids and protein and activities of protease and nuclease of *Nostoc muscorum* (96-hr treated culture).

Measurement	Control	Benthiocarb (mg/L)			Methylparathion (mg/L)		
		2	4	6	5	10	20
DNA[a]	4.92±0.10	4.10±0.07**	3.45±0.05**	2.96±0.04**	4.80±0.10	3.90±0.07**	3.20±0.05**
RNA[a]	38.70±0.75	31.20±0.56**	24.20±0.39**	19.50±0.40**	38.10±0.78	30.80±0.55**	25.20±0.50**
Protein[b]	234.50±4.46	200.00±4.01**	142.50±2.60**	98.00±2.00**	230.30±4.90	175.00±2.95**	150.30±2.70**
DNase[c]	0.80±0.01	0.87±0.03*	0.94±0.02**	1.04±0.05**	0.81±0.07	0.93±0.04**	1.00±0.02**
RNase[c] X 10^2	2.60±0.015	3.10±0.020**	3.64±0.029**	4.00±0.03**	2.60±0.05	3.30±0.03*	3.70±0.03**
Protease[d]	0.47±0.04	0.62±0.06*	0.75±0.06**	0.89±0.02**	0.46±0.09	0.52±0.08	0.59±0.03*

The values are the means ± SD of four sets of experiments.

*differ significantly $p<0.01$ when compared with control; **differ significantly $p<0.001$ when compared with control.

[a]DNA and RNA content expressed µg/mg protein; [b]protein content expressed µg/mL culture;

[c]Specific activity expressed $\Delta OD_{260\ nm}$/mg protein/hr at 37°C;

[d]Specific activity expressed $\Delta OD_{280\ nm}$/mg protein/hr at 37°C.

content of benthiocarb treated cells may result from higher protease activity under experimental condition. Retarded growth of algae under both methylparathion and benthiocarb treatment could be explained through reduced protein content, decreased nucleic acid level, and increased nuclease activities. Furthermore, the present study also correlates the changes of macromolecules and hydrolytic enzymes with the pesticide treatment. Elucidation of mechanisms for differential response of algae towards these two herbicides and insecticides however needs further studies.

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