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Response of Enzymes Involved in the Processes of Antioxidation towards Benthiocarb and Methylparathion in Cyanobacteria *Nostoc muscorum*

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Cyanobacteria are the most important and abundant nitrogen-fixing microorganism in rice paddy fields (Roger and Kulasooria 1980) where they contribute significant amount to fertility (Hermelink and Kramer 1981). *Nostoc muscorum*, among the most ubiquitous nitrogen-fixing cyanobacteria in Indian soil, is one of the most promising biological system (Tandon *et al* 1988), and contributes nitrogen 15-49 kg/ha, through nitrogen fixation (Watanabe 1962). In the modern agriculture organophosphate and carbamate pesticides are now extensively used due to low persistance and high effectiveness (Fest 1977). Therefore, application of such pesticides for plant protection and in increasing productivity also bring about adverse effects on the algal population affecting soil fertility (Tandon *et al* 1988). The cyanobacterial cell possesses an antioxidant defence system which causes removal of peroxides, free radicals such as superoxide ($O_2^{\bullet-}$) anions generated during photosynthesis and other metabolic process (Karni *et al* 1984). Normally this system provides the conditions required for nitrogen fixation and other metabolic events by removing peroxides (Karni *et al* 1984). Recently, it has been observed in our laboratory that growth, nitrogen fixation, protein content of cyanobacteria *Nostoc muscorum* were reduced by methylparathion and benthiocarb treatment (Bhunia *et al* 1990). Though many works on toxicity of pesticides on cyanobacteria, specially on growth, photosynthesis and nitrogen fixation are available (Tandon *et al* 1988; Singh and Tiwari 1988), the effects of pesticides on antioxidant enzyme levels is still unclear. In this communication, studies have been presented on the effects of organophosphate insecticide methyl-parathion and carbamate herbicide benthiocarb, on glutathione content, glutathione reductase (GR) and superoxide dismutase (SOD) activities of filamentous, nitrogen-fixing cyanobacteria *Nostoc muscorum*.

MATERIALS AND METHODS

A pure strain of *Nostoc muscorum* ISU (formerly known as *Anabaena*

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ATCC 27893), a generous gift from Dr. D. N. Tiwari, Algal Research Laboratory, Banaras Hindu University, India, was used in this study. The herbicide benthiocarb (90% purity) and insecticide methylparathion (84% purity) were supplied by Pesticide India, India. Oxidised glutathione (GSSG), reduced glutathione (GSH), N-ethylmaleimide (NEM), O-phthaldialdehyde (OPT), NADPH and Bovine serum albumin (BSA), were purchased from Sigma Chemical Company, USA. All other reagents used in the studies were of analytical grade.

The cultures were grown in combined nitrogen free CHU-10 medium (Saffermann and Morris 1964) with fluorescent light intensity of nearly 2500 lux with 14-hr photoperiod and 10-hr nyctoperiod and temperature maintained in an air conditioned growth chamber at 26 ± 2°C under controlled humidity. On the sixth day of growth (at 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 benthiocarb). The observed I_{50} values for methylparathion and benthiocarb were 20 mg/L and 6 mg/L respectively. Furthermore, no change in pH of the medium was observed with the addition of pesticides to the medium. After 96-hr of pesticide exposure, cells were harvested by centrifugation of culture medium at 5000 x g for 5 min and washed repeatedly with distilled water. The most prominent effects of these pesticides were observed at 96-hr of pesticide treatment. To prepare the enzyme solution of glutathione reductase, cyanobacterial cells were homogenised in chilled 75% acetone and filtered. Residues were dried. The enzyme was extracted from the dried residue (acetone dry powder) with 67 mM phosphate-EDTA (ethylene diamine tetraacetic acid) buffer (pH 7.4) (Esterbauer and Grill 1978). All operations were carried out at 4°C. Glutathione reductase activity was assayed spectrophotometrically (Hitachi, Japan, model U2000), following the formation rate of 2-nitro-5-thiobenzoic acid from 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB), with reduced glutathione (GSH), at 412 nm (Tietze 1969). The standard reaction mixture, at a final volume of 1 mL contained : cell free extracts containing 0.1-0.3 mg protein, 1 mM GSSG, 100 mM Na-phosphate buffer (pH 8.0), 5 mM EDTA, 0.6 mM DTNB. The reaction was started by adding 1 mM NADPH. Specific activity was expressed as nM GSH formed/min/mg protein. Glutathione content was measured fluorometrically by the method of Hissin and Hilf (1976). A measured amount (fresh weight) of the cyanobacterial cell pellet was suspended in a medium of 25% metaphosphoric acid and potassium phosphate buffer (pH 8.0), sonicated for 10 min and centrifuged at 30,000 x g for 30 min. The supernatant, kept at 0°C, was used for the GSH and GSSG assays on the same day. For the GSH assay, fluorescence was determined with a Hitachi (Japan) F-3010 fluorescence spectrophotometer at 420 nm (excitation at 350 nm) after incubating 0.2 mL of supernatant with 1.7 mL potassium phosphate-EDTA buffer (pH 8.0) and 0.1 mL of the fluorescence reagent O-phthaldialdehyde (OPT) (1 mg/mL) for 15 min. For the GSSG assay, 0.5 mL of the

supernatant was incubated with 0.2 mL of 40 mM NEM for 30 min at room temperature. After adding 4.3 mL NaOH (0.1 M), 0.2 mL of mixture was incubated with 1.7 mL 0.1 M NaOH and 0.1 mL OPT solution, 1 mg/mL, for 15 min. Fluorescence was determined similarly to that of GSH assay. The results of the assays were calculated against a standard calibration curve for GSH and GSSG. The SOD enzyme extract preparation and assay was carried out as described by Giannopolitis and Ries (1977). Protein was estimated by Folin phenol reagent method (Lowry *et al* 1951) using BSA as standard.

RESULTS AND DISCUSSION

As seen from the Table 1 with the increase of pesticide concentration glutathione reductase activity was stimulated. Even at 2 mg/L benthiocarb treated cells this increment was significant. In case of methylparathion treated cells 10 mg/L and above concentrations glutathione reductase activity was significantly enhanced. Enhancement of glutathione reductase activity on pesticide treatment of cyanobacterial cells, was also reported earlier (Tozum and Gallon 1979). Glutathione level was reduced in a dose dependent manner of both methylparathion and benthiocarb exposed cyanobacterial cells. GSH content of benthiocarb treated cells was much reduced than that of methyl-parathion treated cells. The high glutathione reduction rate indicates a potential physiological role for GSH in cyano-bacterial cells (Karni *et al* 1984). Low content of GSH inspite of higher GSH production may be attributed to the rapid utilisa-tion of GSH under pesticide toxic condition. High utilisation of GSH under pesticide toxic condition was also reported earlier (Tozum and Gallon 1979). Conversely oxidised glutathione content was not reduced but total glutathione (GSH + GSSG) content was decreased in a dose dependent manner of pesticide treatment. The significant increase of GSSG content was observed in the cells except 5 mg/L methylparathion treated cyanobacterial cells as shown in Table 1. It indicates that the rate of reduction of GSSG was decreased in the dose dependent manner of pesticide treatment. This may be due to the limitation of NADPH. GR utilises NADPH as a cofactor for the GSSG reduction (Karni *et al* 1984). Normally, photo-synthesis provides this NADPH but photosynthesis was decreased in presence of methylparathion and benthiocarb as was reported earlier from this laboratory (Bhunia *et al* 1990).

The SOD activity was also increased in a dose dependent manner of pesticide treatment. The increase was much more in case of benthiocarb treated cells compared to methylparathion treated cells. This means that peroxide formation was increased under the both benthiocarb and methylparathion treated cells. In photosynthetic organism peroxide was produced by the superoxide dismutase of dismutation of free radicals such as superoxide and hydroxylradicals and this may generate from the action between oxygen and reduced intermediates of the

Table 1. Activities of glutathione reductase and superoxide dismutase and level of glutathione of *Nostoc muscorum* after 96-hr exposure to benthiocarb and methylparathion

	Control	Benthiocarb (mg/L)			Methylparathion (mg/L)		
		2	4	6	5	10	20
Glutathione reductase[a]	38.30 ± 0.88	54.88 ± 1.09**	67.02 ± 1.35**	70.10 ± 1.38**	40.50 ± 0.87	56.02 ± 1.10**	65.21 ± 1.21**
Superoxide dismutase[b]	15.25 ± 0.29	24.40 ± 0.45**	35.50 ± 0.64**	42.30 ± 0.80**	16.10 ± 0.35*	25.72 ± 0.49**	33.01 ± 0.48**
GSH[c]	87.20 ± 2.20	69.44 ± 1.48**	53.56 ± 1.12**	34.20 ± 0.70**	81.00 ± 1.92*	70.80 ± 1.56**	58.31 ± 1.18**
GSSG[c]	22.30 ± 0.45	23.73 ± 0.44*	25.00 ± 0.43**	25.60 ± 0.48**	22.89 ± 0.45	23.95 ± 0.41*	24.30 ± 0.44**

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 specific activity expressed nM GSH/min/mg protein
b specific activity expressed unit/min/mg protein
c n gm/250 mg cell.

photosynthetic electron transport (Tozum and Gallon 1979). Halliwell and Foyer (1978) reported that H_2O_2 (peroxide), produced in higher plant chloroplast, may be removed with in the chloroplast themselves by reaction with endogenous GSH. Since no glutathione peroxidase was observed in this species, H_2O_2 was decomposed directly to water by nonenzymatic reaction between H_2O_2 and endogenous GSH. Absence of glutathione peroxidase in *Nostoc muscorum* cells was also reported earlier (Karni *et al* 1984).

The employment of GSH for peroxide removal from cyanobacterial preparations was further tested. A spontaneous reaction between GSH and H_2O_2 showed that 0.1 mM GSH interacted with 0.1 mM H_2O_2 and 10 nM GSH were oxidised/min. In pesticide toxic condition observed low content of GSH may be attributed to the employment of GSH at spontaneously remove the peroxide radicals. No effect of methylparathion and benthiocarb were noted on the enzyme (GR and SOD) activities derived either from homogenates of the cells of the control or from cells immediately after treatment with pesticides. Therefore, it may be concluded that increase GR activity and SOD activity and depletion of glutathione content occurred *in vivo* in the cyanobacterial cells under the experimental condition of benthiocarb and methylparathion exposure.

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