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Biochemical Effect of Carbaryl on Oxidative stress, Antioxidant enzymes and Osmolytes of Cyanobacterium *Calothrix brevissima*

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Abstract Carbaryl is used in Indian agriculture for control of rice field pests and it is next to Benzene hexachloride in pesticide consumption. In present study, carbaryl (0, 10, 20, 30 and 40 mg/L) induced toxic effects were observed after 21 days exposure on a non target rice field biofertilizer *Calothrix brevissima* with special reference to oxidative stress, antioxidant enzymes and osmolytes. At 40 mg/L carbaryl the decrease in carotenoid, chlorophyll, phycobilin and protein were 63%, 43%, 40% and 40% respectively in comparison to control. Total carbohydrate, malondialdehyde, superoxide dismutase, ascorbate peroxidase, catalase and osmolytes showed enhancement at all the treated concentration. Increased amount of MDA (46% at 40 mg/L) indicated free radical mediated deleterious effect of carbaryl. Enhancement of SOD, APX, CAT and osmolytes in presence of carbaryl indicated their involvement in free radical scavenging. SOD, CAT and APX showed maximum activities (79%, 64% and 39% respectively) at 40 mg/L carbaryl. The order of enhancement in osmolytes was glycine-betaine (66%) > proline (54%) > sucrose (50%) at 40 mg/L which might be another adaptive defense strategy of the cyanobacterium against the pesticide.

Keywords Carbaryl · *Calothrix brevissima* · Photosynthetic pigments · Lipid peroxidation · Antioxidative enzymes · Osmolytes

Cyanobacteria have been used to increase the yield in paddy fields and for the reclamation of barren and alkaline soil (Relwani 1963). *Calothrix* sp. is one of the dominant algae of wetland rice fields (Watanabe and Roger 1984) and salt affected rice soils. It result in an overall improvement of soil health e.g. decrease in soil pH, electrical conductivity, exchangeable sodium, enrichment of soil with carbon, nitrogen and phosphorus, improvement in soil aggregation and hydraulic conductivity. *Calothrix* sp. usually occurs as second dominant flora in rice fields after *Nostoc* (Kaushik 1998).

It is very essential to understand factors which regulate negatively towards N_2 fixation in rice fields. Weedicides, fungicides and insecticides are used for plant protection in rice fields and they affect the cyanobacterial population adversely (Kolte and Goyal 1990). After the restricted use of organochloro pesticides, carbamate pesticides are extensively used in modern agriculture due to their low persistence and high effectiveness.

Under different environmental stresses (physical, chemical and biological) reactive oxygen species are formed. The imbalance between the production of active oxygen species and the quenching activity of antioxidants results in oxidative damages. The main cellular components susceptible to be damaged by free radicals are lipids, protein, carbohydrates and nucleic acids (Olga et al. 2003). Under normal circumstances, concentrations of oxygen radicals remain low because of the activities of protective enzymes, including superoxide dismutase, catalase and ascorbate peroxidase (Asada 1984). Many organisms accumulate osmolytes under osmotic stress in addition to antioxidant enzymes. These compounds are small, electrically neutral molecules and are non toxic even at high concentrations. According to Somero et al. (1992) osmolytes include amino acids (e.g. proline); quaternary ammonium compounds (e.g. glycine–betaine); diverse sugar alcohols and sugars (e.g. sorbitol, sucrose and trehalose).

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Though considerable work has been done on chlorinated and organophosphate pesticides induced toxicity in cyanobacteria (Babu et al. 2001; Lakshmi and Annamalai 2007). But, carbamate pesticideinduced changes are not yet explored except work done by Bhunia et al. (1994) that covered only a few aspects of nitrogen assimilating enzymes of *Nostoc muscorum*. Thus in present study effect of carbaryl – a carbamate pesticide, which is used for control of rice field pests (Rajagopal et al. 1984) was studied thoroughly on free radicals, antioxidant enzymes and osmolytes of one of the most common cyanobacterial biofertilizer (*Calothrix brevissima*).

Materials and Methods

C. brevissima was procured from National Centre for Conservation and Utilization of Blue Green Algae, Indian Agriculture Research Institute, New Delhi, India. The test organism was inoculated in BG-11 medium (pH 7.3) without sodium nitrate (Stainer et al. 1971) and allowed to grow for 21 days at 30 ± 2°C under light intensity of 2000 ±200 lux provided by 20 W fluorescent tubes following a 16:8 h light/dark regime. The biomass was harvested by centrifugation at 10,000 rpm for 10 min. Each experiment was conducted in triplicate.

Commercial grade carbaryl (99.9%) was supplied by Bayer Crop Science Limited, Bhopal, India. All other reagents used were of analytical grade. The dilutions of carbaryl were made in the media for the final concentration of 0, 10, 20, 30 and 40 mg/L.

Chlorophyll was extracted from the biomass using 95% methanol and heating at 65°C (Mackinney 1941). Carotenoid was extracted from biomass with the help of 85% acetone through freezing and thawing method (Jensen 1978). Total phycobiliprotein was also extracted by freezing and thawing using 0.1 M phosphate buffer (Siegelman and Kycia 1978). Protein was estimated by the method of Lowry et al. (1951). Carbohydrate was estimated by anthrone reagent method (Spiro 1966).

Lipid peroxidation was determined by measuring malondialdehyde (MDA) using the thiobarbituric acid (TBA) method (Heath and Packer 1968). The absorbance was read at 532 nm and correction for specific turbidity was done by subtracting the absorbance at 600 nm. 0.5% TBA in 20% TCA served as blank. The MDA content was calculated according to its extinction coefficient of 155 mM/cm.

Superoxide dismutase (SOD) activity was assayed spectrophotometrically by the method of Dhindsa et al. (1981). 50 mg dry biomass was homogenized in 2 mL 0.5 M phosphate buffer (pH 7.5). Supernatant obtained after centrifugation (15,000 rpm at 4°C) was used for the enzyme assay. SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium chloride (NBT), using reaction mixture consisting of 1 M Na_2CO_3, 200 mM methionine, 2.25 mM NBT, 3 mM EDTA, 60 µM Riboflavin and 0.1 M phosphate buffer (pH 7.8). Absorbance was read at 560 nm. Ascorbate peroxidase (APX) activity was determined according to the method of Nakano and Asada (1981). Fifty mg algal biomass was taken and homogenized with 2 mL of extraction buffer (0.5 M phosphate buffer, pH 7.5). Ascorbate oxidation was read at 290 nm against blank. The concentration of oxidized ascorbate was calculated using extinction coefficient (2.8 mM/cm). One unit of APX was defined as nmol/mg ascorbate oxidized per minute. Catalase (CAT) activity was assayed by measuring the initial rate of disappearance of H_2O_2 by Aebi (1984). Fifty mg algal biomass was taken and homogenized with 2 mL of extraction buffer (0.5 M phosphate buffer, pH 7.5) and the absorbance of the supernatant was observed at 240 nm against blank. An enzyme activity was calculated by using extinction coefficient 0.036 per mM/cm and was expressed as enzyme unit/mg protein. One unit of enzyme is the amount necessary to decompose 1 µL of H_2O_2 per minute at 25°C.

The analytical method for proline quantification was rapid colorimetric procedure described by Bates et al. (1973). The amount of proline in the sample was calculated in µg/g dry weight of samples. Glycine-betaine (GB) estimation was done in dried biomass by the method of Grieve and Grattan (1983).The absorbance was measured at 365 nm with UV–visible spectrophotometer. Reference standards of GB (50–200 µg/mL) were prepared in 1 N sulphuric acid and the procedure for sample estimation was followed. Amount of glycine betaine was expressed as µg/g dry weight. Sucrose was determined in algal biomass using anthrone reagent by the method of Van Handel (1968). The absorbance was recorded at 620 nm spectrophotometrically. Amount of sucrose was expressed as µg/g dry weight with the help of sucrose standard graph.

The experimental values were tabulated as the mean ± standard error (SE) of three replicates. Test of significance was performed by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Range test with control ($p < *0.01$, $p < **0.001$, $p < ***0.0001$ and ns for not significant respectively) by Graph Pad Prism version 5.00 for windows (Graph Pad Software San Diego, CA, USA).

Results and Discussion

The photosynthetic pigments like Chlorophyll, Carotenoids and Phycobilins were adversely affected by carbaryl in *C. brevissima*. Chlorophyll content showed maximum 43%

reduction at 40 mg/L ($p < 0.0001$) carbaryl (Fig. 1a). Bhunia et al. 1994 has reported higher (70%) decrease in chlorophyll content in presence of carbaryl (50 mg/L) in *Nostoc muscorum*. This suggested that *C. brevissima* is more resistant to carbaryl than *N. muscorum*. The reduction of chlorophyll may be due to the degradation of pigments for producing carbon skeleton to satisfy the energy demand of cells (Mohapatra et al. 2003). Carotenoid showed reduction with increasing carbaryl concentration in the medium (Fig. 1b) being 30% ($p < 0.01$), 40% ($p < 0.001$), 50% ($p < 0.0001$) and 63% ($p < 0.0001$) in presence of 10, 20, 30 and 40 mg/L carbaryl. Prasad et al. (2005) have reported 35% carotenoid reduction in *Plectonema boryanum* due to toxic effect of chlorinated pesticide endosulfan (20 µg/mL) that may be due to the pesticide accelerated degradation as well as reduced biosynthesis. As most of the carotenoid synthesizing enzymes are membrane bound, reduction of carotenoid biosynthesis might be interaction of the pesticide with these enzymes (Michelangeli et al. 1990). In case of phycobilin also gradual decrease was observed being 19% ($p < 0.001$), 26% ($p < 0.001$), 38% ($p < 0.0001$) and 40% ($p < 0.0001$) at 10, 20, 30 and 40 mg/L (Fig. 1c). Organochlorine pesticide endosulfan (20 µg/mL) has shown 56% phycobilin decrease in *P. boryanum* (Prasad et al. 2005). The degradation of phycobilin can be attributed to the pesticide interaction with thylakoid membrane (Mohapatra and Mohanty 1992). The decrease in chlorophyll, carotenoid, and phycobilin content is suggested due to active oxygen species at various sites of the photosynthetic electron transport chain during stress (Prasad et al. 2005).

In *C. brevissima* protein content decreased with increase in carbaryl concentration (Fig. 1d).The decrease was 7% (ns), 18% (ns), 27% ($p < 0.001$) and 40% ($p < 0.001$) at 10, 20, 30 and 40 mg/L carbaryl concentration compared with control. Other carbamate pesticide viz. benthiocarb (6 mg/L) and thiobencarb (3 mg/L) also resulted 58% protein decrease in *Nostoc muscorum* (Bhunia et al. 1991) and 50% in *Anabaena variabilis* (Battah et al. 2001) respectively, that may be due to higher protease activity, retarded growth, and decreased carbon and nitrogen assimilation under endosulfan (Babu et al. 2001).

The total carbohydrate content of *C. brevissima* significantly increased in presence of carbaryl 7% (ns), 15% (ns), 36% ($p < 0.01$) and 46% ($p < 0.001$) respectively at 10, 20, 30 and 40 mg/L (Fig. 1e). Battah et al. (2001) had reported 39% carbohydrate increase in presence of another carbamate pesticide thiobencarb (3 mg/L) in *A. variabilis*. This indicated lesser toxicity of carbaryl to non target organism (cyanobacteria). The increase in sugar content may be an adaptive measure aimed for survival under carbaryl stress. It is generally known that when protein synthesis is suppressed by various factors, algal cells, depending on genotype are transformed to synthesize either carbohydrates or lipids (Averamova and Rossler 1975). This unbalanced cell composition could be due to disturbances in nitrogen metabolism and photosynthetic activity (Battah et al. 2001).

MDA increased gradually and significantly to 46% ($p < 0.0001$) at 40 mg/L carbaryl exposure suggesting induction of oxidative stress to *C. brevissima* (Fig. 1f). This is also in agreement with earlier reports of our laboratory with alphamethrin, deltamethrin and endosulfan stress in cyanobacterium, *Westellopsis prolifica*, *Nostoc muscorum*, *Anabaena variabilis* and *Aluosira fertillisma* (Fatma et al. 2007; Kumar et al. 2008). Endosulfan stress also generated free radical in *Plectonema boryanum* due to strong inhibition of PS II and whole chain activities (Prasad et al. 2005). Many pesticides are reported to generate reactive oxygen species, either by direct involvement in radical production or by inhibition of biosynthetic pathways (Kunert et al. 1985). Compounds such as paraquat (also known as methyl viologen) induce light dependent oxidative damage in plants (Dodge 1981). The PS I mediated reduction of the paraquat di-cation results in the formation of mono-cation radical which then reacts with molecular oxygen to produce O_2^- with the subsequent production of other toxic species, such as H_2O_2 and OH (Elstner et al. 1988). These compounds cause severe toxicological problems and result in peroxidation of membrane lipids and general cellular oxidation.

SOD neutralizes the highly reactive superoxide radical generated in the cell especially under stress condition (Elstner et al. 1988). In present study the enzyme SOD was stimulated significantly with increasing concentration of carbaryl. 79% ($p < 0.0001$) increase was observed at 40 mg/L carbaryl (Fig. 1g), while Bhunia et al. (1994) has reported 112% increase in SOD in presence of 50 mg/L carbaryl in *N. muscorum*. The enzyme APX showed 12% (ns), 31% ($p < 0.01$), 44% ($p < 0.001$) and 39% ($p < 0.001$) increase at 10, 20, 30 and 40 mg/L, of carbaryl respectively (Fig. 1h). Prasad et al. (2005) have reported (50%) APX increase in *Plectonema boryanum* in presence of organochlorine pesticide endosulfan (20 µg/mL). APX utilizes the reducing power of ascorbic acid to eliminate potentially harmful H_2O_2 (Kumar et al. 2008). The CAT also showed increase [7% (ns) to 64% ($p < 0.0001$)] with increasing concentration of carbaryl (Fig. 1i). In present study CAT played greater role than APX in H_2O_2 detoxification. Organochlorine pesticide endosulfan (20 µg/mL) also resulted (40%) increase of CAT in *P. boryanum* (Prasad et al. 2005).

The test organism showed 13% (ns), 26% ($p < 0.01$), 45% ($p < 0.0001$) and 54% ($p < 0.0001$) proline increase at 10, 20, 30 and 40 mg/L carbaryl (Fig. 1j). Proline content increase and its involvement in free radical scavenging has been reported from our lab under endosulfan stress in *N. muscurum, A.variabilis* and *A.fertilissima* (Kumar et al.

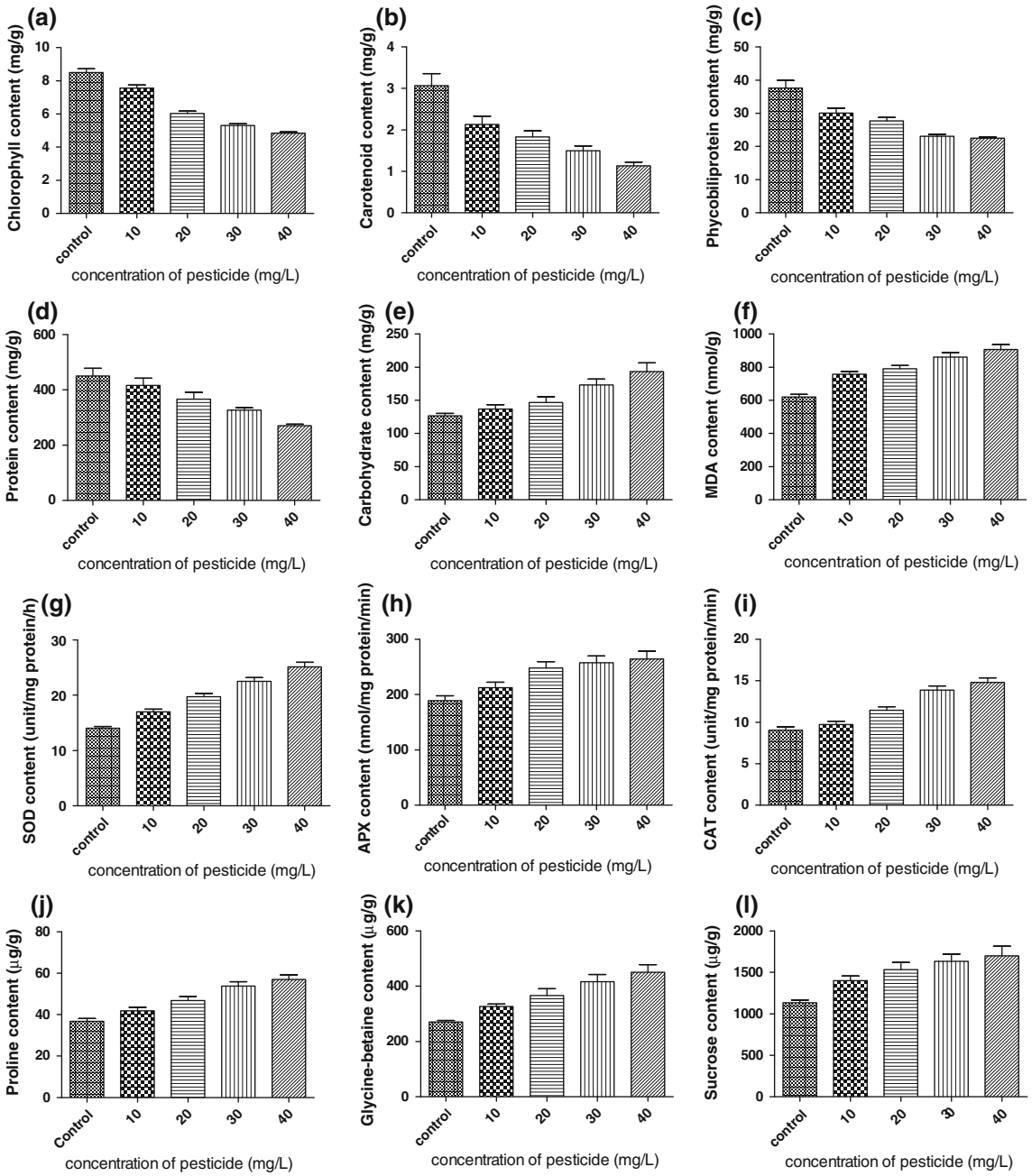

Fig. 1 Effect of carbaryl on **a** chlorophyll, **b** carotenoid, **c** phycobiliprotein, **d** total protein, **e** carbohydrate, **f** MDA, **g** SOD, **h** APX, **i** CAT, **j** proline, **k** glycine-betaine and **l** sucrose content of *Calothrix brevissima* after 21 days treatment. Values are mean ± SE with n = 3

2008) and heavy metal stress in S*pirulina platensis*-S5 (Choudhary et al. 2007). Proline accumulation has been suggested due to decrease in proline degradation, increase in proline biosynthesis, a decrease in protein synthesis or proline utilization and increase hydrolysis of proteins (Hare et al. 1999). In present study Glycine-betaine also increased significantly as 20% (ns), 35% ($p < 0.001$), 54% ($p < 0.001$), and 66% ($p < 0.001$) due to carbaryl stress (Fig. 1k). Glycine-betaine has been implicated in reducing lipids peroxidation in plants (Cushman 2001). It is also suggested that glycine-betaine can protect proteins via a chaperon-like action on protein folding and that glycine-betain may act as a signal molecule that could elicit the expression of genes associated with stress tolerance (Rontein et al. 2002). According to Prasad et al. (2005) protection by glycine-betaine against H_2O_2 induced photo-oxidative stress and enhanced level of antioxidant system; protect the PS II complex against high light–induced damage in transgenic lines. Sucrose content also increased to 23% (ns), 35% ($p < 0.01$), 44% ($p < 0.001$) and 50%

($p < 0.001$) as compared to untreated control (Fig. 1l). The protective role of proline and sucrose has also been observed in higher plants against lipid peroxidation under salinity stress (Juan et al. 2005).

In conclusion, the toxic effect of carbaryl in *C. brevissima* was well pronounced photosynthetic pigments and protein showing declining trend. MDA, an indicator of lipid peroxidation was elevated suggesting free radical mediated toxic responses. To deal with carbaryl induced oxidative stress, the antioxidant enzymes SOD, APX and CAT were activated. Osmolytes (proline, glycine-betaine and sucrose) also contributed to the defense mechanism probably by providing metabolic protection to the cellular machinery of the cyanobacterium. Due to tolerant nature of *Calothrix brevissima* it is advised to always include in rice field cyanobacterial inoculums mixture.

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