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ORIGINAL ARTICLE

Chlorpyrifos-induced changes in the antioxidants and fatty acid compositions of *Chroococcus turgidus* NTMS12

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Significance and Impact of the Study: Chlorpyrifos induces oxidative stress in *Chroococcus turgidus* NTMS12. A strong inference was made on increased activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and proline content and lowering the level of unsaturated fatty acids under the pesticide-exposed condition. These significant changes are the defence mechanisms against the oxidative stress. Thus, this organism holds great promise in resisting toxic pesticide.

Keywords
catalase, chlorpyrifos, *Chroococcus turgidus*, gas chromatography, proline, superoxide dismutase.

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Abstract

This study investigated the effect of pesticide chlorpyrifos (CP) on a freshwater cyanobacterium *Chroococcus turgidus* NTMS12. The changes in chlorophyll-*a*, proline, superoxide dismutase (SOD), catalase (CAT) activities and fatty acid composition of the test organism were analysed. Organism was grown at 6, 9 and 12 mg l^{-1} of CP, and based on the chlorophyll-*a* content, 6 mg l^{-1} of CP was found to be the tolerable concentration. Hence, 6 mg l^{-1} of CP was taken to evaluate the concentration of proline and activities of SOD and CAT at 48-h exposure. The changes in the fatty acid profile were analysed after 7 days of exposure. Upon pesticide exposure, increased concentration of proline and activities of SOD and CAT were found. Significant changes in fatty acid profile have also been observed. However, polyunsaturated fatty acid content was decreased in treated cultures when compared with the untreated control. Changes in biochemical activities indicate that cyanobacteria *C. turgidus* NTMS12 undergo adaptive changes against CP-induced oxidative stress.

Introduction

Nowadays, the use of modern organic synthetic pesticides has increased about 40-fold. Pesticides are used to kill or control pests. Organophosphates, organochlorines, carbamates and pyrethroid are various groups of pesticides used worldwide. Currently, among the various groups of pesticides, organophosphates form a major and most widely used group. The first organophosphate, tetraethyl pyrophosphate, was developed and used in 1937 (Dragun *et al.* 1984). In a quick time, several other organophosphorus pesticides were industrialized and commercialized. Globally, organophosphate pesticides (mostly insecticides) constitute 20–38% of the total pesticides used: others include chlorine, hypochlorites, specialty biocides and wood preservatives (Singh and Walker 2006).

Chlorpyrifos (CP) O, *O*-diethyl *O*-(3,5,6-trichloro-2-pyridinol) (TCP) phosphorothioate is one of the most widely used organophosphorus insecticides (Lee *et al.* 2012). A large number of pesticides are used in paddy fields to protect the rice seedlings and crops and selectively destroy the pests. These pesticides cause adverse effect on nontarget beneficial micro-organism such as cyanobacteria (Rodger *et al.* 1994).

Cyanobacteria are one of the main diazotrophic components of the primary microbiota producers in rice fields and significantly contribute to building-up soil fertility (Kumar *et al.* 2009, 2011; Galhano *et al.* 2011; Rajeshwari

et al. 2011). Application of pesticides in field may cause oxidative stress in cyanobacteria and results in the production of reactive oxygen species (ROS), including superoxide radicals (O_2^-), hydroxyl radicals (^-OH) and hydrogen peroxide (H_2O_2). These products of high biological activity could seriously attack cell membranes, proteins and nucleic acids, cause enzyme inactivation, protein denaturation, lipid peroxidation and DNA mutation and cause ecotoxicity through oxidative damage to cellular components (Imlay *et al.* 1998; Vandana *et al.* 2001).

Every cyanobacterial cell possesses a complex array of enzymatic antioxidant defence system, which comprises mainly the enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione *S*-transferase (GST) (Srivastava *et al.* 2005; Wiktelius and Stenberg 2007). On the other hand, under oxidative stress, the unsaturated fatty acids of the cell membrane get peroxidized and subsequently lead to cell death, whereas organisms employ numerous approaches to limit their damage. Hence, low degree of fatty acid unsaturation may protect the cell against oxidative damage. Such adaptation in fatty acid content under chromium-induced oxidative stress was reported (Kumar *et al.* 2012, 2013).

Previous reports on degradation of organophosphorus pesticide using cyanobacteria and their effect on cyanobacteria are scarce. With this background, this study aimed at investigating the effects of CP on a freshwater cyanobacterium *Chroococcus turgidus* NTMS12 with respect to their antioxidants and fatty acid profile.

Results and discussion

Effect of CP on growth of *C. turgidus* NTMS01 is shown in Fig. 1. The growth of cyanobacteria decreased with increase in CP concentration. This may be due to the damaging effect of the pesticide on pigment synthesis.

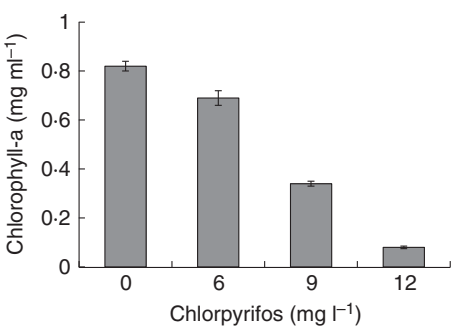

Figure 1 Effect of chlorpyrifos concentrations on chlorophyll-*a* in *Chroococcus turgidus* NTMS12 at 7 days of incubation. Data represent the mean ± standard deviation (w/v) of three separate trials.

Chloroplasts have a complex system of membranes rich in polyunsaturated fatty acids, which are potential targets for peroxidation often resulting in inhibition of chlorophyll synthesis. A study demonstrated that norflurazon caused oxidative damage in *Cucumis sativus* resulted in a reduction in chlorophyll (Halliwell and Gutteridge 1999). Similar study reported the inhibitory effect on the growth of cyanobacteria with other insecticides, carbofuran, phorate and malathion (Kaushik and Venkataraman 1983). A cyanobacterium from paddy field *Nostoc linckia* was reported to exhibit up to 75% reduction in growth when exposed to 1–25 mg endosulfan ml^{-1} (Satish and Tiwari 2000).

Proline

There are reports on the degradation of pesticide using cyanobacteria, but the interaction between them with regard to proline is scarce. The amount of present proline in control and CP-exposed culture was 22·5 and 30 mg g^{-1} of biomass, respectively (Fig. 2). Cyanobacterial proline accumulation is an active energy-requiring process. Intracellular proline content of cyanobacteria *Nostoc muscorum* has been reported to be an important index for stress tolerance capacity due to function as a hydroxyl and singlet oxygen scavenger (Galhano *et al.* 2011). Increased amount of proline was observed in endosulfan-treated nitrogen fixing cyanobacteria *Nostoc muscorum, Anabaena variabilis and Aulosira fertilissima* than control (Kumar *et al.* 2008). Heavy metal stress also increased the level of proline in *Spirulina platensis*-S5 (Choudhary *et al.* 2007). Many studies have been reported that cellular proline accumulation was higher in many higher plant species under variety of stress conditions such as water, salt, drought and heavy metal (Bates *et al.* 1973; Kumar *et al.* 2008). It was proposed that increased accumulation of proline in plants exposed to stress is one of the adaptive

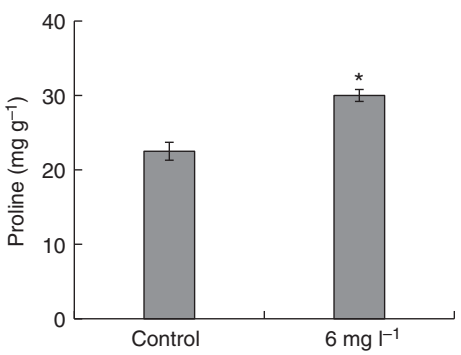

Figure 2 Effect of chlorpyrifos at 6 mg l^{-1} on proline of *Chroococcus turgidus* NTMS12 at 48 h exposure. Data represent the mean ± standard deviation (w/w) of three separate trials. (*) symbol represents that the values are significantly higher at $P < 0·01$.

roles to reduce free radical generation (Alia and Saradhi 1993). Hence, the cyanobacterium *C. turgidus* NTMS12 reacts to the stress in the way to increase the intracellular proline content as an adaptive mechanism.

SOD activity

Figure 3 represents the CP-induced SOD activity in *C. turgidus* NTMS12. Activity of SOD was 11·4 and 16·9 unit mg^{-1} protein in control and CP-exposed cultures, respectively. Organic pollutants such as herbicides could enter micro-organisms and participate in biotransformation that further produces ROS, including superoxide radicals, hydroxyl radicals and hydrogen peroxide (Vandana *et al.* 2001). To prevent the damage caused by superoxide radical, cell system increased the activity of SOD. SODs can catalyse superoxide radicals to oxygen and hydrogen peroxide. Antioxidants are expected to occur in response to enhanced ROS production and may differ according to the duration and severity of the stress (Okamoto *et al.* 2001). Similar studies reported that the SOD activity was increased in *Bacillus subtilis* B19, *B. megaterium* L1 and *Escherichia coli* K12 after being supplemented with bensulfuron-methyl concentration (Lin *et al.* 2009). In other studies, an increased SOD activity in perennial ryegrass, *Conyza bonariensis*, and other bacterial cells was observed when treated with the herbicides paraquat, diquat, diuron, atrazine, quinclorac and acetamiprid. The first line of defence against the generation of toxic oxygen species is the induction of SOD (Lenartova *et al.* 1998).

Catalase activity

Figure 4 represents the CAT activity in *C. turgidus* NTMS12 under normal and pesticide exposure. The

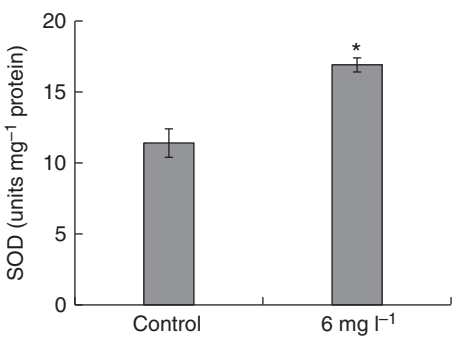

Figure 3 Effect of chlorpyrifos at 6 mg l^{-1} on superoxide dismutase (SOD) in *Chroococcus turgidus* NTMS12 at 48-h exposure. Data represent the mean ± standard deviation of three separate trials. (*) symbol represents that the values are significantly higher at $P < 0{\cdot}01$.

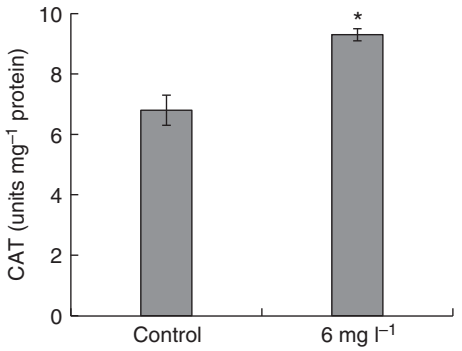

Figure 4 Effect of chlorpyrifos at 6 mg l^{-1} on catalase (CAT) activity in *Chroococcus turgidus* NTMS12 at 48-h exposure. Data represent the mean ± standard deviation of three separate trials. (*) symbol represents that the values are significantly higher at $P < 0{\cdot}01$.

amount of CAT present in control and CP-treated cells was 6·8 and 9·3 unit mg^{-1} protein, respectively. Maintenance of a high antioxidant capacity in cells has been related to increased tolerance against different kinds of environmental stress (Okamoto *et al.* 2001). CAT decomposes the hydrogen peroxide into water and oxygen. Increased production of hydrogen peroxide under pesticide-exposed condition can be assumed to be from an inactivation of water-splitting complex (Das and Bagchi 2010). Hence, it is essential for any cell to overcome such stress. In this way, *C. turgidus* NTMS12 exhibited increased CAT activity under the pesticide stress. Similarly, increased production of CAT was observed in endosulfan-treated *Plectonema boryanum* (Prasad *et al.* 2005). The ability of bacteria to overcome oxidative stress is related to the levels and types of antioxidant enzymes that they possess (Lin *et al.* 2009). The combined action of SOD and CAT is critical in mitigating the effects of oxidative stress. The presence of antioxidant enzymes in control indicates the presence of ROS in normal condition. Under normal condition, the ROS was produced in the aerobic organism, which is involved in signal transduction but level of ROS was increased during stress conditions. The defence system increased the production of antioxidant enzymes to protect them from oxidative damage caused by ROS and to maintain the balance between the antioxidant enzymes and ROS.

Fatty acid profile

Fatty acid profile for *C. turgidus* NTMS12 under normal and pesticide-exposed conditions is shown in Table 1. Fatty acid profile is considered to be a useful biomarker to assess the effect of pesticides on aquatic microbial communities (Galhano *et al.* 2011). Upon exposure to chlorpyriphos, significant changes ($P < 0{\cdot}01$) were

Table 1 Effect of chlorpyrifos (CP) at 6 mg l^{-1} on fatty acid profile in *Chroococcus turgidus* NTMS12

Fatty acid methyl ester		Control† (% w/w)	CP‡ (% w/w)
Caproic	C6:0	0·14	0·39
Caprylic	C8:0	0·23	nd
Capric	C10:0	1·39	nd
Undecanoic	C11:0	0·3	nd
Lauric	C12:0	0·47	1·68
Tridecanoic	C13:0	nd	1·08
Myristic	C14:0	nd	1·66
Palmitic	C16:0	2	3·17
Stearic	C18:0	7·37	nd
Arachidic	C20:0	11·02	7·59
Heneicosanoic	C21:0	nd	8·02
Tricosanoic	C22:0	1·16	nd
Behenic	C22:0	nd	15·2
Lignoceric	C24:0	7·07	nd
Total saturated fatty acids		31·15	38·79*
Elaidic	C18:1	nd	4·32
Oleic	C18:1	44·43	52·77
cis-11-eicosenoic	C20:1	14·12	nd
Linolelaidic	C18:2	3·05	4·12
Linoleic	C18:2	6·06	nd
cis-5, 8, 11, 14, 17-eicosapentaenoic	C20:5	1·19	nd
Total polyunsaturated fatty acids		68·85*	61·21

nd, not detected.

*Represents that the values are significantly higher at $P < 0·01$.

†Culture grown on BG 11 medium without CP.

‡Culture grown on BG 11 medium with 6 mg l^{-1} of CP.

observed in the fatty acid profile of *C. turgidus* NTMS12. Saturated fatty acids such as caprylic, capric, undecanoic, stearic, tricosanoic and lignoceric acid were not detected in chlorpyriphos-exposed cells, whereas, instead, fatty acids such as tridecanoic, myristic, heneicosanoic, behenic acids were detected. While comparing the unsaturated fatty acids, eicosenoic, linoleic and eicosapentaenoic acids were absent upon pesticide exposure. Monounsaturated fatty acid such as elaidic and oleic acid contents was increased under stress condition. On the whole, a significant decrease ($P < 0·01$) in polyunsaturated fatty acid was observed under pesticide-exposed condition. Lipophilic pesticide CP will be incorporated in the membrane lipids of organelles, such as chloroplasts and mitochondria. Under such conditions, generation of ROS will be initiated. Often, these oxygen radicals catalyse lipid peroxidation. Cell membranes, which are structurally made up of large amounts of polyunsaturated fatty acid, are highly susceptible to such oxidative attack (Singh *et al.* 2002). Polyunsaturated fatty acid present in cyanobacterial thylakoid membranes is susceptible to oxidative damage with inevitable changes in membrane fluidity and permeability (Galhano *et al.* 2011). Certain cyanobacterium develops a number of mechanisms to defend themselves against environmental stressors. Changes in fatty acid profiles may be one of the mechanisms against the stress. It was clearly shown from the present results that the cyanobacterium undergoes adaptive mechanisms to lower their degree of unsaturation so as to react to the CP-induced oxidative stress.

In summary, this study investigates the effect of CP on the antioxidant level and changes in the fatty acid profile of a freshwater cyanobacterium *C. turgidus* NTMS12. A noteworthy increase in the activity of antioxidant enzymes proline, SOD and CAT was evident under CP-exposed cells. Under CP-exposed conditions, the organism was also found to change its fatty acid profile by lowering the level of unsaturated fatty acids. The significant changes such as increased activity of cellular antioxidants and lowering the levels of cellular unsaturated fatty acids are considered as strong defence mechanisms against the pesticide-induced stress, by the organism.

Materials and methods

Chemicals

CP was dissolved in acetone to prepare a concentration of 1000 mg l^{-1}, filtered as a stock solution and stored at 4°C before it was added to the BG 11 medium. Various working concentrations were obtained by diluting the stock solution.

Organism and culture condition

Axenic culture of cyanobacterial strain, *C. turgidus* NTMS12, was originally isolated and maintained in Microalgal Repository at Department of Microbiology, Bharathidasan University, Tiruchirappalli, India. Pure culture was grown on BG 11 medium, which was composed of the followings: $NaNO_3$ (1·75 g), K_2HPO_4 (0·02 g), $MgSO_4·7H_2O$ (0·075 g), $CaCl_2·2H_2O$ (0·036 g), Na_2CO_3 (0·02 g), citric acid (0·006 g), ferric ammonium citrate (0·006 g), EDTA (0·001 g) and trace elements solution (1 ml) in 1000 ml distilled water. Trace element solution (1 ml) containing H_3BO_3 (2·86 g), $MnCl_2·4H_2O$ (0·22 g), $Na_2MoO_4·2H_2O$ (0·39 g), $CuSO_4·5H_2O$ (0·079 g) and Co $(NO_3)_2·6H_2O$ (0·0494 g) in 1000 ml distilled water. The pH of the medium was adjusted to 7·0. The cells were grown in sterile flasks containing 100 ml of BG 11 medium. Experimental cultures were incubated at 25 ± 2°C, 14-/10-h light/dark cycle, with illumination of 27 μmol m^{-2} s^{-1} under cool white fluorescent lamps. The cultures were gently shaken by hand on alternate days.

Experimental design

The experiments were carried out in 250-ml Erlenmeyer flask containing the 5 ml of concentrated suspension and the various concentrations of CP (6, 9 and 12 mg l^{-1}) to find the lethal dosage. Chlorophyll-*a* was estimated at the 7 days of incubation. The lethal concentration of 6 mg l^{-1} was used throughout the study to analyse the changes in enzyme activity and fatty acid profile. Cyanobacterial cells without CP in the medium served as a control.

Chlorophyll-*a*

zChlorophyll-*a* was extracted in 80% methanol and measured spectrophotometrically at 663 nm (Mackinney 1945).

Proline

Cells (50 mg) were suspended in 10 ml of 3% sulphosalicylic acid and centrifuged at 5000 g for 10 min to remove cell debris. To 2 ml of supernatant, 2 ml of ninhydrin was added, followed by the addition of 2 ml glacial acetic acid and incubated at boiling temperature for 1 h. The mixture was extracted with toluene. Proline was quantified spectrophotometrically at 520 nm from organic phase (Bates *et al.* 1973).

Superoxide dismutase

Cyanobacterial biomass (50 mg) was homogenized in 0·25 ml cold ethanol and 0·15 ml cold chloroform. Supernatant obtained after centrifugation of the homogenate was used for the enzyme assay. To the supernatant 2 ml Tris (pH 8·2), 0·5 ml pyrogallol and 1·5 ml distilled water were added. Absorbance was read at 470 nm (Marklund and Marklund 1974).

Catalase

Cyanobacterial biomass (50 mg) was homogenized with 2 ml of extraction buffer (0·5 mol l^{-1} phosphate buffer, pH 7·5). The homogenate was centrifuged at 12 000 g for 20 min, and the supernatant (enzyme extract) was separated for assay. To 100 μl of enzyme extract, 1·6 ml phosphate buffer, 0·2 ml 0·3% H_2O_2 and 3 mmol l^{-1} EDTA were added and incubated for 3 min. One unit of enzyme is the amount necessary to decompose 1 μl of H_2O_2 per minute at 25°C. The absorbance of the supernatant was observed at 240 nm against blank (Aebi 1984).

Lipid extraction

After cultivation, culture was centrifuged at 10 000 g, 4°C for 4 min to collect the biomass and freeze-dried under lyophilizer. The cell powder was extracted with methanol/chloroform mixture (2 : 1 v/v) for 20 min under continuous shaking. The mixture was filtered to separate the cell-free organic phase, washed with water, vortex and centrifuged to remove the upper aqueous phase. Further, the lower organic phase was rinsed twice with equal volume of methanol/water (1 : 1 v/v). Finally, the extracted lipid was collected from the solvent phase and evaporated under vacuum (Praveenkumar *et al.* 2012a).

Preparation of fatty acid methyl esters

The weighed lipid and methanolic sulphuric acid (2% H_2SO_4 in methanol) were refluxed for 4 h. The contents were mixed thoroughly with equal volume of distilled water with separating funnel. Then, the aqueous layer was extracted twice with ethyl acetate. The collected ethyl acetate extract containing the fatty acid methyl esters (FAME) was dried over anhydrous sodium sulphate to remove any excess moisture and concentrated under vacuum. The dried FAME of both control and pesticide-treated cells were analysed by gas chromatograph (Praveenkumar *et al.* 2012b).

Gas chromatographic (GC) Analysis

The fatty acid samples were analysed by gas chromatograph (GC2014; Shimadzu, Kyoto, Japan) with flame ionization detector (FID). One microlitre of each sample was injected in the FAME WAX column (Restek, Bellefonte, PA, USA) (30 m × 32 mmID × 25 μm film thickness). Temperature programme was as follows: initial 140°C with 5 min hold; ramp 2°C min^{-1} to 230°C with a 5 min hold. Column flow was set at 22·2 ml min^{-1}. Instrument condition was as follows: carrier gas nitrogen; FID set at 260°C, split ratio of 10 : 1. Run time for a single sample was 55 min (Praveenkumar *et al.* 2012b). Each sample was performed in triplicates, and fatty acid identification was run by comparison with standard certificate, Supelco FAME mix C4-C24 (Sigma-Aldrich, Bellefonte, PA, USA).

Statistical analysis

Data were statistically analysed, and the results were expressed as means ± standard deviation of three independent replicates. A *t*-test for equality of means was carried out to analyse the data at 99% confidence interval of the difference.

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Conflict of interest

No conflict of interest declared.

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