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Alteration in Growth and Physiological Activities in *Chlorella vulgaris* under the Effect of Photosynthetic Inhibitor Diuron

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ABSTRACT

In this study, we describe the effect of diuron, a photosynthesis-inhibiting herbicide, on growth, photosynthetic pigment contents, carbohydrate, protein, amino acids, proline, nitrate reductase and some antioxidant enzymes in cell suspensions of *Chlorella vulgaris* for seven days. The growth (cell number & dry weight) was seriously affected even with lowest diuron dose (0.1 µM). Photosynthetic pigment contents (chl-a, chl-b & caroteniod) of diuron-treated algae, was more sharply decreased as compared to control. Diuron induced oxidative stress as indicated by the alteration of ascorbate oxidase. Nitrate reductase (NR) activity in the volume of algal suspension was significantly decreased with increasing diuron doses. Guaiacol peroxidase was un-detectable in diuron-treated and un-treated, which may be due to hypersensitivity of *C. vulgaris* towards toxic compounds. Proline, total amino acids, carbohydrate and protein contents of algal suspension decreased significantly in response to diuron treatments. All results showed a negative correlation with treatments except ascorbate oxidase activity.

Key Words: Antioxidant enzymes; *C. vulgaris*; Diuron; Growth; Nitrate reductase; Pigments

INTRODUCTION

Herbicides are the principal method of weed control but the introduction of these compounds into the aquatic environment through runoff may have severe consequences for non-target plants. The widespread use of herbicidal chemicals in weed control has created problems of toxic residues in the natural environment (Glotfelty *et al*., 1984; Leonard, 1990). The adverse effects of these pollutants on non-target plants are particularly of concern due to their increasingly worldwide use (Van den Brink & Ter Braak, 1999). Phenylurea herbicides are widely used throughout the world for the protection of several crops and consequently, can have residual effect on crops, soil and surface waters (Peña *et al*., 2002).

Diuron, 3-(3,4-dichlorophenyl)-l,l-dimethylurea), has been shown to inhibit photosystem-II (PSII) electron transport, chlorophyll content and affect growth, cell organelles and metabolism of photosynthetic plants (Oettmeier, 1999; Fayez, 2000a; Eullaffroy & Vernet, 2003). Moreover, the herbicide diuron is commonly incorporated into antifouling paints to boost the efficacy of the compound towards algae (Chesworth *et al.,* 2004).

Algae play an important role in the equilibrium of aquatic ecosystems, being the first level of the trophic chain to produce organics and oxygen (Campanella *et al*., 2000). The use of photosynthetic organisms in toxicity tests is very appropriate because more than 65% pesticides are herbicides (USDA, 1998). Furthermore, about 50% of these herbicides act via inhibition of photosynthesis at the photosystem-I (PSI) and PSII levels by replacing PSI's ultimate electron acceptor (Powles *et al*., 1997) or by blocking PSII-catalyzed photosynthetic electron transport. Pigments have often been used as biomarkers of exposure to different class of herbicides in autotrophic plants including algae (Blaise, 1993; Sandmann, 1993).

In this study, we investigated the response of *Chlorella vulgaris* to diuron (PS II inhibitor) in terms of growth, photosynthetic pigment contents, activity of nitrate reductase, some antioxidant enzymes and metabolites of carbohydrate, protein and amino acids were determined.

MATERIALS AND METHODS

Algal culture conditions. The freshwater unicellular green algae *C. vulgaris* was isolated from Nile water. An axenic culture was maintained in 500 mL glass Erlenmeyer flasks filled with 250 mL of Beijerink medium (Stein, 1966). The pH was adjusted at 6.8 before sterilization. Cultures were maintained at 25°C under a light intensity of 160 µmol m^{-2} s^{-1} with 16 h light period, shaken at 60 rpm on an orbital shaker and kept in suspension by bubbling air. Initial density of the *C. vulgaris* cultures was 273×10^4 cells mL^{-1} (Fig. 1). The optimal pigment (chl- a, b & caroteniod) contents for algal suspension experiments, was found to be 3.03 µg mL^{-1}. Measurements were performed at zero time (prior to algal treated with herbicide).

Application of herbicide. Diuron herbicide stock solutions were made in acetone followed by 0.1 – 0.5 µM concentrations prepared from stock. Algal solutions containing equivalent solvent concentrations in the absence of herbicide were used as controls. All experiments were replicated three times.

Cell number and its dry weight. Hemacytometer, 0.1 mm deep, having improved Naubauer ruling (A.O. Spencer "Brght fine") was used. The mean counts of three replicates were taken in consideration and the data were given as cell

mL^{-1} algal suspension. Dry weight was determined according to Utting (1985). Culture aliquots (50 mL) were filtered through previously dried and weighed Whatman GF/C filters. Thereafter the filters were dried in an oven at 80°C for 72 h.

Photosynthetic pigment extraction. Chlorophyll *a*, *b* and Caroteniod were extracted in 100% methanol at 65°C and their contents were determined spectrophotometrically (Spekol 11, Carl Zeiss, Jena, Germany) according to Metzner *et al*. (1965).

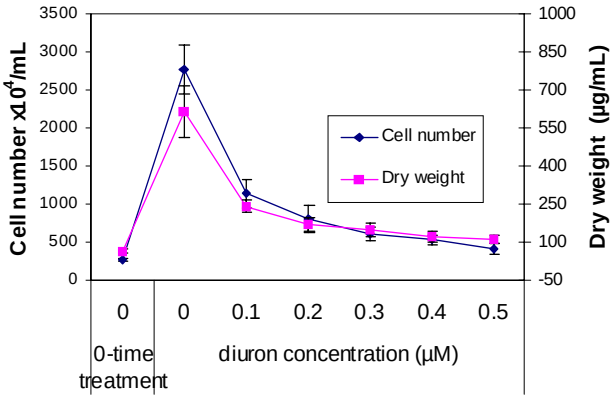
Nitrate reductase assay *in vivo*. For *in vivo* assay of nitrate reductase (EC 1.7.1.1), the method of Jaworski (1971) was used. Algal cells of 10 mL algal suspension of diuron-treated alga and un-treated were precipitated and incubated anaerobically in dark for 1 h in 5 mL of 0.1 M K-phosphate (pH = 7.5) containing 50 mM KNO_3 and 1% (v/v) n-propanol at 28ºC. The reaction was stopped by boiling in water bath for 5 min and then centrifuged. The supernatant of one mL sample mixed well with two mL 1% w/v sulphonilamide in 1 N HCl and 2 mL 0.1% w/v N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water. The absorbency was measured by using spectrophotometer at 540 nm. Nitrate reductase activity was expressed as ng NO_2 mL^{-1} algal suspension h^{-1}.

Ascorbic acid oxidase (EC 1.10.3.3) and peroxidase (EC 1.11.1.7) determination. For ascorbic acid oxidase, the method of Olliver (1967) modified by Chinoy (1976) was used. The reaction mixture consisted of 1 mL of 0.1 mm ascorbic acid, 1 mL of 0.5 mm phosphate buffer (pH 6.8) and 2 mL of enzyme extract. This reaction mixture was incubated at 37°C for 25 min. After incubation the 2, 6-dichlorophenol indophenol was added and the absorbance was taken at 620 nm. The peroxidase activity was measured by following the change of absorption at 470 nm due to guaiacoil oxidation. The activity was assayed for 1 min in a reaction solution (3 mL final volume) composed of 100 mm potassium phosphate buffer (pH 7.0), 20 mm guaiacoil, 10 mm H_2O_2 and 50 µL of crude extract (Polle *et al.*, 1994).

Estimation of proline. Free proline content of algal suspension was determined according to Bates *et al*. (1973). Ten mL of algal suspension was centrifuged and the alga extracted in five mL of aqueous 3% sulfosalicylic acid for 3 h. The extract was centrifuged at 1500 rpm for 10 min. and two mL of the supernatant were mixed with two mL of fresh acid ninhydrin solution and 2 mL glacial acetic acid in a test tube for 1 h at 100ºC. The reaction was terminated in an ice bath and the mixture was extracted with four mL toluene. The extract was vigorously stirred for 20 s using a test tube stirrer. Therefore the chromophore-containing toluene was aspirated from the aqueous phase and its absorbance was measured at 520 nm. Proline was used as a standard.

Protein content. Protein content was determined according to Lowry *et al*. (1951). Ten mL algal suspension was extracted in distilled-water (soluble protein) and in NaOH (total protein) for two h at 90°C. The extract was centrifuged and the supernatants were pooled. The water-soluble protein was estimated by the Folin-phenol reagents spectrophotometrically using bovine serum albumin was used as standard.

Fig. 1. Cell number and dry weight contents of diuron treated and untreated *C. vulgaris* for 7 days as well as the initial cell number and dry weight at zero time treatment. Values are the mean of three replicates. Vertical bars are SD. Statistical significance of differences compared to control. The values of cell number and dry weight are significant at all diuron doses (P > 0.01; r =-0.84 & -0.81 for cell number and dry weight, respectively)



Carbohydrate content estimation. Carbohydrate content was determined in aqueous (soluble carbohydrate) and in HCl solutions (total carbohydrate) with anthrone sulphuric acid reagent according to Fales (1951), using glucose as a standard. The blue green color developed was measured at the 620 nm using spectrophotometer.

Statistical analysis. All data are the mean of three replicates. Data were statistically analyzed by a one-way ANOVA. Means in individual experiments were tested for significance at $P < 0.01$. Correlation coefficient (r) of results was also performed.

RESULTS AND DISCUSSION

After 7 days, the growth of *C. vulgaris* towards increasing concentrations of diuron in terms of cell number and dry weight was significantly decreased (Fig. 1). The cell number increased by tenfold (2760.333 X 10^4) compared to the initial cell number. After seven day of treatment, the cell number at 0.1 µm and 0.5 µm diuron decreased by 2.4 and 6.7 fold, respectively ($P < 0.01$; $r = -0.84$). The dry weight also decreased significantly with increasing diuron doses (Fig. 1). The dry weight of *C. vulgaris* after seven days of 0.1 and 0.5 µm diuron treatment was lowered by 2.5 and 5.5 fold, respectively in comparison with the control ($P< 0.01$; $r = -0.81$), which is inline with previous reports (Shehata *et al*., 1997; Rioboo *et. al*., 2002).

Photosynthetic pigments were significantly decreased ($P < 0.01$; $r = -0.87$) in response to increasing diuron treatments (Table I). After 7 days treatments, the total pigment contents (chl-a, chl-b & caroteniod) at lowest (0.1 µM) and highest (0.5 µM) doses indicated 45.60 and 14.09% respectively of the control (Table I). Pigments deficiency can be caused by photobleaching or by inhibition

Fig. 2. Proline and total amino acid contents of diuron treated and untreated *C. vulgaris*. Values are the mean of three replicates. Vertical bars are SD. Statistical significance of differences compared to control. The values of proline and amino acids are significant at all diuron doses (P > 0.01; r =-0.90 & -0.75 for proline and total amino acids, respectively)

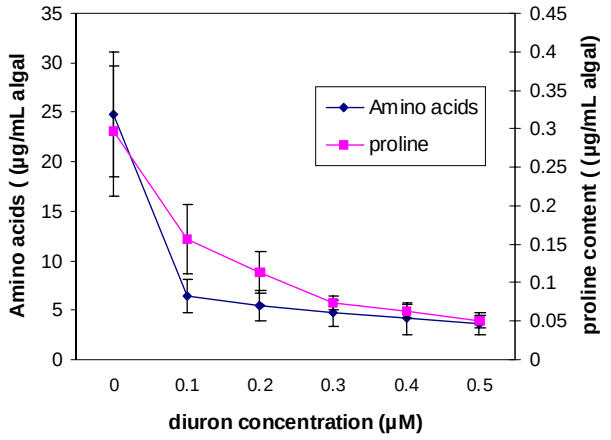
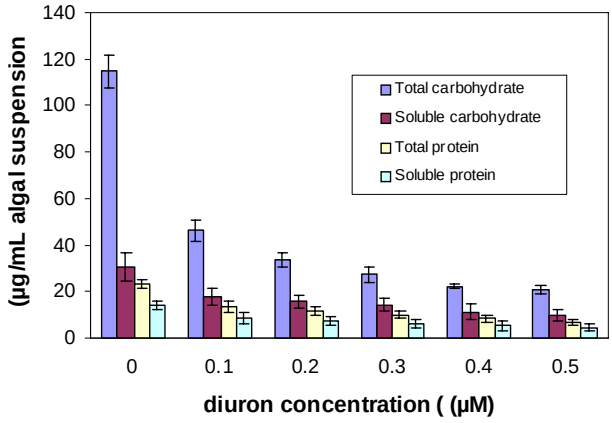


Fig. 3. Total and soluble metabolites of protein and carbohydrates of diuron treated and untreated *Chlorella vulgaris*. Values are mean of three replicates. Vertical bars are SD. Statistical significance of differences compared to control: The values of carbohydrates and proteins are significant at all diuron doses (P > 0.01; r =- -0.82, -0.89, -0.90, -0.91 for total carbohydrate, soluble carbohydrate, total protein & soluble protein, respectively)



of their biosynthesis (Barry *et al*., 1990; Fayez, 2000a; Couderchet & Vernet, 2003). Böger and Sandman (1990) reported that photosynthetic inhibitors inhibited the formation of protoporphrin IX in *Scenedesmus*, soybean and *Lemna*. Photosynthesis inhibition is an important indicator of the toxic effect of pollutants (Gonen-Zurgil, 1997; Hargers *et al*., 1998). Ascorbate oxidase activity (Table II) was significantly enhanced in response to diuron doses comparing with the control ($P < 0.01$; $r = 0.67$). The highest activity (229% of control) was detected with 0.3 µm diuron. Such an effect might be due to blocking of photosynthetic electron transport (Barry *et al*., 1990; Sandmann, 1993). Guaiacoil peroxidase was un-detectable in diuron-treated and un-treated *C. vulgaris* (Table II). The absence of

Table I. Photosynthetic pigments (µg mL^{-1} algal suspension) of diuron treated and untreated of *C. vulgaris* for 7 days. Values are the mean ± SD of three replicates. Statistical significance of differences compared to control: *, significant at P > 0.01; r =- 086, -0.89, and - 0.86 for Chl-a, Chl-b and Caroteniod, respectively

Diuron doses (µM)	µg/ml algal suspension			Total pigments	% of control
	Chl-a Mean ± SD	Chl-b Mean ± SD	Caroteniod Mean ± SD		
0.0	7.45 ± 0.88	2.88 ± 0.38	2.87 ± 0.25	13.2	100
0.1	3.32* ± 0.33	1.42* ± 0.31	1.27* ± 0.19	6.02	45.60
0.2	2.25* ± 0.27	1.05* ± 0.17	0.96* ± 0.25	4.26	32.27
0.3	1.81* ± 0.21	0.79* ± 0.11	0.78*± 0.18	3.39	25.68
0.4	1.41* ± 0.47	0.55* ± 0.15	0.60* ± 0.24	2.57	19.47
0.5	0.98* ± 0.26	0.42* ± 0.12	0.46* ± 0.14	1.86	14.09

Table II. Enzyme activities of diuron treated and untreated *C. vulgaris* for 7 days. Values are the mean ± SD of three replicates. Statistical significance of differences compared to control: *, significant at P > 0.01; r = 0.67 and -0.89 for ascorbate oxidase and nitrate reductase activities, respectively

Diuron doses (µM)	Ascorbate oxidase activity (%) of control	NR activity ng NO_2 mL^{-1} h^{-1}	Guaiacoil peroxidase activity
0	100	26.33 ± 2.08	Nd
0.1	121	20.33* ± 2.08	Nd
0.2	191	05.33* ± 1.52	Nd
0.3	229	04.40* ± 1.80	Nd
0.4	217	02.86* ± 0.40	Nd
0.5	160	02.26* ± 0.41	Nd

Nd = Non- detectable

guaiacoil peroxidase may explain the hypersensitivity of the alga to diuron. Peroxidase is one of antioxidant enzymes scavenge reactive oxygen species (ROS). Oxidative stress occurs when a plant is subjected to biotic and abiotic stresses due to production of ROS (Mittler, 2002; Blokhina *et al*., 2002; Clarke *et al*., 2002; Ernani *et al*., 2003).

In-vivo NR activity was significantly decreased in response to diuron treatments (Table II). The inhibition was most obvious with increasing diuron doses ($P < 0.01$; $r = -0.89$). At 0.1 and 0.5 µM diuron doses, the activity of NR was 77 and 8.6%, respectively of the control value. NR activity of plant was inhibited by various stresses (Aslam *et al*., 1984; Megharaj *et al*., 1993; Fayez, 2000b; Quaggiotti *et al*., 2004; Mallick, 2004).

Proline and total amino acids contents mL^{-1} of algal suspension were significantly decreased (Proline: $P < 0.01$; $r = -0.89$ & total amino acids: $P < 0.01$; $r = -0.74$) with increasing diuron doses to those of the control (Fig. 2). One mL of algal suspension treated with lowest (0.1 µM) and highest dose (0.5 µM) of diuron was 52 and 17%, of control, respectively. Total amino acids contents mL^{-1} of algal suspension at 0.1 and 0.5 µM of diuron were 25 and 15% of the control, respectively. However proline and amino acids contents expressed on cell dry weight basis showed an increase at various diuron doses. For example, the proline content of control and 0.1µM diuron was 481 and 647 µg g^{-1} dry weight of alga, respectively. Such an increase in proline level in plant roots has been reported, indicating modulated metabolic activities of cell under stress conditions (Fayez & Kristen, 1996; Fayez, 2000a). Protein

and carbohydrate contents (total & soluble) of algal also decreased significantly ($P < 0.01$) with increasing diuron doses (Fig. 3). However calculation the concentration of protein and carbohydrate on the basis of dry weight showed an increase in their contents with increasing diuron doses. The soluble protein content at control and lowest dose (0.1 µm) was 23 and 35 mg g^{-1} dry weight, respectively.

We conclude that changes in growth, pigment levels and metabolite constituents and antioxidants in *C. vulgaris* can be used effectively as tools to evaluate the toxicity effects of diuron and possibly of other photosynthetic inhibitors.

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