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Chlorophyll *a* Fluorescence and Photosynthetic Activity as Tools for the Evaluation of Simazine Toxicity to *Protosiphon botryoides* and *Anabaena variabilis*

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On studying the effect of simazine on *Protosiphon botryoides* and *Anabaena variabilis*, data revealed that chlorophyll *a* content and dry weight were decreased with the increase in simazine concentration. High concentration of simazine (0.8 mg L^{-1}) reduced gross photosynthesis and carbohydrate content, whereas protein content and respiration rate were increased. Algal cell recovery from simazine toxic effect occurred after 2 and 4 days for *Anabaena* and *Protosiphon*, *respectively*, which may be attributed to the difference in algal genotype of the tested organisms. © 2001 Academic Press

Key Words: **algae; *Anabaena*; *Protosiphon*; herbicide; simazine; photosynthesis; recovery; respiration; fluorescence.**

INTRODUCTION

The widespread use of herbicides in modern agriculture can have adverse effects on soil algal flora. Many reports indicate interaction between soil algae and herbicides, including effects of herbicides on algal growth, photosynthesis, nitrogen fixation, biochemical composition, and metabolic activities (Singh and Tiwari, 1988; Bhunia *et al*., 1991; El-Sheekh *et al*., 1994; Caux *et al*., 1996). With the increasing and widespread use of agriculture chemicals, it becomes more important to study the effect of these substances on soil algae.

Simazine (princep) is a triazine herbicide widely used for control of algae and submerged weeds in ponds. Its mode of action is a photosynthetic electron transport inhibitor at photosystem II (Rochaix and Erickson, 1988). Bonilla *et al.* (1998) investigated toxicity of paraquat and simazine herbicides to marine microalgal communities using photosynthesis as a test parameter. Simazine was more toxic than paraquat at similar concentrations.

Growth inhibition of algae by simazine has been reported by many authors (Ellis *et al*., 1976; Torres and O'Flaherty, 1976; Mehta and Hawxby, 1979; O'Neal and Lembi, 1983; Fournadzhieva al., 1995).

The present investigation was aimed to elucidate the effect of simazine on *Protosiphon* and *Anabaena* growth, photosynthetic activity, and respiration as important parameters in the evaluation of herbicide toxicity. Algal recovery after simazine removal was also followed in both organisms.

MATERIAL AND METHODS

Organisms and culture conditions. *Protosiphon botryoides* (Kuetzing) Klebs, a simple siphonous chlorophyceae, and the filamentous cyanophyceae *Anabaena variabilis* Keutzing were isolated from paddy field of Qalubia, Egypt (1996). Isolation and purification were made by moist plate method. *P. botyoides* was grown in 250-ml flasks containing 100 ml of sterile Bold's basal medium (BBM) (Nichols, 1973) and incubated in a controlled growth chamber at $24 \pm 2°C$ and 70 $\mu mol\ m^{-2}\ s^{-1}$, while cultures of *A. variabilis* were grown in 100 ml of sterile BG-11 medium free of combined nitrogen source (Stainer *et al*., 1971). Cultures were incubated in a controlled growth chamber at $28 \pm 2°C$ and 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) provided by cool white fluorescent lamps set on 16/8 light/dark photoregime for 16 and 10 days for *P. botryoides* and *A. variabilis*, respectively. Simazine herbicide (formulation, 90%) was obtained from Ciba Geigy. Concentrated herbicide stock was prepared by appropriate dilution in acetone, which was then completely evaporated to dryness. Based on the results of the preliminary experiments, an aliquot of the prepared stock herbicide was added to each culture to obtain final concentrations. All culture flasks (three per treatment) received the same inocula (200 $\mu g L^{-1}$ chlorophyll *a* (Chl *a*) and were incubated under the prescribed growth conditions. Recovery of growth was followed after resuspension of *Protosiphon* cells, which were previously exposed to simazine herbicide and reincubated under the prescribed growth conditions.

Growth measurements. Chlorophyll *a* content was determined fluorometrically with a Turner 111 fluorometer.

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Optimum sensitivity for chlorophyll *a* measurements was obtained at an excitation wavelength of 430 nm and an emission wavelength of 663 nm. The specific growth rate [$\mu m\ day^{-1}$ (d^{-1})] was determined for individual cultures by linear regression through Chl *a* data, and the harvested cells were dried at 105°C for 8 h to obtain dry weight according to APHA (1992). Proteins were determined by Bradford method following extraction of Jones *et al.* (1989). Total carbohydrates were extracted according to Myklestad and Haug (1972) and then determined as described in Dubois *et al.* (1956).

Photosynthetic activity measurements. Gross photosynthesis (P_m) and dark respiration (R_d), were determined as O_2 exchange at 24 ± 2°C in a Hansatech DW3 water-jacketed, 10-ml polarographic electrode chamber according to Henley *et al.* (1991).

Fluorescence measurements at 77 K. A customized, computer controlled fluorometer was used for measurement of fluorescence at 77 K (Henley *et al.*, 1991).

Statistics. Results were tested (SYSTAT 7.0) by one-way analysis of variance (ANOVA), if ANOVA was significant, followed by one-sided nonparametric or parametric Dunnett's post hoc comparisons to corresponding controls (simazine-free) with each herbicide treatment. ANOVA effects and treatment differences were considered significant when $P < 0.05$.

RESULTS

One-way ANOVA revealed a significant effect of simazine on *P. botryoides* for all variables except Chl *a* [$mg\ g^{-1}$ dry weight (DW)] ratio and carbohydrate content (Table 1). Specific growth rate (μm, Days 2–8) significantly increased at low simazine concentration, but significantly decreased at the highest concentration. Chl *a* and DW yields significantly decreased at 0.4 and 0.8 $mg\ L^{-1}$ simazine concentrations, as presented in Table 1. Chl *a* ($mg\ g^{-1}$ DW) increased significantly at 0.2 and 0.4 $mg\ L^{-1}$ simazine. Dry weight normalized protein content increased significantly with increasing simazine concentration, whereas carbohydrate content was not significantly affected by simazine treatment. Variable fluorescence to maximal fluorescence (F_v/F_m), which is an indicator of the photochemical efficiency of photosystem II, significantly decreased with increasing herbicide concentration (Table 1). Gross photosynthesis with respect to chlorophyll *a* (P_m^{Chl}) significantly decreased at the highest simazine concentration, whereas dark respiration normalized in relation to chlorophyll *a* (R_d^{Chl}) decreased significantly at 0.4 $mg\ L^{-1}$ compared to the control. During the recovery period after subculturing in simazine-free medium, none of the variables analyzed for *P. botryoides* were significantly affected by prior simazine exposure (Fig. 1).

On the other hand, one-way ANOVA revealed a significant effect of simazine on all variables in *A. variabilis* except specific growth rate (μm) and variable fluorescence to maximal fluorescence to maximal fluorescence ratio (F_v/F_m) on Day 10, as presented in Table 2. Chl *a* and DW yields, and Chl *a* ($mg\ g^{-1}$DW) significantly decreased with increasing simazine concentrations, as presented in Table (2). Protein content increased significantly at 0.4 and 0.8 $mg\ L^{-1}$ of simazine, whereas carbohydrate content significantly decreased at the highest concentration. F_v/F_m ratio was

TABLE 1
Growth and Photosynthetic Parameters of *Protosiphon botryoides* on Day 16 of Simazine Treatment and after the Recovery Period of 16 Days

Simazine $mg\ L^{-1}$	μm Chl per day (d)	Chl *a* ($mg\ L^{-1}$)	DW ($mg\ L^{-1}$)	Chl *a*	Protein ($mg\ g^{-1}$ DW)	Carbo.	F_v/F_m	Gross P_m ($mol\ O_2 \cdot g^{-1}\ Chl \cdot h^{-1}$)	R_d
Control	0.10 ± 0.01	2.89 ± 0.26	401 ± 36	7.3 ± 1.2	89 ± 13	143 ± 14	0.77 ± 0.01	135 ± 17	− 29 ± 4
0.2	0.15 ± 0.01**	3.35 ± 0.55	315 ± 6	10.7 ± 2.0	96 ± 7	160 ± 15	0.73 ± 0.00***	135 ± 12	− 20 ± 2
0.4	0.09 ± 0.01	1.66 ± 0.31**	149 ± 8***	11.1 ± 2.1	157 ± 9***	169 ± 68	0.68 ± 0.01***	121 ± 15	− 11 ± 8**
0.8	0.04 ± 0.01***	0.66 ± 0.08***	83 ± 5***	8.0 ± 1.4	251 ± 16***	110 ± 6	0.57 ± 0.01***	97 ± 14*	− 33 ± 4
One-way ANOVA	***	***	***	NS	***	NS	***	*	**
Recovery									
Control	0.25 ± 0.04	1.89 ± 0.19	301 ± 6	6.3 ± 0.5	112 ± 6	171 ± 8	0.77 ± 0.02	210 ± 43	− 35 ± 11
0.2	0.23 ± 0.01	1.68 ± 0.29	307 ± 23	5.5 ± 1.0	119 ± 9	190 ± 23	0.78 ± 0.00	160 ± 6	− 35 ± 6
0.4	0.22 ± 0.03	1.92 ± 0.17	299 ± 6	6.4 ± 0.4	109 ± 2	192 ± 36	0.79 ± 0.02	163 ± 9	− 31 ± 9
0.8	0.29 ± 0.02	1.87 ± 0.13	319 ± 21	5.9 ± 0.4	107 ± 7	175 ± 7	0.79 ± 0.01	186 ± 27	− 20 ± 10
One-way ANOVA	NS	NS	NS	NS	NS	NS	NS	NS	NS

Note. μ = specific growth rate over Days 2–8.

Means ± SD ($n = 3$). Results of one-way ANOVA and Dunnett's one-sided comparison of treatments to controls indicate *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

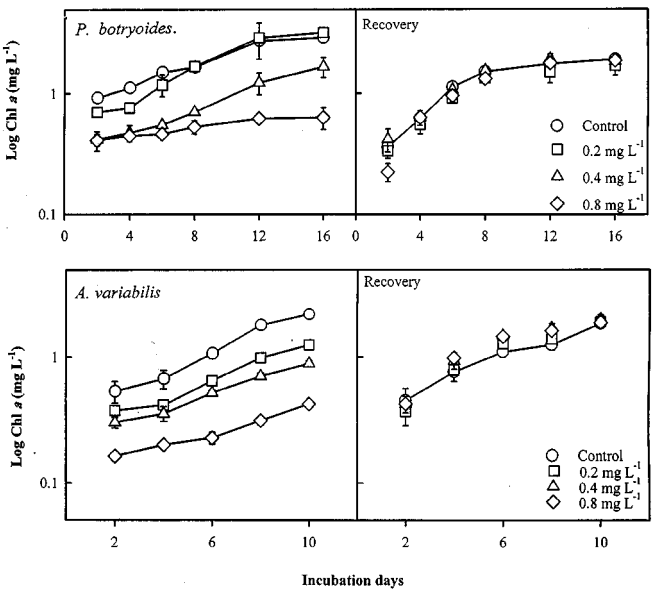

FIG. 1. Effect of simazine on chlorophyll *a* content of *P. botryoides* and *A. variabilies* and its recovery. Means ± SD ($n = 3$).

unaffected by simazine. Gross photosynthesis with respect to chlorophyll *a* (P_m^{Chl}) and dark respiration normalized to chlorophyll *a* (R_d^{Chl}) increased significantly with increasing simazine concentration compared to controls. After 10 days of subculturing of *Anabaena* cells in simazine-free medium, the ANOVA indicated no significant effect of treatment (Table 2).

DISCUSSION

Simazine is a triazine herbicide, which is a photosynthetic electron transport inhibitor that disrupts photosystem II efficiency (Rochaix and Erichson, 1988; Hansson and Wydrzynski, 1990; Tomlin, 1994). Many studies have revealed the effects of simazine on photosynthetic activity of algal species (O'Neal and Lembi, 1983). Results obtained in this investigation indicated that 0.8 mg L^{-1}, simazine significantly reduced biomass yields of both *Protosiphon* and *Anabaena* cultures. Simazine increased cellular protein content but decreased carbohydrate content in both organisms, in agreement with Shabana and Abou-Waly (1995), who stated that the drop in *Nostoc muscorum* carbohydrate content at higher concentrations of triazine herbicides may be attributed to a respective inhibition of algal photosynthesis due to blocked photosynthetic electron transport. Carbohydrate content reduction due to simazine treatment revealed that it has an effect on algal photosynthesis, the process responsible for carbohydrate synthesis (Tomlin, 1994).

Simazine significantly reduced photosystem II efficiency (F_v/F_m) of *P. botryoides*, whereas it was unaffected in *A. variabilis*, which is in agreement with Hansson and Wydrzynski (1990). Brack and Frank (1998) reported that, if the functional state of the photosynthetic apparatus changes, the extent of fluorescence emission also changes. When a toxic compound acts on this system, the change in fluorescence should be different, depending on whether the absorption of light, the electron-transport chain, the generation of proton-motive force, or the ATPase was inhibited.

TABLE 2
Growth and Photosynthetic Parameters of *Anabaena variabilis* on Day 10 of Simazine Treatment and after the Recovery Period of 10 Days

Simazine mg L^{-1}	μm Chl (d^{-1})	Chl *a* (mg L^{-1})	DW (g L^{-1})	Chl *a*	Protein (mg g^{-1} DW)	Carbo.	F_v/F_m	Gross P_m (mol $O_2 \cdot g^{-1}$ Chl $\cdot h^{-1}$)	R_d
Control	0.18 ± 0.06	2.23 ± 0.11	283 ± 36	7.9 ± 0.6	167 ± 13	140 ± 26	0.38 ± 0.02	233 ± 28	− 19 ± 8
0.2	0.14 ± 0.02	1.25 ± 0.00***	195 ± 13***	6.4 ± 0.4**	198 ± 13	133 ± 6	0.41 ± 0.02	407 ± 42***	− 54 ± 10**
0.4	0.14 ± 0.01	0.88 ± 0.05***	153 ± 5***	5.8 ± 0.4***	238 ± 13**	111 ± 14	0.42 ± 0.05	533 ± 29***	− 39 ± 10*
0.8	0.08 ± 0.02	0.42 ± 0.02***	91 ± 6***	4.7 ± 0.4***	337 ± 29***	100 ± 2*	0.39 ± 0.03	380 ± 33***	− 49 ± 10**
One-way ANOVA	NS	***	***	***	***	*	NS	***	**
Recovery									
Control	0.33 ± 0.06	1.85 ± 0.16	344 ± 14	5.4 ± 0.3	139 ± 10	115 ± 12	0.42 ± 0.02	673 ± 20	− 55 ± 30
0.2	0.32 ± 0.06	1.92 ± 0.07	355 ± 12	5.4 ± 0.1	132 ± 2	119 ± 25	0.40 ± 0.02	624 ± 35	− 32 ± 19
0.4	0.32 ± 0.03	2.01 ± 0.18	335 ± 18	6.0 ± 0.5	145 ± 11	133 ± 19	0.41 ± 0.02	715 ± 88	− 36 ± 33
0.8	0.31 ± 0.01	1.89 ± 0.11	337 ± 14	5.6 ± 0.5	145 ± 7	125 ± 11	0.39 ± 0.02	772 ± 35	− 19 ± 5
One-way ANOVA	NS	NS	NS	NS	NS	NS	NS	NS	NS

Note. μ = specific growth rate over Days 2–6.

*Means ± SD ($n = 3$). Results of one-way ANOVA and Dunnett's one-sided comparison of treatments to controls indicate *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant.

Gross photosynthetic capacity of *P. botryoides* decreased and increased in *A. variabilis* with respect to increase in simazine concentrations, a phenomenon that is contradictory to findings of Ellis *et al.* (1976) and Mehta and Hawxby (1979). The most consistent physiological effect of simazine on *A. variabilis* was an increase in respiration rate (Table 2). Elevated respiration rate may be a general stress response and/or may be involved in simazine catabolism. This stimulation of respiration rates under herbicide treatment has been observed by many investigators (Singh *et al.*, 1983; Singh and Tiwari, 1988; Bhunia *et al.*, 1994; Mohapatra *et al.*, 1997). The low concentration of photosynthetic pigment in *Anabaena* could be due to photoxidation arising from the inability of chlorophyll *a* to dissipate its absorbed energy when electron transport is inhibited. Inhibition of electron transport limits the availability of NADPH and chemical energy from ATP (Moreland, 1980). Moreover, any possible suppressive activity on the photosynthetic ATP generation might cause the organism to depend on endogenous carbon reserved (polyglucose and poly- $\propto$ -hydroxy butyrate) and oxidative phosphorylation (respiration) to meet the extra energy demands under stress conditions. The overall increase in respiration rate may be due to a series of mechanisms that microorganisms possess to counteract the effect of toxic chemicals. These mechanisms include uptake, accumulation, biodegradation, and transport of chemicals from the cell (Tiwari *et al.*, 1985).

All measures of growth and physiological parameters indicated that recovery occurred after 4 days in *P. botryoides* and after 2 days in *A. variabilis* when transferred to simazine-free medium, which revealed that simazine has a temporary toxic effect to both organisms. Fournadzhieva *et al.*, (1995) reported that simazine-treated algal cells recovered their growth after transfer to herbicide-free medium.

CONCLUSIONS

High concentrations of simazine in both *Protosiphon* and *Anabaena* cultures reduced biomass yield and carbohydrate contents, which were accompanied by a drop in photosynthetic activity. Cellular protein content and respiration rate were elevated. Recovery of these two algal organisms from toxic effects of simazine depends principally on genotype.

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