Scientific Research and Essay Vol. 4 (11), pp. 1331-1341, November, 2009
Available online at http://www.academicjournals.org/SRE
ISSN 1992-2248 © 2009 Academic Journals

Full Length Research Paper

Metabolic detoxification as a viable mechanism for triazine herbicide resistance in ametryn resistant biotype of the fresh water chlorophyte, *Scenedesmus vacuolatus*

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Accepted 28 September, 2009

The quantitative determination of ametryn, atrazine and prometryn as well as their metabolites in the culture medium of herbicide-treated *Scenedesmus vacuolatus* was undertaken using high-performance liquid chromatography (HPLC). Resistant biotype metabolized about 60% of biologically active herbicides tested into two metabolites for ametryn and three metabolites for atrazine and prometryn. Chromatographic analysis data of the three herbicides tested were strongly correlated with their inhibitory effects on algal growth and reproduction. Susceptible culture of *S. vacuolatus* was able to metabolize about 5% of the applied concentration of ametryn and atrazine into one metabolite (for ametryn) and two metabolites (for atrazine). However, the same biotype was better able to metabolize 15% of applied concentration of prometryn into two metabolites. The bioassay and biochemical data confirmed the role of herbicide metabolic degradation as a main mechanism of resistance toward herbicides. In addition, surface area of resistant cell was about four times greater than susceptible cell; however, no clear difference in the thickness of cell was achieved. Weed resistance toward herbicides could be managed by adding suitable synergists to herbicide formulations and also by integrating herbicides with negative cross resistance in rotation with non-chemical means of weed control.

Key words: Triazine herbicides, resistance phenomenon, resistance metabolic mechanism.

INTRODUCTION

In our complementary study (Ibrahim et al, In press), ametryn selected *Scenedesmus vacuolatus* exhibited great resistance to ametryn and also to other triazine and triazinone herbicides. Metabolic detoxification of herbicides as a major mechanism of resistance was stated by some researchers. Gressel et al. (1983a) reported that atrazine resistant biotype of *Brachypodium distachyon* (*Trachynia distachya*) metabolized 14-C-labeled atrazine more rapidly than the sensitive biotype. Prado et al. (2000) found that atrazine resistant biotypes of *Scenedesmus faberi* and *Scenedesmus viridis* were cross-resistant to all the triazine herbicides used; these biotypes metabolized atrazine to conjugate atrazine faster than the susceptible biotypes. In contrast to the findings of the previously mentioned authors, degradative metabolism for herbicides, as reported by Tang et al. (1998b) and Fedtke (1991) have not been shown to be a major mechanism of resistance. Tang et al. (1998a) extracted glutathione-*S*-transferase (G*S*T) from the freshwater algae and found significant differences in G*S*T activity between species, but reduced activity was not correlated with increased sensitivity to atrazine exposure. Fedtke (1991) investigated the possible resistance mechanism in the mefenacet-adapted strain of the green alga, *Chlamydomonas reinhardtii*. His result showed that neither uptake, metabolism nor lipid or amino acid composition could explain the observed resistance behaviour. In addition to the role of metabolic detoxification in the

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resistance phenomenon, previous studies confirm the importance of target site insensitivity. Lundegardh (1992 a, b, c) stated that atrazine *Monoraphidium pusillum* resistant biotypes have altered electron transport through photosystem II (PSII). Schwenger and Barz (2000) noticed that metribuzin resistant *Chenopodium rubrum* cell cultures were known to possess different double and triple mutations in the photosystem II D1 protein. As reported by Mengistu et al. (2000), metribuzin and diuron resistant biotype of *Poa annua* had a substitution from valine to isoleucine at position 219 of the D1 protein encoded by the psbA gene. Insensitive ACCase was detected in weed resistant to acetyl-CoA carboxylase (ACCase) inhibitors (Cocker et al., 2001) and mutation at the same proline residue in the acetolactate synthase (ALS) gene with higher ALS activity may play a role in the resistance detected in acetolactate synthase (ALS) inhibitors (Sibony and Rubin, 2003; Yu et al., 2003).

The DNA sequence analysis of the psbA gene, which is the target site of PSII inhibitors, demonstrated a single amino acid substitution from serine to glycine in the resistant biotypes at residue 264 in the D1 protein [Kohno et al., 2000; Kumata et al., 2001; Diebold et al., 2003; Patzoldt et al., 2003; Sibony and Rubin, 2003; Chodova and Salava, 2004; Park and Mallory-Smith, 2005). However, atrazine resistant *Amaranthus tuberculatus* did not contain the amino acid substitution that is typically found in triazine-resistant plants which confirm the existence of two distinct triazine resistance mechanisms in *A. tuberculatus*. Also, *P. annua* resistant to diuron or metribuzin, but lacking mutations in the herbicide-binding region indicates that resistance to these herbicides can also be attained by other mechanisms (Mengistu et al., 2000). Moreover, Maertens et al. (2004) measured multiple resistance to triazine and acetolactate synthase (ALS)-inhibiting herbicides in three populations of *Amaranthus hybridus* L. and gene sequencing revealed that a glycine for serine substitution at residue 264 of the D1 protein, and a leucine for tryptophan substitution at residue 574 of ALS were the causes of resistance.

In an attempt to evaluate the mechanisms involved in the resistance of ametryn resistant *S. vacuolatus* toward ametryn and other triazine herbicides, biodegradation of ametryn, atrazine and prometryn in laboratory and resistant culture medium of *S. vacuolatus* were assayed. Also, susceptible and resistant cells were scanned and photographed using the scanning electron microscope to clarify the differences between resistant and susceptible cells.

MATERIALS and METHODS

Herbicides and alga

Three herbicidies belonging to triazine group were assayed (Table 1). All herbicide samples were kind gifts from Weed Research Central Laboratory, Agricultural Research Center, Giza, Egypt. Ametryn susceptible and resistant biotypes of the fresh water chlorophyte, S. vacuolatus, formerly Chlorella fusca var vaculata (Kessler et al., 1997) were used in the current study. The medium of the algae culture consists of five macroelements and seven microelements (Tantawy and Grimme, 1982).

Solvents used in the chromatographic analysis and pigment extraction

Acetonitrile, methanol and tetrahydrofurane (99.99% purity) were purchased from Fisher Scientific International Company and used in the quantitative determination of herbicides and their metabolites. Petrolium ether and acetone (99.99% purity) were purchased from the same company and used in pigment extraction.

Apparatuses used in the present study

These inlude: Centrifuge [VEB MLW MEDIZINTE (HNK T54), rotary evaporator (BÜCHI HB-140), Ultrasonic Cleaning System Model FS9 (Fisher Scientific, 711 Forbes Ave., Pittsburgh Pa. 15219), Spectrophotometer (Motic CL 45240-00), and a High Performance Liquid Chromatography (HPLC) that was purchased from Micromeritics Instrument Corporation which consists of: 1- Model 750 Solvent Delivary System; 2- Model 788 Dual Variable Detector; 3- 3392A Integrator Hewlett. Packard; 4- Model 730 Universal Injector; 5- Series "C" Pressure-Lok Liquid Syringe with Fixed Needle and 6- A Lichrosorb 5 RP-18 column (250 x 4.60 mm).

Herbicide extraction

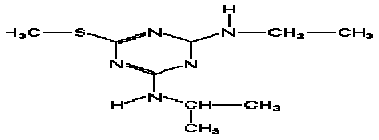
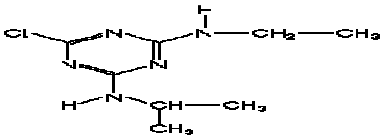
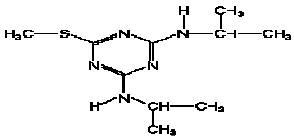
Three triazine herbicides (ametryn, prometryn and atrazine) were selected to conduct this study. The biotypes named S0 and S5 were synchronized to obtain homogeneous cells in the first stage of the growth cycle (details cited in Hassanien, 2007). Selected herbicides were added at 4 µM in 400 µl methanol to the culture medium of S0 and S5. For control treatments, the same amount of each herbicide was dissolved in 400 µl methanol and added to the same volume of algal free medium to study the non-enzymatic degradation of tested herbicides. For each herbicide treatment, three replicates from 4 µM ametryn treated-culture free medium, -S0 culture medium and -S5 culture medium were incubated for 96 h under constant conditions (Hassanien, 2007). Samples (100 ml) of the growth medium (for control treatment) and algal suspension (for S0 and S5 treatments) were taken from the three replicates of each treatment just after adding herbicide to the growth medium and every 24 h up to 96 h post treatment. These samples were centri-fuged at 5000 rpm for 5 min. The S. vacuolatus–free supernatant was completely transferred into the cartridge using a 50 ml syringe with a Luer-lock tip connected to a SEP-PAK cartridge (Waters, Milford, Mass) containing octadecyl-silica as the stationary phase, which was pre-equilibrated first with 5 ml methanol and then with 5 ml doubly-distilled water. The culture medium was passed dropwise through the cartridge to adsorb the lipophilic compounds on the stationary phase. Another 5 ml doubly-distilled water was used to clean the cartridge from all water-soluble compounds, including salts.

The herbicide and its metabolites were eluted from the cartridge with 5 ml methanol. The methanol extract was evaporated to dryness in a rotatory evaporator, and the residue was dissolved in 0.5 ml methanol and used for chromatographic analysis.

Chromatographic analysis

The quantitative determination of herbicides and their metabolites was carried out using high performance liquid chromatography

Table 1. Herbicides used.

Common name	Chemical name	Chemical structure
Ametryn	**IUPAC:** *N*2-ethyl-*N*4-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine. **CAS:** *N*-ethyl-*N'*-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine	
Atrazine	**IUPAC:** 6-chloro-*N*2-ethyl-*N*4-isopropyl-1,3,5-triazine-2,4-diamine. **CAS:** 6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine	
Prometryn	**IUPAC:** *N*2,*N*4-di-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine. **CAS:** *N,N'*-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine	

according to Tantawy et al. (1984). To conduct the experiment, a volume of 5 µl of the methanol extract (0.5 ml) was injected with a precision syringe (Series "C" Pressure-Lok Liquid syringe with Fixed Needle) (vol: 0-25.0 µl). The mobile phase for ametryn and prometryn consisted of eluent 60% (in doubly distilled water) of acetonitrile, methanol and tetrahydrofurane (85:5:15; v/v/v), respectively. While atrazine mobile phase consisted of eluent 65% and 35% acetonitrile in doubly distilled water. The flow-rate for ametryn and prometryn was 1.5 ml/min under 1.91 MPa pressure, while the flow-rate for atrazine was 1 ml/min under 1.71 MPa pressure.

Pigment extraction and determination

The pigment content was determined in 1-10 ml of the algal suspension using the method of Metzner et al. (1965). Cells were harvested by centrifugation at 5000 rpm/min for 10 min. Pigments in the harvested cells were extracted by mixing well with 4 ml of methanol-petroleum ether (10:3, v/v) and incubated in a water bath at 50°C for 15 min, and subsequently left to attain room temperature. The mixture was centrifugated at 5000 rpm/min for 10 min. Then solvents in the mixture were evaporated using the rotatory evaporator. After dryness was attained, the pellet was dissolved in 4 ml acetone 85% then chlorophyll and carotenoids were estimated in the extraction using the spectrophotometer at 663, 644 and 452 nm. Pigment concentrations were calculated according to equations (i) to (iii):

Chl. a (µg/ml acetone) = (10.3 *OD663) – (0.918 *OD644) (i)

Chl. b (µg/ml acetone) = (19.7 *OD644) – (3.87 *OD663) (ii)

Caroteniods (µg/ml acetone) = (4.2 * OD452) – [0.0264 * Chl. (a) – 0.496 * Chl. (b)]. (iii)

The reduction in any of three pigments was calculated with the aid of equation (iv):

% Reduction in pigment concentration (pigment) = [(pigment control) – (pigment treatment)] / (pigment control)]*100. (iv)

Statistical analysis

SPSS and MSTAT-C computer programs were used for analyzing the available data employing analysis of variance followed by Duncan multiple comparison test (Gomez and Gomez ,1984).

RESULTS

After 3 years of preserving S0 and S5 on agar, out of any herbicide stress, the 2 biotypes were evaluated for their ability to degrade the triazine herbicides and also for their tolerance toward herbicides tested. A discriminating concentration for inducing more than 90% inhibition in the growth and reproduction of S0 was selected for this study. The quantitative determination of ametryn, atrazine and prometryn in the culture medium of herbicide-treated *S. vacuolatus* was determined using high-performance liquid chromatography (HPLC). Herbicides tested were also incubated with *S. vacuolatus* free medium as a control treatment to detect the percentages of parental recovery and the metabolites resulting from the non-enzymatic degradation in absence of the alga. For the three herbicides tested, concentrations recovered after applying 4 µM herbicide to culture free medium, laboratory culture medium and resistant culture medium were calculated and data are recorded in Table 2. Percentages of herbicides recovered in the laboratory and resistant culture medium were calculated considering the recovery in the culture free medium as a control (100% recovery, Table 3). In the treatments of ametryn, atrazine and prometryn at 4 µM, using culture free medium, concentrations recovered after 96 h treatment were 3.71, 3.69 and 3.72 µmol/l, respectively (Table 2), which represent 92 to 93% of the applied concentration (Table 3). About 7 - 8% of the applied concentration was lost and

Table 2. Concentrations of ametryn, atrazine and prometryn recovered from the culture medium of *Scendesmus vacuolatus* laboratory and resistant biotypes after 0.0 to 96 h treatments with 4 µM.

Incubation period (h)	Treatments	Concentrations recovered (Mean ± SD) of		
		Ametryn	Atrazine	Prometryn
0	Control	3.98 ± 0.01 a	3.87 ± 0.07 a	3.92 ± 0.05 a
	S0-biotype	3.95 ± 0.04 a	3.80 ± 0.09 a	3.82 ± 0.21 a
	S5-biotype	3.94 ± 0.03 a	3.78 ± 0.08 a	3.76 ± 0.14 a
	F; df; LSD 0.05	1.69 (NS); 2, 6; -	1.03 (NS); 2, 6; -	0.95 (NS); 2, 6; -
24	Control	3.86 ± 0.05 a	3.86 ± 0.06 a	3.82 ± 0.09 a
	S0-biotype	3.78 ± 0.04 a	3.77 ± 0.13 a	3.53 ± 0.16 b
	S5-biotype	2.92 ± 0.06 b	2.82 ± 0.06 b	2.52 ± 0.06 c
	F; df; LSD 0.05	367.90; 2, 6; 0.09	122.79; 2, 6; 0.18	111.48; 2, 6; 0.23
48	Control	3.83 ± 0.06 a	3.82 ± 0.08 a	3.80 ± 0.09 a
	S0-biotype	3.69 ± 0.19 a	3.72 ± 0.03 a	3.43 ± 0.08 b
	S5-biotype	2.66 ± 0.10 b	2.52 ± 0.05 b	2.13 ± 0.07 c
	F; df; LSD 0.05	75.58; 2, 6; 0.25	473.06; 2, 6; 0.11	381.31; 2, 6; 0.15
72	Control	3.72 ± 0.09 a	3.75 ± 0.04 a	3.72 ± 0.10 a
	S0-biotype	3.58 ± 0.16 a	3.61 ± 0.07 a	3.29 ± 0.05 b
	S5-biotype	1.70 ± 0.06 b	1.60 ± 0.17 b	1.89 ± 0.03 c
	F; df; LSD 0.05	315.46; 2, 6; 0.22	372.09; 2, 6; 0.22	679.18; 2, 6; 0.13
96	Control	3.71 ± 0.07 a	3.69 ± 0.06 a	3.72 ± 0.11 a
	S0-biotype	3.53 ± 0.18 a	3.50 ± 0.07 b	3.15 ± 0.11 b
	S5-biotype	1.36 ± 0.14 b	1.44 ± 0.10 c	1.47 ± 0.05 c
	F; df; LSD 0.05	278.19; 2, 6; 0.27	762.56; 2, 6; 0.15	458.20; 2, 6; 0.19

For each herbicide at each incubation period, means followed by the same letter are not significantly different.

there is no evidence that this loss is due to non-enzymatic degradation; HPLC chromatograms of the three compounds in algal free medium did not show any metabolites (Set A in Figures 1, 2 and 3).

When ametryn at 4 µM was incubated with the laboratory culture medium for 24, 48, 72 and 96 h, percentages of ametryn recovery were 97.8, 96.4, 96.3 and 94.7% of the applied concentration, respectively compared to 99.3% initial recovery just after adding the herbicide to the culture medium (Table 3 and Figure 4, set A). Laboratory biotype metabolized 0.8, 2.2, 3.6, 3.7 and 5.3%, of ametryn and there is a peak, appeared at a retention time of 2.06 min, that may be a minor metabolite of ametryn (Set B in Figure 1). This possible metabolite has not been identified because of the unavailability of its reference standard. The differences in the percentages of ametryn recovered from culture free medium and those from laboratory culture medium were only significant at 24 and 96 h treatments. However, the pattern of ametryn degradation was different among laboratory and resistant biotypes at all time intervals. Percentages of ametryn recovered from resistant culture medium were significantly less compared to those recovered from laboratory culture medium at 24, 48, 72 and 96 h treaments [Table 3, Figure 4 (Set A)]. When ametryn was incubated for 96 h with *S. vacuolatus* laboratory culture medium, only 5.3% of the applied concentration was possibly metabolized into a one metabolite having a retention time of 2.06 min, however it was evident that 94.7% of the biologically active herbicide was seperated at a retention time of 4.04 min (Figure 1, Set B). The degradation of ametryn was much pronounced by the resistant biotype. In the 96 h treatment, resistant biotype metabolized 63.4% of the applied concentration into two possible metabolites, a minor metabolite was seperated at 2.06 min, however, a major metabolite was seperated at 1.28 min. Ametryn itself was seperated at 4.04 min (Figure 1, Set C). A possible major metabolite was completely missed in the chromatogram of laboratory biotype reflecting the absence of specific degradative enzyme in this biotype.

Compared to ametryn, atrazine had similar pattern of degradation by laboratory and resistant biotypes. When atrazine was incubated in the laboratory culture medium, percentages of recovery were 98.2, 97.6, 97.3, 96.3 and 94.9 in the treatments of 0, 24, 48, 72 and 96 h, respectively [Table 3; Figure 4 (Set B)]. After an incubation period of 96 h, laboratory biotype might be metabolized only 5.1% of the applied concentration into two metabolites, having a retention time of 1.48 and 6.21 min (Figure 2, Set B). Percentages of atrazine recovered from the resistant biotype culture medium were 97.8,

Table 3. Percentages of herbicides detected by HPLC in the culture medium of *Scendesmus vacuolatus* laboratory and resistant biotypes after 0.0 to 96 h treatments with 4 µM.

Incubation Period (h)	Treatments	% Recovery (Mean ± SD) of		
		ametryn	atrazine	prometryn
0	Control	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
	S0-biotype	99.25 ± 0.67 a	98.18 ± 0.94 b	97.42 ± 4.46 a
	S5-biotype	99.08 ± 0.39 a	97.75 ± 0.50 b	95.74 ± 3.17 a
	F; df; LSD 0.05	3.65 (NS); 2, 6; -	10.67; 2, 6; 1.26	1.38 (NS); 2, 6; -
24	Control	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
	S0-biotype	97.76 ± 0.78 b	97.57 ± 1.99 a	92.30 ± 2.38 b
	S5-biotype	75.50 ± 1.13 c	73.08 ± 0.81 b	65.97 ± 0.67 c
	F; df; LSD 0.05	876.09; 2, 6; 1.58	428.73; 2, 6; 2.49	469.54; 2, 6; 2.85
48	Control	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
	S0-biotype	96.40 ± 3.45 a	97.32 ± 1.47 a	90.18 ± 0.50 b
	S5-biotype	69.44 ± 1.54 b	66.01 ± 2.70 b	56.06 ± 1.46 c
	F; df; LSD 0.05	175.95; 2, 6; 4.36	340.80; 2, 6; 3.54	201.64; 2, 6; 1.78
72	Control	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
	S0-biotype	96.30 ± 2.21 a	96.28 ± 2.74 a	88.41 ± 3.37 b
	S5-biotype	45.78 ± 2.68 b	42.67 ± 4.57 b	50.47 ± 1.43 c
	F; df; LSD 0.05	685.83; 2, 6; 4.00	326.36; 2, 6; 6.15	450.51; 2, 6; 4.22
96	Control	100.00 ± 0.00 a	100.00 ± 0.00 a	100.00 ± 0.00 a
	S0-biotype	94.68 ± 2.81 b	94.87 ± 2.29 b	84.65 ± 4.31 b
	S5-biotype	36.58 ± 2.88 c	38.96 ± 3.24 c	39.63 ± 1.94 c
	F; df; LSD 0.05	690.18; 2, 6; 4.63	653.20; 2, 6; 4.58	397.03; 2, 6; 5.45

For each herbicide at each incubation period, means followed by the same letter are not significantly different.

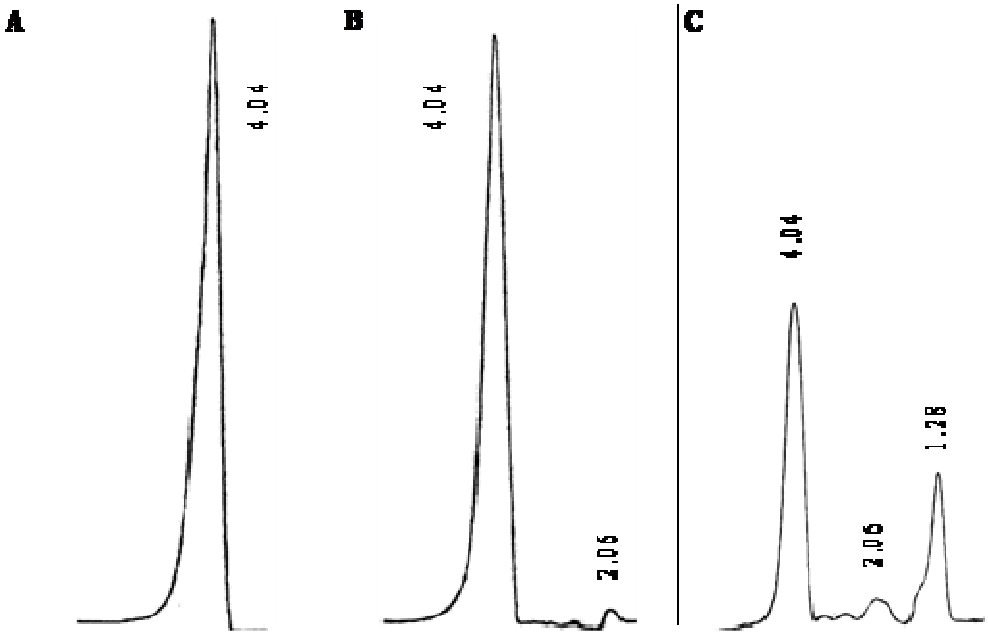


Figure 1. HPLC chromatograms of ametryn and its metabolites extracted from the culture medium of ametryn-treated *Scenedesmus vacuolatus* after 96 h treatment. Isocratic mobile phase consists of 60% of a mixture of acetonitrile, methanol and tetrahydrofuran (85 : 5 : 15 v/v/v) in double distilled water; flow rate 1.5 ml/min; detector adjusted at 230 nm. A: Control (algal free medium), B: Laboratory biotype, C: Resistant biotype.

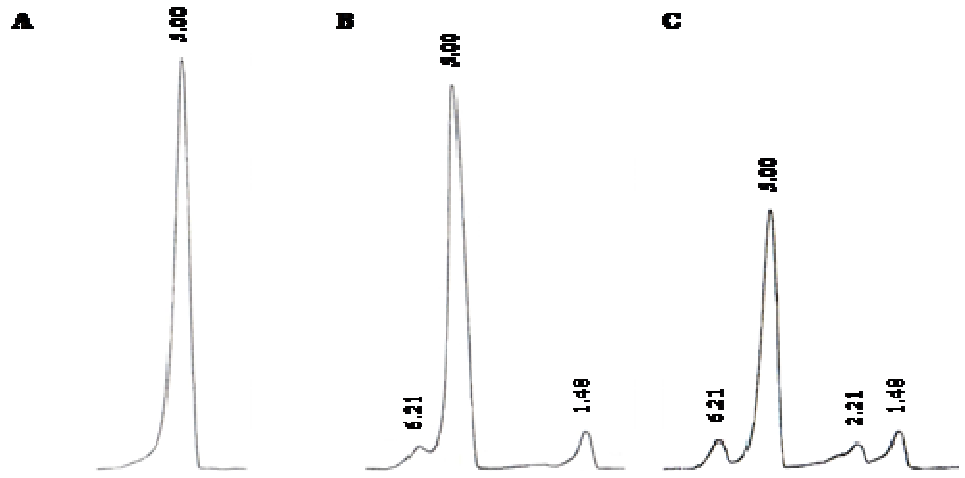


Figure 2. HPLC chromatograms of atrazine and its metabolites extracted from the culture medium of atrazine-treated *Scenedesmus vacuolatus* after 96 h treatment. Isocratic mobile phase consists of acetonitrile in double distilled water (65: 35%); flow rate 1 ml/min; detector adjusted at 220 nm. A: Control (algal free medium), B: Laboratory biotype, C: Resistant biotype.

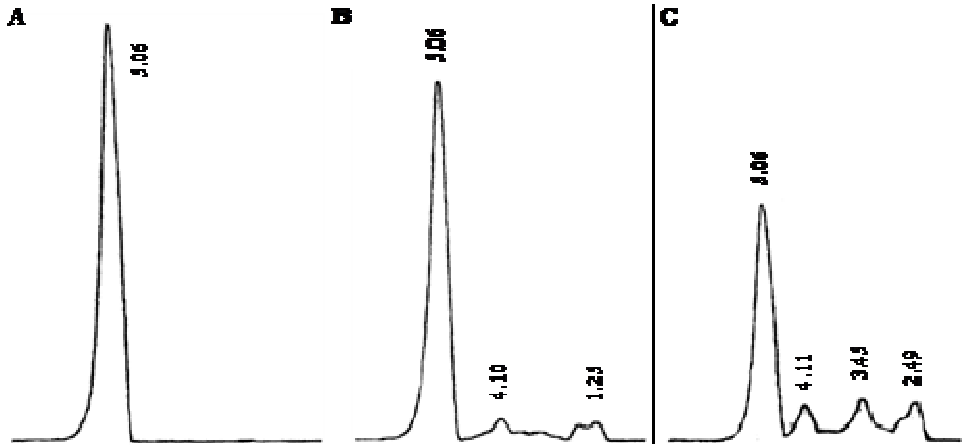


Figure 3. HPLC chromatograms of prometryn and its metabolites extracted from the culture medium of prometryn-treated *Scenedesmus vacuolatus* after 96 hr treatment. Isocratic mobile phase consists of 60% of a mixture of acetonitrile, methanol and tetrahydrofuran (85 : 5 : 15 v/v) in double distilled. water; flow rate 1.5 ml/min; detector adjusted at 230 nm. A: Control (algal free medium), B: laboratory biotype, c: resistant biotype.

73.1, 66.0, 42.7 and 38.9 at 0, 24, 48, 72 and 96 h, respectively [Table 3, Figure 4 (Set B)]. With the exception of 0 h interval, the differences in %atrazine recovered from laboratory and resistant culture medium were significant (Table 3). Resistant biotype metabolized about 61% of atrazine into three possible metabolites. The retention time of the three metabolites was 1.48, 2.21 and 6.22 min. The possible metabolite that ap-peared after 2.21 min in the chromatogram of resistant biotype (Set C in Figure 2) was absent in the chromatogram of laboratory biotype (Set B in Figure 2).

Compared to ametryn and atrazine, laboratory biotype was more active in metabolizing prometryn (Table 3). Percentages of prometryn recovery from the culture medium of laboratory biotype were 97.4, 92.3, 90.2, 88.4 and 84.7 in the treatments of 0, 24, 48, 72 and 96 h, respectively [Table 3 and Figure 4, (Set C)]. After 96 h exposure to prometryn, laboratory biotype metabolized about 15.3% of applied concentration into two possible metabolites; their retention times were 1.25 and 4.1 min.

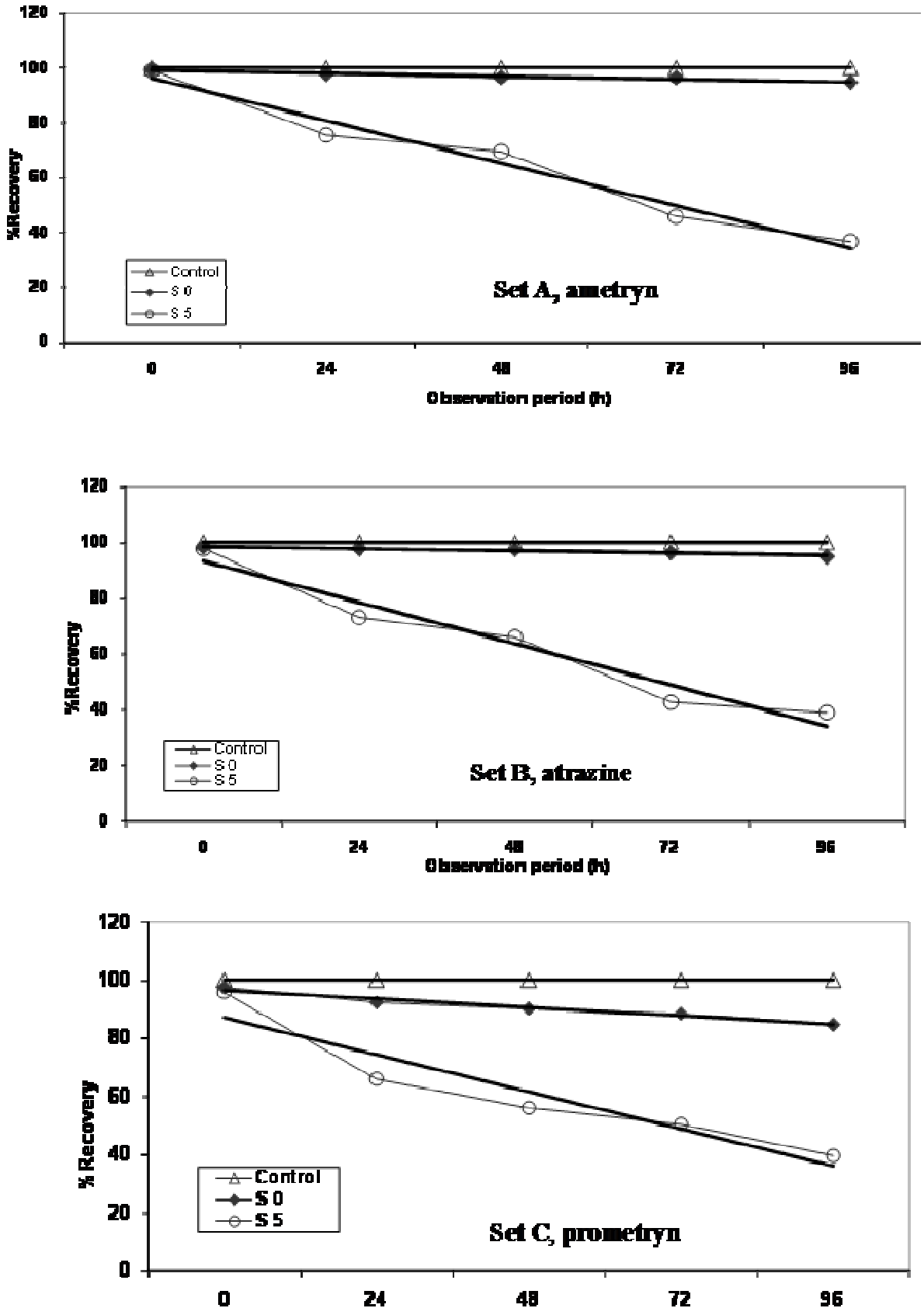


Figure 4. Percentages of ametryn, atrazine and prometryn recovered from the culture medium of 4 µM herbicide-treated *Scenedesmus vacuolatus*.

Table 4. Percentages of herbicides recovered from the culture medium of *Scenedesmus vacuolatus* laboratory and resistant biotypes after 0.0 to 96 h treatments with 4 µM.

Incubation period (h)	Laboratory biotype			Resistant biotype			Statistical analysis		
	Ametryn	Atrazine	Prometryn	Ametryn	Atrazine	Prometryn	F	df	LSD 0.05
0	99.25 a	98.18 a	97.42 a	99.08 a	97.50 a	95.74 a	0.17 (NS)	2, 12	-
24	97.76 a	97.57 a	92.30 b	75.50 c	73.08 c	65.97 d	2.95	2, 12	2.58
48	96.40 a	97.32 a	90.18 b	69.44 c	66.01 c	56.06 d	4.49	2, 12	3.71
72	96.30 a	96.28 a	88.41 b	45.78 cd	42.67 d	50.47 c	11.52	2, 12	5.33
96	94.68 a	94.87 a	84.65 b	36.58 c	38.96 c	39.63 c	8.15	2, 12	5.35

For each row, data followed by the same letter are not significantly different.

About 84.7% of applied concentration of the parental compound was seperated at 5.06 min (Set B in Figure 3). Compared to laboratory biotype, resistant biotype was more active in metabolizing prometryn, %recovery was 95.7, 65.9, 56.1, 50.5 and 39.6 in the treatments of 0, 24, 48, 72 and 96 h, respectively [Table 3 and Figure 4, (Set C)]. About 60.4% of applied concentration of prometryn was metabolized by resistant biotype into three possible metabolites. Their retention times were 2.49, 3.45 and 4.11 min. Only 39.6% of the parental compound was seperated at 5.06 min (Set C in Figure 3).

Data for the comparison between the three triazine herbicides are given in Table 4, revealing that laboratory culture was more active in metabolizing prometryn than ametryn and atrazine. Percentages of recovery of ametryn, atrazine and prometryn from laboratory culture medium in the 24 h treatments were 97.8, 97.6 and 92.3%, respectively. After 96 h, these percentages dropped to 94.7, 94.9 and 84.7, respectively. For the time intervals ranged from 24 to 96 h, prometryn recovery from laboratory culture medium was significantly less compared to that of ametryn and atrazine. At the end of the run, laboratory biotype was able to metabolize about 5% of the applied concentration of ametryn and atrazine into one possible metabolite (for ametryn) and two possible metabolites (for atrazine). However, the same biotype was better able to metabolize 15% of applied concentration of prometryn into two possible metabolites. The same pattern with different values was achieved with the resistant biotype. Percentages of prometryn recovered from the resistant culture medium were 95.7, 65.9, 56.1, 50.5 and 39.6% in the treatments of 0, 24, 48, 72 and 96 h, respectively (Table 4). The corresponding value of ametryn treatments were 99.1, 75.5, 69.4, 45.8 and 36.6%, respectively. Atrazine exhibited the same pattern as ametryn, %recovery was 97.5, 73.1, 66.0, 42.7 and 38.9%. During the first 2 days, percentage of prometryn recovered from the resistant culture medium was signficantly less compared to ametryn and atrazine. However, after 4 days, percentages of the three herbicides recovered from the resistant culture medium was statistically similar. Resistant biotype metabolized about 60% of biologically active herbicides tested into two possible metabolites for ametryn and three possible metabolites for atrazine and prometryn. In our study, we could not identify the metabolites because of the unavailability of reference standards. In control treatment having the herbicide and growth medium, the metabolite peaks were not seen.

In an attempt to link the chromatographic analysis data of the three herbicides tested with the inhibitory effects on both photosynthesis and cell division, chlorophyll a, b, carotenoids and cell number were measured in the same culture medium used in the chromatographic study. After 96 h treatment with 4 uM of the three herbicides tested, bioassay data (Table 5) revealed that there was a highly significant difference in the susceptibility of the two biotypes (S0 and S5) toward the three herbicides tested when using any of the previous mentioned parameters as indicator of phytotoxicity. For atrazine, ametryn and prometryn treatments, respectively, %reduction in chlorophyll a; chlorophyll b; carotenoids and algal count of S0 versus S5 were [(98.11, 98.02 and 97.88) versus (28.19, 27.18 and 25.45)] for chlorophyll a; [(98.44, 98.33 and 97.94) versus (22.83, 16.54 and 13.16)] for chlorophyll b; [(95.70, 95.61 and 95.33) versus (24.28, 19.34 and 17.57)] for carotenoids and [(94.78, 94.77 and 94.76) versus (11.83, 11.05 and 10.39)] for cell numbers. For any of the three herbicides, data exhibited significant differences among S0 and S5 when any of the four biomarkers was used as an indicator of herbicide phytotoxicity. In the assay with S5, no significant difference was reported among the three herbicides when any of the four parameters was compared (Table 5). In the assay with S0, ametryn and atrazine exhibited significantly greater reduction in chlorophyll a and b than prometryn. However, they were insignificantly different when the reduction in carotenoids and algal count was considered. These data confirm the role of herbicide biodegradation as a main mechanism of weed resistance toward herbicides. Weed resistance toward herbicides could be managed by adding suitable synergists to herbicide formulations and also by integrating herbicides with negative cross resistance in rotation with non-chemical

Table 5. Reduction percentages in pigment concentrations and algal count measured in 1 ml of *Scenedesmus vacuolatus* laboratory and resistant biotype suspensions after 96 h incubation with 4 µM of herbicides tested.

Treatments	Parameter	S0 (Mean ± SD)	S5 (Mean ± SD)
Atrazine		98.11 ± 0.056 a	28.19 ± 3.155 a
Ametryn	Chlorophyll (a)	98.02 ± 0.050 a	27.18 ± 1.433 a
Prometryn		97.88 ± 0.061 b	25.45 ± 2.189 a
F; df; LSD 0.05		**13.00; 2,6; 0.11**	**1.03 (NS); 2,6; -**
Atrazine		98.44 ± 0.215 a	22.83 ± 6.931 a
Ametryn	Chlorophyll (b)	98.33 ± 0.174 a	16.54 ± 6.826 a
Prometryn		97.94 ± 0.252 b	13.16 ± 2.945 a
F; df; LSD 0.05		**8.06; 2.6; 0.32**	**2.10 (NS); 2,6; -**
Atrazine		95.70 ± 0.851 a	24.28 ± 7.752 a
Ametryn	Carotenoids	95.61 ± 0.695 a	19.34 ± 6.225 a
Prometryn		95.33 ± 1.273 a	17.57 ± 3.896 a
F; df; LSD 0.05		**0.12 (NS); 2,6; -**	**0.95 (NS); 2,6; -**
Atrazine		94.78 ± 1.055 a	11.83 ± 5.372 a
Ametryn	Cell number	94.77 ± 1.140 a	11.05 ± 4.731 a
Prometryn		94.76 ± 1.208 a	10.39 ± 4.718 a
F; df; LSD 0.05		**0.00 (NS); 2,6; -**	**0.06 (NS); 2,6; -**

For each biotype and parameter, data followed by the same letter are not signficantly different.

means of weed control.

DISCUSSION

The current study has revealed that resistance toward triazine herbicides was evident even after 3 years of storage of the resistant biotype (S5) of *S.vacuolatus* on herbicide free agar. Previous study (Kasai and Hanazato, 1995) revealed a marked difference in the susceptibility of *Scenedesmus gutwinskii* var. heterospina toward the triazine herbicide, simetryn, between the strains isolated from the treated and the control ponds, moreover, tolerance of the isolated strains remained for nearly 2 years in absence of simetryn, which indicated that changes in genetic composition occurred. Researchers focused on the detoxification of atrazine by different microorganisms, atrazine mostly N-dealkylated by some fungi (Giardina et al., 1982), by *Senecio vulgaris* (Gressel et al., 1983b), by white rot fungus, *Pleurotus pulmonarius* (Masaphy et al., 1993), and by *Rhodococcus* strain (Behki and Khan, 1994). Also, Coupe et al. (2005) detected two degradates of atrazine, one degradate of prometryn in water samples collected from five sites in the Yazoo River Basin.

To decide whether rapid metabolic detoxification of three triazine herbicides tested represents the basis for the ability of the resistant biotype of *S.vacuolatus* to survive herbicide treatment, HPLC studies were conducted to compare the chromatograms of herbicide treated laboratory and resistant biotypes. In the current study, resistant biotype rapidly metabolized the three triazine herbicides tested confirming that metabolic mechanism is one of the major mechanisms involved in the resistance of green alga, *S.vacuolatus* toward herbicides as previously confirmed by Tantawy et al. (1984) with the pyridiazinone herbicide, metflurazon, by the same algal species (formerly named, *C. fusca*). Using this technique with the green alga is considered more valuable than the pharmacokientic studies using radiolabeled herbicides in higher plants because radiola-beled material are firmly bound to cellular structures and %recovery of the biologically active herbicide and its metabolites do not exceed 70% (Motooka et al., 1977). It has been shown that resistant *S. vacuolatus* rapidly metabolized triazine herbicides compounds into two or three possible metabolites. In contrast, laboratory culture was not able to metabolize triazine herbicides and was therefore extremely susceptible. Metabolic detoxification as a major mechanism of plant (algae and weeds) resistance toward triazine herbicides was confirmed in previous studies with atrazine using resistant biotype of *B. distachyon* (*T. distachya*) (Gressel et al., 1983 a) and the resistant biotypes of *Setaria faberi* and *S. viridis* (Prado et al., 2000). In contrast to our results and the findings of the previously mentioned authors, degradative metabolism for herbicides, as reported by Tang et al. (1998a) and Fedtke (1991), has not been shown to be a major mechanism of resistance.

In addition to the metabolic mechanism, reduced penetration into resistant cells may be another mecha-nism, as reported by John (1976) and confirmed by Szigeti and

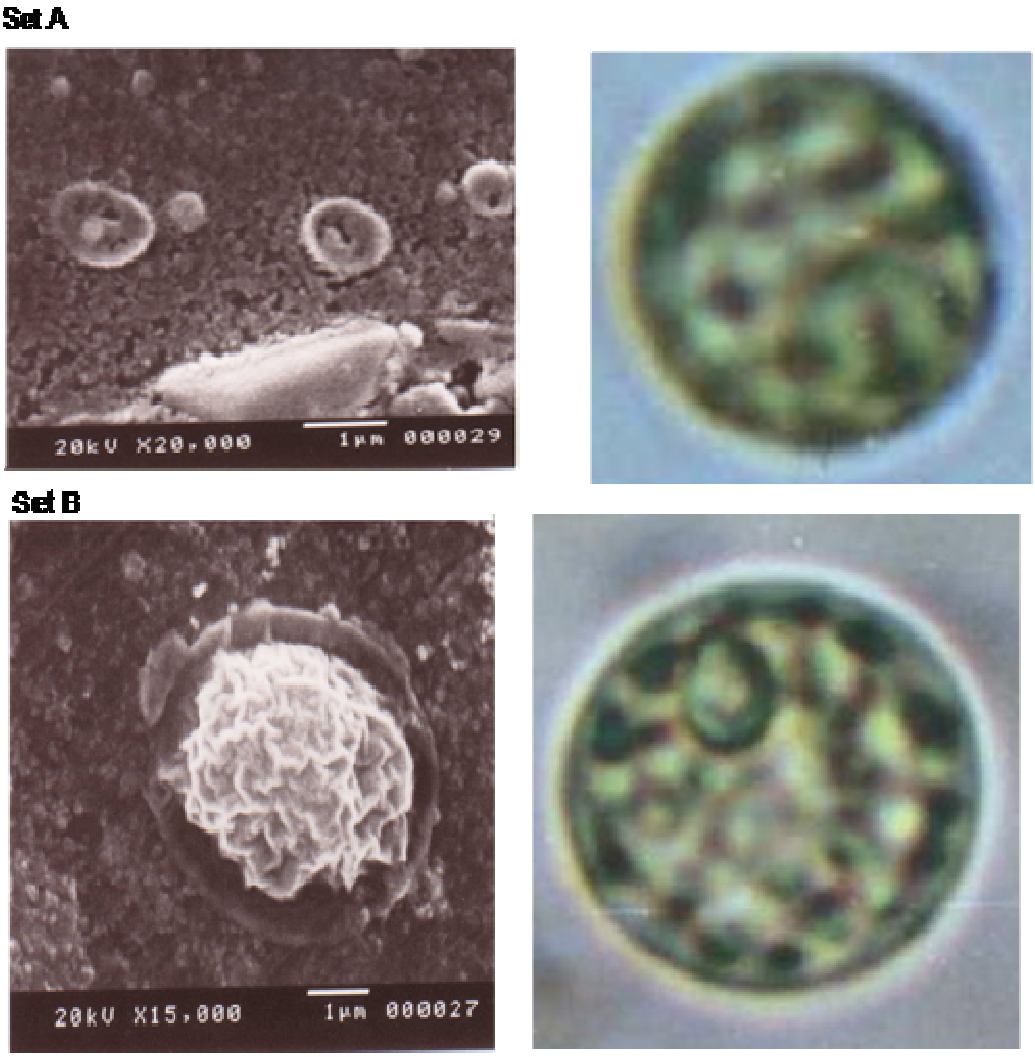


Figure 5. Electronic photographs (right side, Scanning Electron Microscope (JSM5400LV, Electron Microscope Unit, Assiut University) and regular photographs (left side, Carl Zeiss Light Microscope, X = 1250) for *Scenedesmus vacuolatus* laboratory (Set A) and resistant (Set B) cells.

Lehoczki (2003), who found in atrazine-resistant *Conyza canadensis*, that the thylakoid mem-brane lipids contained a lower amount of polar lipid and the fatty acid content exhibited a higher degree of unsaturation. In the current study, electronic photo-graphs using Scanning Electron Microscope (Figure 5) revealed clear difference in the cell surface area among laboratory and resistant cells. The diameter of resistant cell was four times greater than that of the susceptible cell. However, these photographs did not show a dif-ference in cell wall thickness between the susceptible and resistant cells. On this score, we believe that herbicide penetration is more likely a minor mechanism and the resistance is mostly due to metabolic detoxification and target site insensitivity. We observed in some treatments that growth inhibition had occurred at the beginning of treat-ment which confirmed the arrival of herbicide at lethal concentration to the site of action, however, treated cells regenerated and restored their activity. In agreement with the current study, Park and Mallory-Smith (2005) neglected the uptake mechanism and determined the mechanism of acetolactate synthase-inhibitor resistance in a downy brome (*Bromus tectorum*) biotype to be metabolism and an altered target site.

Target site insensitivity as an additional mechanism of resistance toward herbicides in the green algae and higher plants is not included in the present study due to lack of facilities. However, previous studies confirm the importance of this mechanism in the resistance pheno-menon (Lundegardh, 1992 a, b, c; Schwenger and Barz, 2000; Mengistu et al., 2000; Cocker et al., 2001; Sibony and Rubin, 2003; Yu et al., 2003). A single amino acid substitution from serine to glycine was found in resistant biotypes at residue 264 in the D1 protein (Kohno et al.,

2000; Kumata et al., 2001; Diebold et al., 2003; Patzoldt et al., 2003; Sibony and Rubin, 2003; Chodova and Salava, 2004; Park and Mallory-Smith, 2005). However, this amino acid substitution was not found in other resistant biotypes which indicates that resistance to these herbicides can also be attained by other mechanisms (Mengistu et al., 2000; Maertens et al., 2004).

Current study must be complemented in the future as follows: (1) linking the herbicidal phytotoxicity data on the green alga and higher plants, (2) determining the enzymes involved in the metabolic mechanism of resistance and also the components of plant cell wall in susceptible and resistant cells, (3) studying the role of different synergists in enhancing the phytotoxicity of commonly used herbicides and, (4) identifying the bands of resistance in the resistance biotypes. The DNA sequence analysis of the psbA gene in resistant and susceptible biotypes must be identified.

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