

## Molecular Basis for Differential Metabolic Responses to Monosulfuron in Three Nitrogen-Fixing Cyanobacteria

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Nitrogen-fixing cyanobacteria are vital photosynthetic microorganisms that contribute to soil fertility by fixing atmospheric nitrogen and are also important for maintaining ecosystem stability. These microorganisms can be very sensitive to herbicides because they possess many characteristics of higher plants. Six days after the application of monosulfuron at 0.03 to 0.3 nmol L<sup>-1</sup> under laboratory conditions, growth of the nitrogen-fixing cyanobacteria *Anabaena flos-aquae*, *Anabaena azollae*, and *Anabaena azotica* was stimulated, but at higher concentrations (30 to 300 nmol L<sup>-1</sup>) protein synthesis was inhibited. The production of 16 amino acids in *A. flos-aquae* was reduced from 7 to 69% with increasing monosulfuron concentration. Application of monosulfuron at 3 to 300 nmol L<sup>-1</sup> substantially inhibited in vitro acetolactate synthase (ALS) activity as indicated by 50% inhibition index values of 3.3, 65.2, and 101.3 nmol L<sup>-1</sup> for *A. flos-aquae*, *A. azollae*, and *A. azotica*, respectively. In contrast, extractable ALS activity was not affected in these algal species with monosulfuron treatments ranging from 0.03 to 300 nmol L<sup>-1</sup> except in *A. flos-aquae* at higher concentrations (30 to 300 nmol L<sup>-1</sup>). The most sensitive species to monosulfuron was *A. flos-aquae*, followed by *A. azollae* and *A. azotica*. Molecular analyses showed that the genomic DNA of *A. azollae* and *A. azotica* differed in only one amino acid. Results from photogenetic analyses revealed a high degree of homology between these algae. In contrast, the genomic DNA of *A. flos-aquae* differed from that of *A. azollae* and *A. azotica* in 44 and 45 amino acids, respectively. Our findings support the view that monosulfuron toxicity in these three nitrogen-fixing cyanobacteria is due to interference with protein metabolism via inhibition of branch-chain amino acid biosynthesis, and particularly ALS activity.

**Nomenclature:** Monosulfuron; *Anabaena flos-aquae* (Lyngb) Breb; *Anabaena azollae* Strasb; *Anabaena azotica* Ley.

**Key words:** Acetolactate synthase, amino acids, *Anabaena*, DNA, herbicide, nitrogen-fixing cyanobacteria, nontarget effects, protein content.

Nitrogen-fixing cyanobacteria are vital photosynthetic microorganisms that contribute to soil fertility by fixing atmospheric nitrogen (Habte and Alexander 1980; Sinha and Kumar 1992) and maintaining ecosystem stability (Irisarri et al. 2001; Shen and Lu 2005). The agronomic importance of free-living symbiotic cyanobacteria as biofertilizers has been recognized (Habte and Alexander 1980). Some cyanobacterial strains that thrive in fields release small quantities of ammonia, as well as other small nitrogenous polypeptides during active growth. Recent research by Shen and Lu (2005) demonstrated that the application of a mixture of 12 species of nitrogen-fixing cyanobacteria (cultured for 90 d; 1 g dry algae weight per liter) after the sowing of a rice (*Oryza sativa* L.) crop was more beneficial to rice yield and soil fertility than the application (0.78 g L<sup>-1</sup>) of a urea fertilizer [CO(NH<sub>2</sub>)<sub>2</sub>] or a nonfertilizer control treatment. After the addition of the algal mixture to the rice paddies, total nitrogen content of rice stems and leaves increased by 27 and 39%, and rice yields increased by 9 and 12% relative to the urea fertilizer and control treatments, respectively. Three of the most common cyanobacteria in agricultural fields in China are the filamentous nitrogen-fixing species *Anabaena azotica* Ley (a free-living soil cyanobacterium with a high nitrogen-fixing capability), *Anabaena flos-aquae* (Lyngb) Breb. (a free-living soil cyanobacterium that may be toxic to some organisms), and *Anabaena azollae* Strasb (a cyanobacterium that forms a symbiotic relationship with the water fern, *Azolla* spp.) (Li 1962; Shen and Li 1993; Wu and Zhou 2004).

Since the late 1940s, herbicides have played an important role in increasing the productivity of cropping systems in many regions of the world. However, cyanobacteria are very sensitive to herbicides because they possess many characteristics of higher plants (El-Sheekh et al. 1994). In fact, several nitrogen-fixing cyanobacteria (e.g., *A. azollae*, *A. azotica*) commonly found in rice fields of the Shanghai region of China before the extensive use of herbicides such as bensulfuron, propanil, benthocarb, and butachlor are now absent from these systems (Shen et al. 2005; Shen and Lu 2005). Numerous effects of herbicides on nontarget cyanobacteria have been reported, including effects on growth, photosynthesis, nitrogen fixation, and metabolic activities (Bueno et al. 2004; Kaur et al. 2002; Miquel and Ivo 2006; Shen et al. 2004, 2005). These studies have focused largely on the impact of older herbicides on algal growth and metabolism including atrazine, benthocarb, bipyridylum, molinate, metribuzin, and 2,4-D. To date, few studies have investigated the effect of relatively newer classes of herbicides (e.g., sulfonylureas) on nitrogen-fixing cyanobacteria found in fields.

The discovery in the late 1970s of sulfonylurea herbicides capable of suppressing weeds at extremely low application rates was viewed as an important advancement in chemical weed control (Pillmoor 1989; Saari et al. 1994). Sulfonylureas inhibit the enzyme acetolactate synthase (ALS), which is responsible for catalyzing the biosynthesis of the branch-chain amino acids valine, leucine, and isoleucine (Durner et al. 1991; Whitcomb 1999). The ALS enzyme is present in all plants, bacteria, fungi, algae (including cyanobacteria), and yeasts (LaRossa and Schloss 1984; LaRossa and Van Dyk 1988; Milano et al. 1992), but is not present in animals (Whitcomb 1999). Because of their relatively low mammalian toxicity, high efficacy, and environmental safety (Saari et al. 1994), the use of sulfonylurea herbicides has increased rapidly since their introduction. Currently, over 30 sulfonylurea

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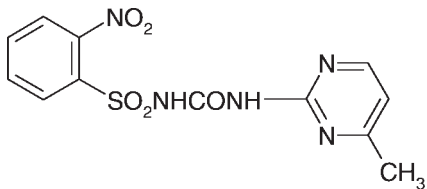


Figure 1. Chemical structure of monosulfuron [*N*-[(4'-methyl) pyrimidin-2'-yl]-2-nitrophenylsulfonamide].

herbicides are applied worldwide for selective control of weeds in a variety of crops including corn (*Zea mays* L.), rice, wheat (*Triticum aestivum* L.), and potatoes (*Solanum tuberosum* L.) (Tomlin 2003).

Monosulfuron is a relatively new sulfonamide herbicide that was developed by the National Pesticide Engineering Research Center in Tianjin, China (Figure 1) (Li et al. 1994). This herbicide exhibits low mammalian toxicity and is very effective at POST rates of 15 to 30 g ai ha<sup>-1</sup> in a wide range of crops including corn, wheat, rice, and millet (*Panicum miliaceum* L.). At these application rates, monosulfuron provides effective control of many weeds including Chinese sprangletop [*Leptochloa chinensis* (L.) Nees], redroot pigweed (*Amaranthus retroflexus* L.), common lambsquarters (*Chenopodium album* L.), and water barnyardgrass [*Echinochloa phyllopogon* (Stapf) Koss] (Fan et al. 2000). Results from bioassays and in vitro ALS activity studies indicate that high concentrations (3.4 to 171.4 μmol L<sup>-1</sup>) of monosulfuron severely inhibit the growth of corn roots and ALS activity, but at lower concentrations (e.g., 0.3 nmol L<sup>-1</sup>) stimulate root growth and enhance ALS activity (Fan et al. 2005).

The effect of monosulfuron on nitrogen-fixing cyanobacteria capable of enhancing the fertility of agricultural soils has not been investigated. Also, no studies have used molecular analyses to assess the differential metabolic responses of nitrogen-fixing cyanobacteria to sulfonamides. The objectives of this research were to determine the effect of a range of monosulfuron concentrations on: (1) protein content and amino acid production of three common but distinct nitrogen-fixing cyanobacteria (*A. flos-aquae*, *A. azollae*, and *A. azotica*); (2) in vitro and extractable specific activity of ALS in these three cyanobacteria; and (3) to use molecular analyses of genomic DNA of the ALS enzyme to determine the molecular basis for any differential response to this herbicide in the three species.

## Materials and Methods

**Source of Chemicals.** Monosulfuron of 99.4% purity was synthesized at the National Pesticide Engineering Research Center in Tianjin, China. Monosulfuron was dissolved using dimethylformamide (DMF)<sup>1</sup> and Triton X-100<sup>2</sup> with no observed effect on concentration (i.e., ratio of DMF 0.5% [v/v] and Triton X-100 0.005% [v/v]). The stock solution was freshly prepared and filter sterilized by a molecular lab water purifier<sup>3</sup> before being added to the culture medium.

**Algal Cultures Used.** Cultures of *A. azotica*, *A. flos-aquae*, and *A. azollae*, three nitrogen-fixing algal species in the Nostocaceae, were obtained from the Institute of Hydrobiology of the Chinese Academy of Sciences in Wuhan, China. Axenic cultures were grown in a liquid sterilized medium as

described by Kratz and Myers (1955) at 30 ± 2 C under fluorescent light at an intensity of 36 μmol m<sup>-2</sup> s<sup>-1</sup>. The experimental cultures were first grown in 250-ml flasks containing 100 ml of medium with 0.5 to 1 million cells per milliliter under the same conditions as described above. The cultures were shaken 30 min three times daily on a shaker table. At the exponential growth phase of the algal cultures, monosulfuron from the stock solution was added to the culture medium at 0.003, 0.03, 0.3, 3, 30, and 300 nmol L<sup>-1</sup>. Sterilized water was added to some of the culture media rather than the herbicide and these cultures served as controls. Each treatment concentration was replicated three times and all experiments were conducted twice. Culture samples were collected 6 d after herbicide treatment to determine protein and amino acid content, and ALS activity.

**Algal Growth.** Growth of algae was measured by recording light absorbance of the culture at 485 nm using a spectrophotometer. Standard curves relating spectrophotometric absorbance readings (485 nm) with cell numbers were developed for continuous cultures of *A. azotica*, *A. flos-aquae*, and *A. azollae*. These curves were used to determine cell numbers in continuous culture samples used as inoculum for the screening bioassay. Data used to produce the standard curves were obtained from absorbance measurements (Schraeder et al. 1998). To determine the dry weight of cells, corresponding cultures in triplicate were pelleted (centrifuged at 10,000 × g for 30 min using Sorvall centrifuges) and the pellet washed using distilled water three times to remove impurities before drying to constant weight at 105 C for 8 h (Abou-Waly et al. 1991).

**Protein Assay.** The protein content of the cultures was determined using the procedure described by Bradford (1976) using bovine serum albumin<sup>4</sup> as a standard. One-hundred nanomoles of Coomassie Brilliant Blue G-250<sup>5</sup> was first dissolved in 50 ml of 95% ethanol and then 100 ml of 85% (w/v) phosphoric acid was added to this solution. The resulting solution was diluted to a final volume of 1 L. The volume in the test tube was adjusted to 0.1 ml with the appropriate buffer. Five milliliters of protein reagent was added to the test tube and the contents mixed by either inversion or vortexing. The absorbance of the sample at the 595 nm wavelength was measured after 2 min at 20 C and before 1 h in 3-ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent.

**Amino Acid Analysis.** Amino acid components were analyzed using an amino acid automatic analyzer<sup>6</sup> with an ion exchange column after the algal sample was hydrolyzed in 6 mol L<sup>-1</sup> HCl at 110 ± 1 C for 24 h. The absorbance of the sample was measured at 570 nm except for proline (440 nm) after color development using ninhydrin reaction at 100 C.

**In Vitro ALS Assay.** ALS was extracted from 6-d-old nitrogen-fixing cyanobacteria of the three test species. The cyanobacteria were centrifuged at 10,000 × g for 30 min (4 C) using Sorvall centrifuges<sup>7</sup> after cells were broken by an ultrasonic cell disruptor (250 mV, 3 s, 3 s) in the dark at 4 C. The protein in the supernatant was precipitated with

ammonium sulfate, and then centrifuged at  $10,000 \times g$  for 30 min (4 C) after 2-h incubation at 0 C. The pellet was dissolved in phosphate buffer (pH 7.5) containing  $200 \text{ mmol L}^{-1}$  sodium pyruvate,  $5 \text{ mmol L}^{-1}$   $\text{MgCl}_2$ ,  $10 \text{ mmol L}^{-1}$  thiamine pyrophosphate (TPP),<sup>8</sup>  $200 \text{ } \mu\text{mol L}^{-1}$  flavin adenine dinucleotide (FAD),<sup>9</sup> and  $1 \text{ mol L}^{-1}$  DL-dithiothreitol.<sup>10</sup> This enzyme solution was kept in ice water in the dark before data collection.

The enzyme solution was dissolved in a solution of  $50 \text{ mmol L}^{-1}$  phosphate buffer (pH 7.5) containing  $20 \text{ mmol L}^{-1}$  sodium pyruvate and  $0.5 \text{ mmol L}^{-1}$   $\text{MgCl}_2$ . A volume of 0.1 ml of monosulfuron was added to 0.4 ml of enzyme and 0.5 ml of enzyme reaction solution, which contained  $50 \text{ mmol L}^{-1}$  pH 7.5 phosphate buffer with  $24 \text{ mmol L}^{-1}$  sodium pyruvate,  $0.6 \text{ mmol L}^{-1}$   $\text{MgCl}_2$ ,  $1 \text{ mmol L}^{-1}$  TPP, and  $0.02 \text{ mmol L}^{-1}$  FAD. The mixture was subsequently heated to  $37 \pm 1 \text{ C}$  using an electrothermal water tank for 1 h in the dark. After adding a 0.1-ml aliquot of  $3 \text{ mmol L}^{-1}$  sulfuric acid, the mixture was incubated at  $60 \pm 1 \text{ C}$  for 15 min to decarboxylate the acetolactate to acetoin. A 0.5-ml freshly prepared solution of 5.0%  $\alpha$ -naphthol in  $2.5 \text{ mol L}^{-1}$  sodium hydroxide and 0.5 ml of 0.5% creatine was then added to the mixture. Color development was then conducted at  $60 \pm 1 \text{ C}$  for 15 min. The red color complex was cooled to room temperature ( $\sim 25 \text{ C}$ ) in a water bath and absorbance was measured at 525 nm using a spectrophotometer.<sup>11</sup> The specific activity of ALS was expressed as nanomoles of acetoin per nanomoles of protein per hour (Fan et al. 2005).

**Extractable ALS Assay.** To determine extractable ALS activity, 0.1 ml of monosulfuron and 0.4 ml of the enzyme were substituted with 0.5 ml of the enzyme solution extracted from nitrogen-fixing cyanobacteria that had been cultivated in the culture medium containing monosulfuron (Fan et al. 2005). Six concentrations of monosulfuron were used as outlined previously and the specific activity of ALS was determined and expressed as described above.

**Calculation of ALS 50% Inhibition Index ( $I_{50}$ ) Value.** The  $I_{50}$  value for inhibition of ALS is defined as the concentration of monosulfuron that inhibits 50% of the specific activity of ALS in 1 h on the basis of the methodology described above. The percentage inhibition of the specific activity of ALS was calculated using the equation

$$I = A_1 - A_2/A_1 \times 100 \quad [1]$$

where  $I$  is the percentage inhibition of the specific activity of ALS,  $A_1$  is the average specific activity of ALS in the nonherbicidal control treatment, and  $A_2$  is the average specific activity of ALS for the monosulfuron treatment. The  $I_{50}$  value was then calculated by regressing the logarithm of the monosulfuron concentration against the probability corresponding to the percentage inhibition of the specific activity of ALS for the regression.

**Genomic DNA of ALS.** Genomic DNA of the ALS enzyme was extracted from algae as described by Wu et al. (2000) and based on the published sequence of the ALSIII gene from GenBank. The sequences of the up primers with length 22 and molecular weight (MW) 6668 were designed to 5'GTGACTGTGAGCTTGCCCTTCCC3'. The sequences

of the low primers with length 23 and MW 7097 were designed to 5'GGTCTTTGGTGGTTGCTTCGGTA3'. The primers were synthesized by Songni Biotechnology Company (Shanghai, China). Each 50- $\mu\text{l}$  reaction volume contained 5  $\mu\text{l}$  of  $10\times$  reaction buffer for *Taq* DNA polymerase, 5  $\mu\text{l}$  of  $\text{MgCl}_2$  (25 mM), 1  $\mu\text{l}$  of deoxynucleotide triphosphates (0.2 mM of each), 1.5  $\mu\text{l}$  of each primer, 3 units of *Taq* DNA polymerase, and 1  $\mu\text{l}$  of DNA. First, 94 C, 3 min, 1 cycle; then 94 C, 30 s; 56 C, 30 s; 72 C, 2 min; 30 cycles; and 72 C, 5 min. Polymerase chain reaction products were determined using agarose gel electrophoresis.

**Statistical Analyses.** A completely randomized design with three replications was used in all experiments. Experiments were repeated. The data represent the average of the two trials because of a nonsignificant trial-by-treatment interaction and homogeneity of variance according to Bartlett's test. ANOVAs were performed on nontransformed data. Significant differences were determined using Duncan's test at the  $P = 0.05$  level of significance (PROC GLM, SAS Institute 2001). Regression equations for protein content and ALS-specific activity and the logarithm of the monosulfuron concentration were obtained using the regression feature in Sigmaplot 2001.<sup>12</sup>

## Results and Discussion

**Effects on Algal Growth.** In general, monosulfuron applied at low concentrations (i.e., 0.03 to  $0.3 \text{ nmol L}^{-1}$ ) stimulated growth in the three algal species, but the converse was observed at higher concentrations (i.e., 30 to  $300 \text{ nmol L}^{-1}$ ) (Figure 2). Cell numbers in *A. flos-aqua* were increased by 23 and 10% at 0.03 to  $0.3 \text{ nmol L}^{-1}$ , and reduced by 63, 94, and 97% at 3, 30, and  $300 \text{ nmol L}^{-1}$ , after 144 h, respectively, relative to the control treatment. However, cell numbers in *A. azollae* and *A. azotica* were increased by 120 to 135% and 80 to 103% at 0.03 to  $3 \text{ nmol L}^{-1}$ , and reduced by 93 and 69% at  $300 \text{ nmol L}^{-1}$ , after 144 h, respectively, relative to the control treatment. Thus, the three algae had different degrees of sensitivity to monosulfuron, with the most sensitive species being *A. flos-aquae*, followed by *A. azollae* and *A. azotica*.

The observed effects of monosulfuron on algal growth in this study are similar to those reported by Shen et al. (2005) for butachlor and acetochlor on several *Anabaena* species. Butachlor and acetochlor stimulated growth of these cyanobacteria under low concentrations ( $1$  to  $8 \text{ mg L}^{-1}$ ), but showed high toxicity at concentrations above  $16 \text{ mg L}^{-1}$ . Shen and Lu (2005) reported that MCPA stimulated the growth of nitrogen-fixing algae at concentrations as high as  $30 \text{ mg L}^{-1}$ . However, exposure of several *Anabaena* species to prometryne even at the relatively low concentration of  $4 \text{ mg L}^{-1}$  reduced growth by 79, 71, and 69% respectively, whereas no growth was observed at a concentration of  $8 \text{ mg L}^{-1}$ . Blanck et al. (1984) reported that the sensitivities of different strains and geographical races of algae to a toxicant can vary as much as 200-fold. Therefore, to maintain ecological diversity of the resident microflora and improve crop yields within agricultural systems, it is critical to select herbicides and dosages that are effective and safe for these important microorganisms. In field tests using high-performance liquid chromatography-UV residue analysis, for example, monosulfuron residues have been shown to dissipate

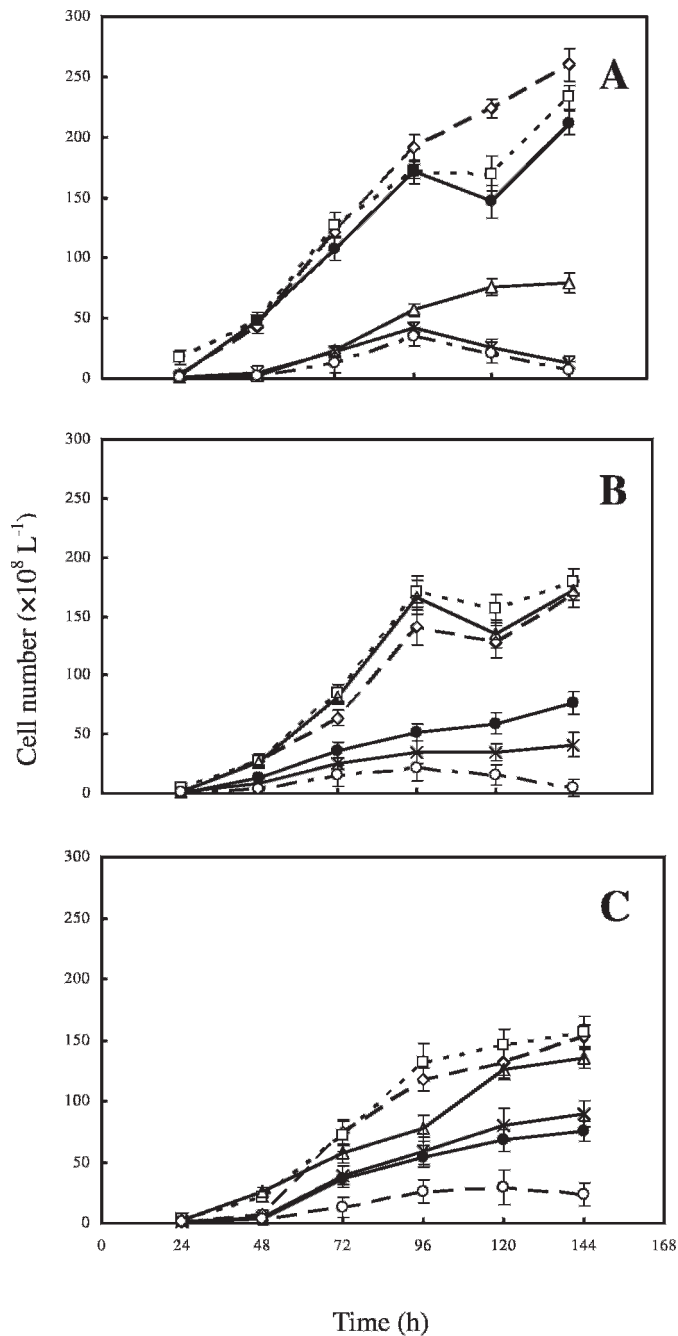


Figure 2. Effects of monosulfuron on growth of three cyanobacteria. (A) *Anabaena flos-aquae*, (B) *Anabaena azollae*, (C) *Anabaena azotica*; (□) 0.03 nmol L<sup>-1</sup>, (◻) 0.3 nmol L<sup>-1</sup>, (◻) 3 nmol L<sup>-1</sup>, (⊠) 30 nmol L<sup>-1</sup>, (○) 300 nmol L<sup>-1</sup>, and (●) control. Vertical bars represent mean ±SE (n = 3).

rapidly, with half-lives of less than 14 d (Fan et al. 2004). Moreover, monosulfuron provides effective control of many weeds under field conditions at application rates of 1.2 nmol L<sup>-1</sup> (Shen et al. 2008). On the basis of the effects of monosulfuron on growth of the three nitrogen-fixing cyanobacteria, we suggest that monosulfuron be applied at low concentrations (i.e., less than 3 nmol L<sup>-1</sup>) in the field to control numerous weeds but also to minimize the negative impact on these beneficial microorganisms.

**Effects on Protein Content.** The effect of monosulfuron on protein content of cells for the three algal species differed in a

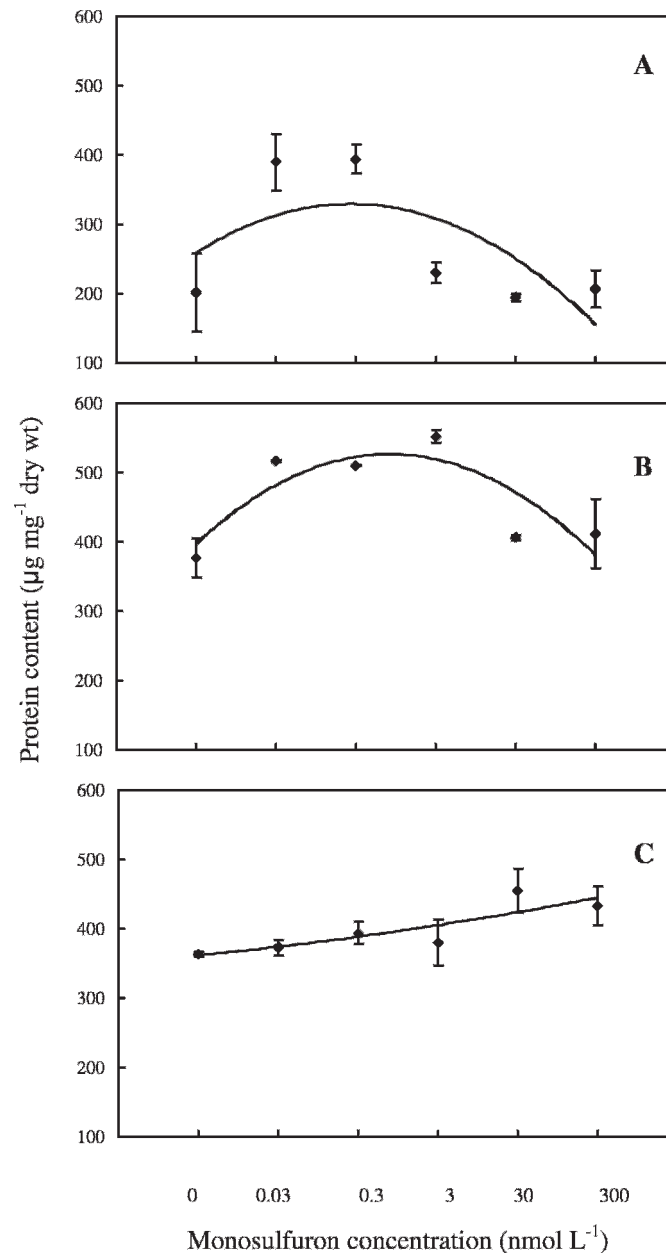


Figure 3. Effects of monosulfuron on protein content of three cyanobacteria (*Anabaena flos-aquae*, *Anabaena azollae*, and *Anabaena azotica*) for 6 d (n = 3). Regression equations are: (A, *A. flos-aquae*)  $y = -0.018x^2 - 0.10x + 0.47$ ,  $r^2 = 0.83$ ; (B, *A. azollae*)  $y = -0.021x^2 - 0.057x + 0.90$ ,  $r^2 = 0.67$ ; (C, *A. azotica*)  $y = 0.00043x^2 + 0.033x + 0.73$ ,  $r^2 = 0.66$ .

similar manner to that of algal growth (Figure 3). *Anabaena flos-aquae* exhibited the greatest sensitivity to monosulfuron. The protein content of *A. flos-aquae* cell cultures treated with monosulfuron at 0.03 and 0.3 nmol L<sup>-1</sup> increased 94 and 95%, respectively, relative to the nonherbicidal control treatment (Figure 3A). However, the protein content of cells decreased sharply as monosulfuron concentration increased. For example, cells of *A. flos-aquae* subjected to the 30 nmol L<sup>-1</sup> monosulfuron concentration had only 4% of the protein content in comparison with cells receiving the nonherbicidal treatment (P < 0.05). The protein content of *A. azollae* cell cultures treated with monosulfuron 0.03 and 3 nmol L<sup>-1</sup> increased 37 and 47%, respectively, relative to the control treatment (Figure 3B). This increase in protein

Table 1. Quantity of various amino acids produced by three nitrogen-fixing cyanobacteria subjected to different monosulfuron concentrations.

Species	Concentration (nmol L <sup>-1</sup> )	Amino acid (mg L <sup>-1</sup> ) <sup>a</sup>															
		Val	Ile	Leu	Lys	Thr	Phe	Ser	Pro	His	Tyr	Arg	Ala	Met	Asp	Glu	Gly
<i>Anabaena azotica</i>	Control	73.8 <sup>b</sup>	61.7	111.7	62.5	77.3	58.0	59.4	4.8	12.7	13.5	81.5	132.4	31.3	138.6	147.4	68.8
	0.003	199.9	154.3	274.6	172.0	218.8	155.1	165.7	12.5	37.6	25.3	319.2	382.5	95.7	462.5	455.4	213.1
	±% <sup>c</sup>	+171	+150	+146	+175	+183	+167	+179	+160	+196	+87	+292	+189	+206	+234	+209	+210
	0.3	151.8	138.0	246.2	145.0	175.4	115.1	124.7	9.3	32.8	57.3	241.1	284.4	76.1	310.4	334.1	154.1
	±%	+106	+124	+120	+132	+127	+98	+110	+94	+158	+324	+196	+115	+143	+124	+127	+124
	30	74.1	60.5	107.8	62.0	78.1	54.1	57.5	5.0	11.2	18.9	95.7	130.8	61.9	150.2	145.1	69.4
±%	0	-2	-3	-1	+1	-7	-3	+4	-12	+40	+17	-1	+98	+8	-2	+1	
<i>Anabaena flos-aquae</i>	Control	250.6	291.2	547.1	298.9	380.6	239.6	271.8	0.0	87.6	173.0	492.1	608.3	147.9	676.1	727.0	329.7
	0.003	205.0	263.0	487.8	270.8	331.5	219.2	237.5	9.4	77.6	147.0	459.1	542.4	136.6	608.8	635.9	294.5
	±%	-18	-10	-11	-9	-13	-9	-13	/	-11	-15	-7	-11	-8	-10	-13	-11
	0.3	194.7	165.5	307.6	171.7	208.8	141.4	149.2	11.6	47.7	85.2	274.6	345.0	82.7	371.2	403.8	185.0
	±%	-22	-43	-44	-43	-45	-41	-45	/	-46	-51	-44	-43	-44	-45	-44	-44
	30	167.5	151.8	256.9	173.2	207.0	138.5	145.1	12.1	43.9	63.0	352.0	351.6	95.1	448.6	395.9	186.3
±%	-33	-48	-53	-42	-46	-42	-47	/	-50	-64	-28	-42	-36	-34	-46	-43	
<i>Anabaena azollae</i>	Control	179.9	165.7	299.1	161.0	197.9	123.7	149.2	8.1	40.2	92.8	249.4	341.1	83.6	357.7	373.5	173.5
	0.003	114.1	99.3	176.7	98.3	122.3	84.5	92.8	5.9	23.0	31.3	147.0	208.0	49.2	221.6	229.5	111.3
	±%	-37	-40	-41	-39	-38	-32	-38	-27	-43	-66	-41	-39	-41	-38	-39	-36
	0.3	112.3	96.3	171.1	98.7	121.4	82.0	90.4	5.1	22.9	41.7	162.5	206.3	52.2	234.8	228.7	111.0
	±%	-38	-42	-43	-39	-39	-34	-39	-37	-43	-55	-35	-40	-38	-34	-39	-36
	30	106.3	91.7	167.1	92.9	116.2	80.7	87.6	5.4	20.3	29.0	142.2	195.7	46.0	216.3	212.4	102.0
±%	-41	-45	-44	-42	-41	-35	-41	-33	-50	-69	-43	-43	-45	-40	-43	-41	

<sup>a</sup> Amino acid is expressed using milligram per liter hydrolytic solution.

<sup>b</sup> Sampling was carried out 6 d after herbicide treatment.

<sup>c</sup> ±% is percentage decrease (-) or increase (+) compared with control.

content decreased markedly as monosulfuron concentration increased. For instance, at a monosulfuron concentration of 300 nmol L<sup>-1</sup>, the protein content of *A. azollae* cells increased by only 8%. However, the protein content of *A. azotica* cell cultures treated with monosulfuron concentrations from 0.03 to 300 nmol L<sup>-1</sup> increased between 3 and 25% relative to the control treatment, indicating that *A. azotica* was less sensitive to monosulfuron than the two other cyanobacteria (Figure 3C).

Several studies have assessed the effects of herbicides on metabolic pathways of protein synthesis in nontarget organisms. For example, Gruenhagen and Moreland (1971) evaluated the effects of 22 herbicides on adenosine triphosphate (ATP) content, ribonucleic acid (RNA), and protein synthesis in soybean [*Glycine max* (L.) Merr.] hypocotyl tissue. These workers reported that at 0.6 mM, 8 of the 22 herbicides tested (dinoseb, ioxynil, propanil, chlorpropham, pyriclor, propachlor, 2,4,5-T, and fenac) reduced RNA and protein synthesis, and that this inhibition was due to a reduction in ATP production. Our findings at low monosulfuron concentrations (0.03 to 3 nmol L<sup>-1</sup>) indicate that the protein content of cells from the three target nitrogen-fixing cyanobacteria increased substantially. The increase may have resulted from stimulation of protein synthesis via increased production of RNA. Alternatively, at lower monosulfuron concentrations, algal cells may have assimilated more organic carbon and nitrogen (Hoare et al. 1967; Kamiya and Kowallik 1987), which interfered with herbicide uptake through the formation of an inactive herbicide complex with organic carbon and nitrogen. This process can reverse herbicide toxicity or can convert the herbicide into hydrate of carbon and amino acid metabolites, thus decreasing the toxicity of the herbicide (Mishra and Pandey 1989). Interestingly, at monosulfuron concentrations of 30 to 300 nmol L<sup>-1</sup> in our study, protein metabolism differed markedly from that at lower rates. This change in response with increasing herbicide concentration may be due to

differences in the biological properties of the three cyanobacteria (Chen et al. 2001; Wu and Zhou 2004) and may be exploited in cropping systems to select for cyanobacteria that are more resistant to monosulfuron.

The finding that *A. flos-aquae* exhibited greater sensitivity to monosulfuron may be due to this species having a greater capacity to take up the herbicide. This feature may be exploited by using monosulfuron to reduce *A. flos-aquae* populations in lakes that have experienced serious eutrophication problems because of this species (Wu and Zhou 2004). Our finding that monosulfuron concentrations of 30 to 300 nmol L<sup>-1</sup> reduced the protein content of *A. flos-aquae* cells may be also be related to an increase in protease activity (Bhunia et al. 1991). Monosulfuron toxicity may also have been due to an inhibition in the synthesis of the ALS enzyme, thereby interfering with production of the three branched-chain amino acids (BCAAs) valine, leucine, and isoleucine, which are essential precursors of proteins (Fan et al. 2005).

**Effects on Amino Acid Production.** The effect of monosulfuron concentration on the production of amino acids in the three algal species tested is shown in Table 1. Monosulfuron applied at low concentrations (0.003 to 0.3 nmol L<sup>-1</sup>) stimulated the production of valine, leucine, and isoleucine in *A. azotica*, but production of these amino acids decreased markedly as monosulfuron concentration increased. Hormesis, the stimulatory effect of subtoxic concentrations of a toxin, has been documented following the application of other herbicides and allelochemicals (Calabrese and Baldwin 2003; Calabrese 2005; Prithiviraj et al. 2007). In contrast to *A. azotica*, the production of these BCAAs in cells of *A. flos-aquae* and *A. azollae* decreased with increasing monosulfuron concentration. Therefore, cells of *A. flos-aquae* and *A. azollae* exhibited greater sensitivity to monosulfuron than cells of *A. azotica*. This dose-dependent reduction in the amino acid content of the three algal test species is consistent with findings from previous studies on *Escherichia coli* [(Migula)

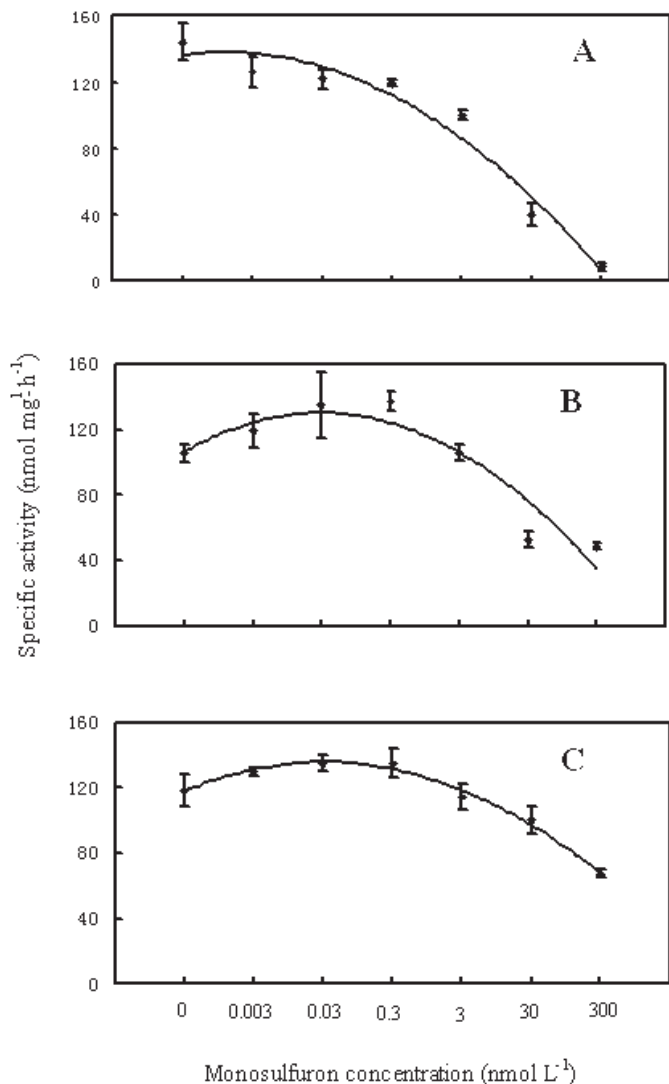


Figure 4. In vitro specific activity of acetolactate synthase for three nitrogen-fixing cyanobacteria (*Anabaena flos-aquae*, *Anabaena azollae*, and *Anabaena azotica*) subjected to different concentrations of monosulfuron for 6 d ( $n = 3$ ). Regression equations are: (A, *A. flos-aquae*)  $y = -6.55x^2 - 30.93x + 91.19$ ,  $r^2 = 0.97$ ; (B, *A. azollae*)  $y = -5.67x^2 - 23.67x + 105.82$ ,  $r^2 = 0.87$ ; (C, *A. azotica*)  $y = -4.36x^2 - 16.87x + 119.15$ ,  $r^2 = 0.99$ .

Castellani and Chalmers] and *Corynebacterium glutamicum* [(Kinoshita, Nakayama and Akita) Abe, Takayama and Kinoshita] subjected to applications of the sulfonyleurea herbicide sulfometuron (ALSIII) (Barak 1988; Leyval et al. 2003; Vyazmensky et al. 1996).

In our study, we demonstrated that the quantity of BCAAs decreased as monosulfuron concentration increased. However, we also found that the response to monosulfuron of 13 additional amino acids (alanine, asparagine, glutamine, phenylalanine, glycine, histidine, lysine, methionine, proline, arginine, serine, threonine, and tyrosine) was similar to that of the three BCAAs (Table 1). We hypothesize that monosulfuron inhibits ALS activity, and interferes with protein synthesis, resulting in a reduction in the quantity of other amino acids produced.

**Effects on ALS Activity.** The in vitro specific ALS activity varied between the three algal species after monosulfuron application (Figure 4). For *A. flos-aquae*, the specific activity

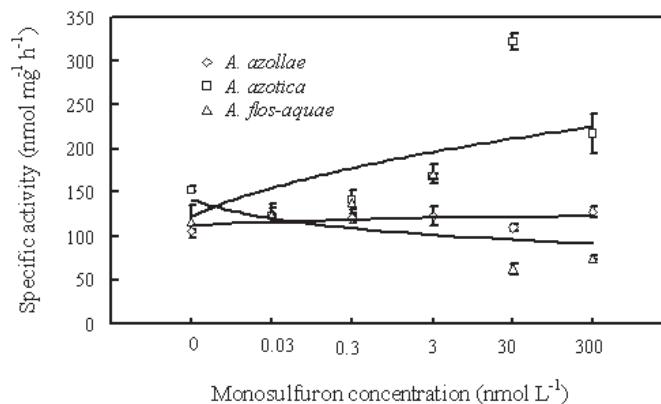


Figure 5. Extractable specific activity of acetolactate synthase for three nitrogen-fixing cyanobacteria (*Anabaena flos-aquae*, *Anabaena azollae*, and *Anabaena azotica*) subjected to different concentrations of monosulfuron for 6 d ( $n = 3$ ). Regression equations are: (*A. flos-aquae*)  $y = -10.13x^2 - 17.19x + 134.46$ ,  $r^2 = 0.55$ ; (*A. azollae*)  $y = 2.19x^2 - 0.79x + 117.02$ ,  $r^2 = 0.54$ ; (*A. azotica*)  $y = -8.62x^2 + 36.77x + 211.41$ ,  $r^2 = 0.58$ .

of ALS was reduced from 13 to 17% at monosulfuron concentrations of 0.003 to 0.3  $\text{nmol L}^{-1}$  compared with the control ( $P < 0.05$ ). Reductions in activity rose to 31, 72, and 94% as the concentration of monosulfuron increased to 3, 30, and 300  $\text{nmol L}^{-1}$ , respectively. There was a significant difference in activity between each concentration ( $P < 0.05$ ). In contrast, the specific ALS activity of *A. azollae* at low monosulfuron concentrations (i.e., 0.003 to 3  $\text{nmol L}^{-1}$ ) did not differ significantly compared with the nonherbicidal control. However, ALS activity in this algal species was drastically reduced by 51 and 55%, as the concentration of monosulfuron increased from 30 to 300  $\text{nmol L}^{-1}$ , respectively, compared with the control. Of particular interest was the observation that the specific activity of ALS in *A. azotica* at monosulfuron concentrations ranging from 0.003 to 0.3  $\text{nmol L}^{-1}$  showed a significant increase (10 to 14%) relative to the control ( $P < 0.05$ ), followed by a slight decrease at the 3  $\text{nmol L}^{-1}$  concentration, and subsequently decreasing by 16 and 43% at the 30 and 300  $\text{nmol L}^{-1}$  concentrations. These results indicate that monosulfuron does inhibit ALS activity in the three nitrogen-fixing cyanobacteria tested, particularly at higher concentrations. This strong in vitro inhibition of ALS activity confirms that the mode of action of monosulfuron is inhibition of the ALS enzyme, as has been reported for other sulfonyleurea herbicides (Chaleff and Mauvais 1984; LaRossa and Schloss 1984; Ray 1984). Calculated  $I_{50}$  values for *A. flos-aquae*, *A. azollae*, and *A. azotica* were 3.3, 65.2, and 101.3  $\text{nmol L}^{-1}$ , respectively, supporting the view of a negative impact of this herbicide on the growth of these algal species. Shen et al. (2005) reported that several nitrogen-fixing cyanobacteria (e.g., *A. azollae*, *A. azotica*) that were common in rice fields of the Shanghai region of China before the overuse of herbicides are now absent from these systems. Therefore, excessive application of selective herbicides such as monosulfuron either through overuse or misuse can substantially reduce microbial diversity in these cropping systems.

At monosulfuron concentrations 0.03 to 300  $\text{nmol L}^{-1}$ , the extractable ALS activity in the three algal species was not inhibited except in *A. flos-aquae* at higher concentrations (30 to 300  $\text{nmol L}^{-1}$ ) (Figure 5). Monosulfuron actually stimulated ALS activity, except for *A. flos-aquae*, where the specific

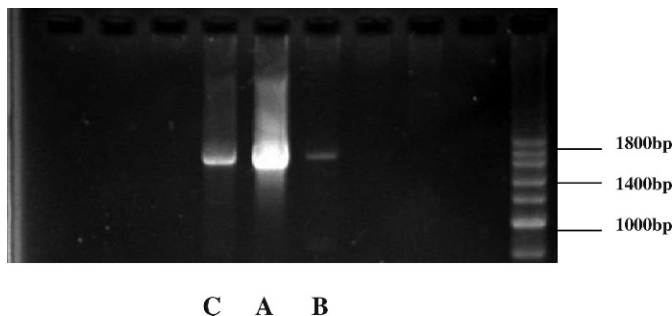


Figure 6. Agarose gel electrophoresis of the polymerase chain reaction product of the acetolactate synthase gene generated from three nitrogen-fixing cyanobacteria. (A) *Anabaena flos-aquae*; (B) *Anabaena azollae*; and (C) *Anabaena azotica*.

activity decreased from 35 to 47% as the concentration of monosulfuron increased to 30 to 300 nmol L<sup>-1</sup> compared with the control ( $P < 0.05$ ). It is possible that differences in specific activity between in vitro and in vivo responses of the algal cultures were due to differences in the levels of herbicide detoxification enzymes present (e.g., glutathione *S*-transferase and mixed-function oxidase) (Dastgheib and Field 1998; Koeppe et al. 2000). Another possible reason for the markedly different effects of the same monosulfuron concentration on extractable ALS specific activity between the algal species is that absorption of this herbicide as well as the degree of ALS inhibition varied between them.

**Genomic DNA Analyses.** Agarose gel electrophoresis of DNA for the three algal species is shown in Figure 6. The nucleotide alignments of ALS were not identical in three nitrogen-fixing algal species (Figure 7). The genomic DNA of *A. azollae* and *A. azotica* differed in only one amino acid, the substitution of an Ala<sub>63</sub> in *A. azollae* by a Thr<sub>68</sub> in *A. azotica*. The results of photogenetic analyses displayed high homology between both algae (Figure 8). The homology matrix of the two sequences was 99.8%. However, the genomic DNA of *A. flos-aquae* differed from that of *A. azollae* and *A. azotica* for 44 and 45 amino acids, respectively (e.g., Ala<sub>55</sub>, Ser<sub>58</sub>, Gln<sub>152</sub>, Ile<sub>196</sub>, Arg<sub>199</sub>, Gly<sub>237</sub>, Leu<sub>271</sub>, Thr<sub>287</sub>, Asn<sub>358</sub>, Ser<sub>476</sub>, Val<sub>519</sub>, and so on). The homology matrix of the two sequences with *A. azollae* and *A. azotica* were 92 and 91%, respectively.

Although enhanced herbicide detoxification was also reported as a resistance mechanism in some weed populations, resistance to the ALS-inhibiting herbicides is mostly due to an altered target site, namely point mutations that occur within discrete conserved domains of the ALS gene (Sibony and Rubin 2003). A substitution of Ser<sub>264</sub> by Gly in the D<sub>1</sub> protein was responsible for triazine resistance in several smooth pigweed (*Amaranthus hybridus* L.) biotypes (Hirschberg and McIntosh 1983). A prostrate pigweed (*Amaranthus blitoides* S. Wats.) population resistant to ALS-inhibiting herbicides and triazines in which domain A of the ALS gene differed in one nucleotide, resulting in substitution of Pro by Ser<sub>188</sub>, was found to be 6- to 790-fold more resistant to ALS inhibitors than the wild type because of an altered target site (Sibony and Rubin 2003). A mutation at the same site in kochia [*Kochia scoparia* (L.) Schrad.] where Pro was substituted by either Thr, Ser, Arg, Leu, Gln, or Ala resulted in resistance to both sulfonylurea (31- to 138-fold) and imidazolinone (2- to 11-fold) herbicides (Guttieri et al. 1995). These results indicate that mutation at the same positions in a gene may confer

Anabaena azollae	.....SSVWTHRRITGGFALLDSLRRHVEYIFGYFG	32
Anabaena flos-aquae	HTQSVNSSEVVVTFPRTGGFALLDSLRRHVEYIFGYFG	40
Anabaena azotica	...QSAACSSEVWTHRRITGGFALLDSLRRHVEYIFGYFG	37
Consensus	s vvtpr r tggfalldsllrhgveyifgyfg	
Anabaena azollae	GAILFIYDLYKVESTCALHHIIVRHEQGAHAADGYARA	72
Anabaena flos-aquae	GAILFIYDLYKVEATSLKHHIIVRHEQGAHAADGYARA	80
Anabaena azotica	GAILFIYDLYKVESTCALHHIIVRHEQGAHAADGYARA	77
Consensus	gailpiyddlykve tg lkhilvrheqga haadgyara	
Anabaena azollae	TGKVCVCFGTSGPGATNLVGTIATAYMDSIPMVIITGQVP	112
Anabaena flos-aquae	TGKVCVCFGTSGPGATNLVGTIATAYMDSIPMVIITGQVP	120
Anabaena azotica	TGKVCVCFGTSGPGATNLVGTIATAYMDSIPMVIITGQVP	117
Consensus	tgkvgvcfgtsgpgatnlvtgiataymdsipmviitgqvp	
Anabaena azollae	RRMIGTDAFQETDIYGITLPIVKHSYVVRDPCMARIVAE	152
Anabaena flos-aquae	RRMIGTDAFQETDIYGITLPIVKHSYVVRDPCMARIVAE	160
Anabaena azotica	RRMIGTDAFQETDIYGITLPIVKHSYVVRDPCMARIVAE	157
Consensus	rkmigtdafqetdiygitlpivkhsyvvrp cmarivae	
Anabaena azollae	AFHIASTGRGPEVLIIVPKIVAFEDYDVEVEEGIVKLG	192
Anabaena flos-aquae	AFHIASTGRGPEVLIIVPKIVAFEDYDVEVEEGIVKLG	200
Anabaena azotica	AFHIASTGRGPEVLIIVPKIVAFEDYDVEVEEGIVKLG	197
Consensus	afhiastrgpgpvlidvpkivafe fdy pvepg kl g	
Anabaena azollae	YRPTVKNRPRCINAAILLIRESRRLYVGGGAIASGAF	232
Anabaena flos-aquae	YRPTVKNRPRCINAAILLIRESRRLYVGGGAIASGAF	240
Anabaena azotica	YRPTVKNRPRCINAAILLIRESRRLYVGGGAIASGAF	237
Consensus	yrptvknprcinaai l lrerprllyvqgqaias ga	
Anabaena azollae	EVKQLAELFNIFVITTMGIGAFDEHHPISVGLMGHGTA	272
Anabaena flos-aquae	EVKQLAELFNIFVITTMGIGAFDEHHPISVGLMGHGTA	280
Anabaena azotica	EVKQLAELFNIFVITTMGIGAFDEHHPISVGLMGHGTA	277
Consensus	e laelfnfpvttimgigafdehnpis vglmghgta	
Anabaena azollae	YANFAVSCDILICVGARFEDRVTKLDEFASRAKVIHID	312
Anabaena flos-aquae	YANFAVSCDILICVGARFEDRVTKLDEFASRAKVIHID	320
Anabaena azotica	YANFAVSCDILICVGARFEDRVTKLDEFASRAKVIHID	317
Consensus	yanfav dcdllicvgarfedrvtkldefasrakvihid	
Anabaena azollae	ICPAEVGKNRIPEVPIVGVVRLVLLLRCKQSGVQVETP	352
Anabaena flos-aquae	ICPAEVGKNRIPEVPIVGVVRLVLLLRCKQSGVQVETP	360
Anabaena azotica	ICPAEVGKNRIPEVPIVGVVRLVLLLRCKQSGVQVETP	357
Consensus	idpaevgknripevpivgvr vlldllrckq g k tp	
Anabaena azollae	NQNQEWLNLIWRWREYPLVYFCHPDSISQEQIIVVESHQ	392
Anabaena flos-aquae	NQNQEWLNLIWRWREYPLVYFCHPDSISQEQIIVVESHQ	400
Anabaena azotica	NQNQEWLNLIWRWREYPLVYFCHPDSISQEQIIVVESHQ	397
Consensus	nqnqewlnlinrwr e yplv yfchpdsi sqe iver v q	
Anabaena azollae	AFHAFYTTDVGQHCWMAAQFLKNGPRRWISSAGLGTMGEG	432
Anabaena flos-aquae	AFHAFYTTDVGQHCWMAAQFLKNGPRRWISSAGLGTMGEG	440
Anabaena azotica	AFHAFYTTDVGQHCWMAAQFLKNGPRRWISSAGLGTMGEG	437
Consensus	aphafyttvgqhcwmaaqflkngprrwissagltmgfg	
Anabaena azollae	LFAAIGAPVAIFENFVICISGDASFQMCQLQELGTAQYGI	472
Anabaena flos-aquae	LFAAIGAPVAIFENFVICISGDASFQMCQLQELGTAQYGI	480
Anabaena azotica	LFAAIGAPVAIFENFVICISGDASFQMCQLQELGTAQYGI	477
Consensus	lpaaignakvaf e vicisgdasfcmqlqelgta qygi	
Anabaena azollae	NVKTIVINNGQGVVRCWQQAQFHGERYSNMEVGMPEDE	512
Anabaena flos-aquae	NVKTIVINNGQGVVRCWQQAQFHGERYSNMEVGMPEDE	520
Anabaena azotica	NVKTIVINNGQGVVRCWQQAQFHGERYSNMEVGMPEDE	517
Consensus	nvktvi nngqgvvrqwqaqfhgeryscnmevgmpe e	
Anabaena azollae	LLAKAYGIGIVVREQLKDAIAMLAHNGFVILNHFVI	552
Anabaena flos-aquae	LLAKAYGIGIVVREQLKDAIAMLAHNGFVILNHFVI	560
Anabaena azotica	LLAKAYGIGIVVREQLKDAIAMLAHNGFVILNHFVI	557
Consensus	llakeyigigivv reqlkdaia mla hngfvil nhfvi	
Anabaena azollae	RCENCYPNVA	562
Anabaena flos-aquae	RCENCYPNVAL	571
Anabaena azotica	RCENCYPNVAL	568
Consensus	dencypnva	

Figure 7. Deduced amino acid sequence of the acetolactate synthase gene from the three nitrogen-fixing cyanobacteria (*Anabaena flos-aquae*, *Anabaena azollae*, and *Anabaena azotica*).

different responses in different plant species. The genomic DNA data obtained for the ALS enzyme in the three algal species we tested were consistent with our findings on algal growth, protein, amino acid, and ALS activity. This may be the main reason why *A. flos-aquae* exhibited greater sensitivity to monosulfuron than did *A. azollae* and *A. azotica*. For a better understanding of this mechanism, additional research using microarray analyses is needed that focuses on the action of these different DNA gene points on algal growth and the detoxification mechanism. To our knowledge, this is the first

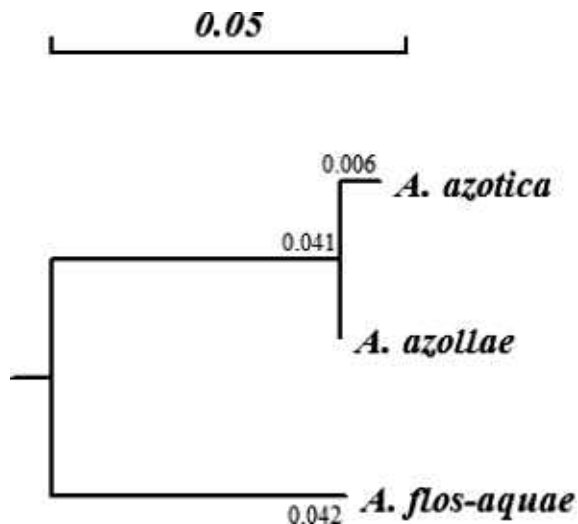


Figure 8. Phylogenetic tree of deduced acetolactate synthase amino acid sequences in three nitrogen-fixing cyanobacteria constructed using the neighbor-joining method.

study to characterize the differential responses to monosulfuron of these three nitrogen-fixing cyanobacteria in terms of production of amino acids and proteins, ALS activity, and their molecular basis.

In summary, our findings suggest that under laboratory conditions various nitrogen-fixing cyanobacteria may exhibit differential sensitivity to the presence of the sulfonylurea herbicide, monosulfuron, due to DNA genetic point differences. Therefore, in rice cropping systems where the herbicide monosulfuron is applied at low concentration ( $3 \text{ nmol L}^{-1}$ ), use of the beneficial nitrogen-fixing cyanobacteria *A. azotica* and *A. azollae* may be most suitable as “biofertilizers” given their relative high tolerance to this herbicide. From an agronomic perspective, we propose that rather than selecting for herbicides that will have the least negative impact on the growth of important nitrogen-fixing cyanobacteria, it may be more valuable and efficient to develop or select for nitrogen-fixing cyanobacteria that exhibit a greater tolerance to herbicides used in cropping systems.

### Sources of Materials

<sup>1</sup> Dimethylformamide, Jianshan Chemicals Ltd., 38 Jinxing Road, Jianshan, Zhejiang Province, 324100, China.

<sup>2</sup> Triton X-100, Jibishi Gene Technology Ltd., 99 Gualing Road, Shanghai, 200233, China.

<sup>3</sup> Molecular Lab Water Purifier, Molecular Instrument Shanghai Co, Ltd., 2988 Ningduo Road, Shanghai, 201108, China.

<sup>4</sup> Bovine serum albumin, Pufi Biological Technology Ltd., 825 Zhaojiaoban Road, Shanghai, 200032, China.

<sup>5</sup> Coomassie Brilliant Blue G-250, Sigma Company, 3050 Spruce St. Box: 14508, St. Louis, MO 63178.

<sup>6</sup> Model 835-50 amino acid automatic analyzer, Hitachi (Shanghai), 205 Maoming Road, Shanghai 200020, China.

<sup>7</sup> Sorvall RC26 Plus, DJB Labcare Ltd., 20 Howard Way, Interchange Business Park, Newport Pagnell, Buckinghamshire, MK16 9QS, UK.

<sup>8</sup> Thiamine pyrophosphate, Sigma Company, 3050 Spruce St. Box: 14508 St. Louis, MO 63178.

<sup>9</sup> Flavin adenine dinucleotide, Sigma Company, 3050 Spruce St. Box: 14508 St. Louis, MO 63178.

<sup>10</sup> DL-Dithiothreitol, GL Biochem Ltd., 351 Guo Shoujing Road, Shanghai, 201203, China.

<sup>11</sup> UV-722 Spectrophotometer, Shanghai Biotechnic Ltd., 15 Keyuan Road, High technology Park, Zhanjian, Putong, Shanghai, 201203, China.

<sup>12</sup> Sigmaplot 2001 for Windows®, SPSS Inc., Headquarters, 233S, Wacker Drive, Chicago, IL 60606.

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