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Differential Responses of Two Freshwater Cyanobacteria, *Anabaena variabillis* and *Nostoc commune*, to Sulfonylurea Herbicide Bensulfuron-methyl

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Abstract The effect of bensulfuron-methyl on the non-targeted cyanobacteria was greater on *A. variabillis* than *N. commune*. Both *A. variabillis* and *N. commune* were initially able to utilize low concentrations of the herbicide, bensulfuron-methyl, whereas higher concentrations of bensulfuron-methyl or the hydrolyzed products of the herbicide were found to be toxic. Growth and photosynthesis inhibitions of over 50% were observed, when 8 to 10 ppm of the herbicide was applied. Nitrogenase activities of the cyanobacteria were decreased by 94–98% in *A. variabillis* and by 85–86% in *N. commune* after 24 h of incubation with 10 ppm and 20 ppm of bensulfuron-methyl. Nitrogenase activities were also inhibited by the addition of ammonium salts as low as 0.05 mM. Furthermore, the toxic effect of the herbicide was the highest at pH 4–6, showing approximately 42–60% toxicity, whereas much lower toxicity (9–28%) was observed at higher pH of 7–10, due to base-catalyzed hydrolysis of bensulfuron-methyl.

Key words: *Anabaena variabillis*, *Nostoc commune*, nitrogen fixation, herbicide, bensulfuron-methyl

The nitrogen-fixing cyanobacteria form a prominent component of microbial population in wetland soils, especially in rice paddy fields, since they significantly contribute to fertility as a natural biofertilizer [14]. Some cyanobacteria strains that thrive in rice fields release small quantities of the major fertilizing product, ammonia, and small nitrogenous polypeptides during active growth, whereas most of the fixed products become available mainly through autolysis and decomposition [10]. However, herbicides have a detrimental effect on microorganisms that play an important role in plant growth, crop productivity, and soil fertility [7]. The effect of herbicides on the population of nitrogen-fixing organisms varies with characteristics of herbicide, water availability, temperature, and soil type [3, 6, 15]. A great deal of attention has been paid on the importance of monitoring the effect of herbicides on non-target organisms. In particular, soil algae and cyanobacteria are potentially susceptible to herbicides.

With the development of agriculture, sulfonylurea herbicides have widely been used in rice paddy fields in Korea to manage broadleaf and grass weeds [13]. Sulfonylurea herbicides inhibit acetolactate synthase (ALS), the first enzyme that catalyzes the biosynthesis of branched-chain amino acids such as valine, leucine, and isoleucine [1, 4, 19]. They exist almost exclusively in the anionic form, which are much less susceptible to their main degradation pathway, hydrolysis [9, 11, 12]. Many researches on the toxicity of sulfonylurea herbicides on weeds and crops have been reported [5, 8]. However, there have been only a limited number of investigations on their environmental behavior and physicochemical properties, as well as their effects on aquatic organisms. The present study, therefore, focused on the assessment of the effect of a sulfonylurea herbicide, bensulfuron-methyl, on N_2-fixing cyanobacterial strains *Anabaena variabillis* and *Nostoc commune*, and elucidated the degree of toxicity.

Materials and Methods

Microorganisms and Culture Conditions

The filamentous cyanobacteria, *Anabaena variabillis* KJ-013 and *Noctoc commune* KJ-018, were isolated from rice paddy fields in Korea and grown in batch cultures at 28°C in the nitrogen-free BG-11 liquid medium [20]. The growth was determined by measuring the culture density at 800 nm, and chlorophyll *a* concentration was calculated after extraction with 90% methanol according to the method of Parsons and Strickland [17]. Cyanobacteria filtered by GF/C (Whatman, Uppsala, Sweden) were subjected to

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extraction with 90% methanol at 60°C for 10 min. After extraction, the solid suspension was removed by centrifugation. Then, absorbance of extracts was measured at 665, 645, and 635 nm using a spectrophotometer (model HP8453, Hewlett Packard, MI, U.S.A.). The concentration of chlorophyll *a* was calculated using the following equation.

$$\text{Chl-}a\ (\text{mg/l}) = 11.6\ A_{665} - 1.31\ A_{645} - 0.14\ A_{515}$$

The liquid medium was adjusted to the desired pH (between 4 and 10) before inoculation and treatment. The cultures were treated with 10 ppm of bensulfuron-methyl for 24 h.

Herbicide Preparation

The herbicide, bensulfuron-methyl (commercial herbicide grade), kindly supplied by Dongbu-Hannong Co., Ltd. in Korea, was prepared in stock solution and added aseptically to the culture medium to final concentrations indicated for each treatment.

Nitrogenase Activity

To determine the response to the addition of ammonium, the acetylene reduction assay (ARA) was performed in 25-ml aliquots of cell suspensions placed in 50 ml vials, 24 h after the addition of 0.05, 0.2, 0.5, and 1.0 mM NH_4Cl to the media. The incubation time with acetylene was 30 min. Nitrogenase activity was expressed in μmol acetylene/mg of protein/h. Protein contents were measured by the method reported earlier [2]. The effect of herbicide on nitrogenase activity was estimated at 3 and 24 h after the herbicide addition.

Oxygen Production

Photosynthetic activity was measured via O_2 evolution with a Clark-type electrode. Three-milliliter aliquots of cell suspensions with a cell density of 0.1 mg/ml were placed in a 27°C temperature-controlled cuvette and illuminated with 300 $\mu E/m^2/s$.

RESULTS

Growth Responses of the Two Cyanobacteria to Bensulfuron-methyl

The detrimental effect of bensulfuron-methyl on the growth of *A. variabillis* KJ-013 and *N. commune* KJ-018 was negligible at low levels, in particular under 1.0 ppm. However, bensulfuron-methyl at concentrations between 0.2 ppm to 1.0 ppm actually stimulated the growth (final cell concentration) dramatically, and the chlorophyll *a* level of both cyanobacteria was increased (Fig. 1). The stimulating effect of 0.1 ppm bensulfuron-methyl on growth of the two cyanobacteria was not outstanding, and the final cell concentration and chlorophyll *a* concentration were almost identical to those of the control (without the herbicide).

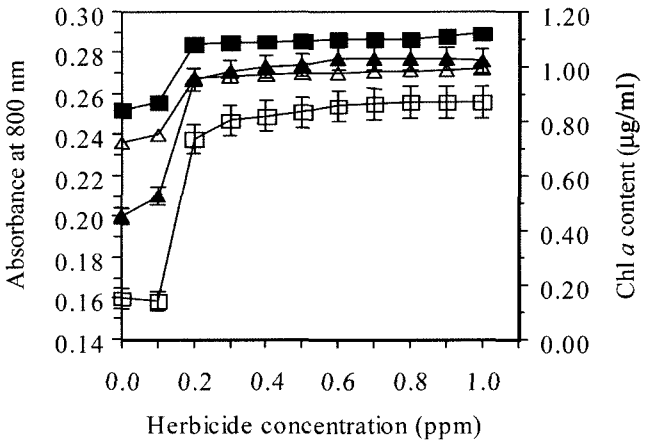

Fig. 1. The growth responses of *Anabaena variabillis* KJ-013 and *Nostoc commune* KJ-018 to the treatments of bensulfuron-methyl with low concentration ranges (0.1–1.0 ppm).
The growth of cyanobacteria was monitored by measuring absorbance at 800 nm (▲, *A. variabillis* KJ-013; ■, *N. commune* KJ-018) and chlorophyll *a* content (△, *A. variabillis* KJ-013; □, N. *commune* KJ-018). Bars represent standard deviation from the mean of three replicates.

However, the growth stimulatory effects leveled off at over 0.5 ppm of the herbicide.

As shown in Table 1, the growth of the cyanobacteria started to be inhibited at 2.0 ppm or higher concentrations of bensulfuron-methyl. The cultures with higher concentration of the herbicide were slightly inhibited initially (6–12 h); however, the chlorophyll *a* contents after 24 h were gradually reduced, and the growth inhibitory effect was noticeably increased as the concentration of the herbicide increased. Concentrations of 8 and 10 ppm inhibited more than 50% of the growth of both cyanobacteria. Toxicity was also observed at concentrations higher than 2.0 ppm. The chlorophyll *a* contents were found to be a more sensitive indicator of the herbicide than the final cell concentration, although the chlorophyll *a* content and the final cell concentration showed similar response patterns in both cyanobacteria. *N. commune* KJ-018 was slightly more sensitive to the herbicide than *A. variabillis* KJ-013 was (Table 1).

Table 1. Effect of bensulfuron-methyl on the growth of *Anabaena variabillis* KJ-013 and *Nostoc commune* KJ-018.

Concentration of bensulfuron-methyl (ppm)	Inhibition (%)[a]			
	A. variabillis KJ-013		*N. commune* KJ-018	
	Growth	Chl *a*	Growth	Chl *a*
2	5.3±0.5	6.8±0.3	8.8±0.1	11.9±0.2
4	16.2±1.1	20.9±1.2	19.2±0.1	24.9±0.3
6	41.1±1.8	62.8±1.4	49.2±0.4	70.3±0.7
8	50.8±1.3	70.2±2.2	58.1±1.5	79.6±1.9
10	52.1±2.1	70.8±2.3	60.1±2.1	80.1±2.4

[a]Values represent mean±standard deviation of three replicates.

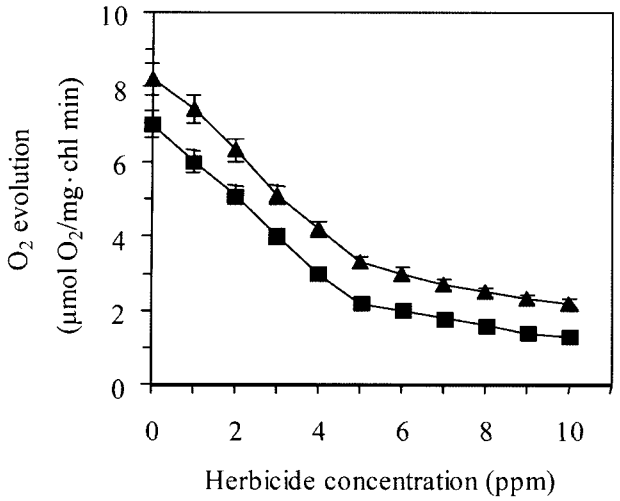

Fig. 2. Effect of different bensulfuron-methyl concentrations on oxygen photoevolution in *A. variabillis* KJ-013 (▲) and *N. commune* KJ-018 (■).
Bars represent standard deviation from the mean of three replicates.

Effect of Herbicide on Photosynthesis

The interference of bensulfuron-methyl with photosynthetic chlorophyll was further clarified by investigating the effect of the herbicide on the photosynthetic electron flow. The effects of the herbicide on the photosynthetic activities (oxygen production) of *A. variabillis* KJ-013 and *N. commune* KJ-018 are shown in Fig. 2. Here, the herbicide was directly added to the oxygen electrode chamber in order to assay the direct responses of photosynthesis to the herbicide. As shown in Fig. 2, bensulfuron-methyl clearly suppressed the photosynthetic activity of *N. commune* KJ-018, and the oxygen evolution was dramatically decreased to 32.3% at 5 ppm and to less than 18% at 10 ppm. For *A. variabillis* KJ-013, the suppression level by bensulfuron-methyl was less dramatic, resulting in decreased oxygen evolution down to 40.2% at 5 ppm and 26.8% at 10 ppm.

Effect of Herbicide on Nitrogenase Activity

Nitrogenase activities under the treatment with bensulfuron-methyl showed different patterns for *N. commune* KJ-018 and *A. variabillis* KJ-013 (Table 2). Acetylene-reducing activity (ARA) was decreased by more than 40% in *N. commune* KJ-018 culture after 3 h of incubation at 5 ppm of bensulfuron-methyl, and the inhibition in ARA was maintained at a similar level after 24 h in all concentrations of the herbicide (Table 2). The inhibitory effect of bensulfuron-methyl on *N. commune* KJ-018 reached the maximum at around 10 ppm and no further inhibition was observed by 20 ppm.

For *A. variabillis* KJ-013, ARA inhibition by bensulfuron-methyl was more detrimental than that for *N. commune* KJ-018. The ARA of *A. variabillis* KJ-013 was decreased by 50% at 5 ppm of bensulfuron-methyl after 3 h of incubation and by 60% after 24 h. Higher concentrations of bensulfuron-methyl almost completely shut down the ARA of *A. variabillis* KJ-013, and only 2–6% of the nitrogenase activity of *A. variabillis* KJ-013 was observed at 10 ppm or higher concentrations of bensulfuron-methyl (Table 2), thus indicating that *A. variabillis* KJ-013 was more sensitive to the herbicide.

Table 2. Effects of different bensulfuron-methyl concentrations on the nitrogenase activities of two cyanobacteria.

Bensulfuron-methyl concentration (ppm)	Nitrogenase activity (μmol/mg protein/h)[a]			
	A. variabillis KJ-013		*N. commune* KJ-018	
	3 h	24 h	3 h	24 h
0	40±2	41±3	38±4	37±5
5	20±3	17±3	22±3	22±3
10	5±2	6±2	18±4	15±2
20	2±1	2±1	19±3	14±3

[a]Values represent mean±standard deviation of three replicates.

The detrimental effect of the herbicide on the nitrogenase activity could be reproduced by the addition of ammonium salts. The nitrogenase activity after the addition of ammonium at 0.05, 0.2, 0.5, and 1.0 mM was also dramatically decreased. ARA at low levels of the herbicide (0.05 mM) was inhibited by 26–37% after 24 h of cultivation, but ARA under 1.0 mM ammonium was almost completely inhibited (Table 3).

Effect of pH on Toxicity of Bensulfuron-methyl in Cyanobacteria

The pH variation is an integrated function in individual metabolism for the cyanobacterial growth. Therefore, the effect of pH on the effectiveness of bensulfuron-methyl in responses of *N. commune* KJ-018 and *A. variabillis* KJ-013 was examined. The above obtained results (Table 1) indicated a significant growth inhibition of higher than 50% at 10 ppm. However, as expected, an adverse effect of the herbicide was shown as a function of pH (Fig. 3): The toxicity of 10 ppm bensulfuron-methyl was higher at acidic pH of pH 4–6, whereas that at basic pH of pH 7–10 was less. The growth of *N. commune* KJ-018 and *A. variabillis* KJ-013 was inhibited at pH 4–5 by 50% and 60%, respectively. On the other hand, the inhibitory effects of

Table 3. Effect of different ammonium concentrations on the nitrogenase activities of two cyanobacteria after 24-h cultivation.

Ammonium (mM)	Nitrogenase activity (μmol/mg protein h)[a]	
	A. variabillis KJ-013	*N. commune* KJ-018
0.00	57±5	42±3
0.05	36±4	31±4
0.20	31±3	19±3
0.50	16±2	5±1
1.00	5±1	3±1

[a]Values represent mean±standard deviation of three replicates.

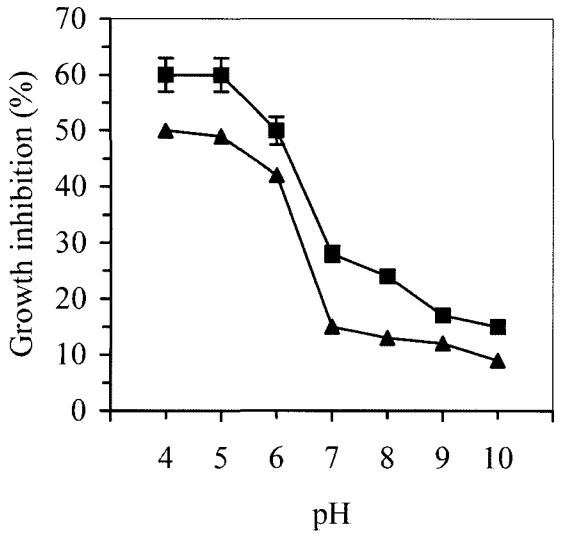

Fig. 3. Effect of pH on toxicity of bensulfuron-methyl in the cultures of *A. variabillis* KJ-013 (▲) and *N. commune* KJ-018 (■) treated with 10 ppm bensulfuron-methyl.
Bars represent standard deviation from the mean of three replicates.

the herbicide was somewhat lessened at pH 6, resulting in 50% and 42% inhibition for *A. variabillis* KJ-013 and *N. commune* KJ-018, respectively. Treatment of KJ-013 and KJ-018 with the same herbicide at the same concentration (10 ppm) at neutral pH inhibited the growth by only 28% and 15%, respectively. Alkaline pH over 8 nearly counteracted the toxic effect of bensulfuron-methyl, and the growth at basic pH was inhibited by only 10–15%.

DISCUSSION

The stimulating effect of less than 0.1–1.0 ppm bensulfuron-methyl suggests initial absorption of the herbicide by *A. variabillis* KJ-013 and *N. commune* KJ-018. However, growth inhibitions by more than 50% were observed after 24 h of incubation, when the higher concentrations of 8 and 10 ppm of bensulfuron-methyl were used. These cyanobacteria seemed to be able to metabolize and then utilize the herbicide to a certain degree (to a certain concentration of the herbicide). However, the toxic effects of bensulfuron-methyl on *A. variabillis* KJ-013 and *N. commune* KJ-018 were obvious at concentrations higher than 1.0 ppm, suggesting that susceptibility of the sheathless cyanobacteria to the herbicide was comparable with recently reported levels for other green algae [21]. The concentrations of bensulfuron-methyl that caused significant detrimental effects on the algal growth ranged from 0.21 to 15.5 ppm for *Chlorella vulgaris*, and from 0.035 to 3.6 ppm for *Scenedesmus subspicatus* [21]. Changes of the nitrogen-fixation ability of the cyanobacterial strains have earlier been reported to be dependent on the concentration of this herbicide, but independent of the incubation period [23]. Similar trends were also observed with *A. variabillis* KJ-013 and *N. commune* KJ-018 (Table 2). However, *N. commune* KJ-018 was slightly more sensitive to the herbicide than *A. variabillis* KJ-013 was (Table 1). This phenomenon was due to the difference of N_2-fixation ability between *A. variabillis* KJ-013 and *N. commune* KJ-018: High efficiency (57.0–85.0 μM C_2H_4/mg of Chl *a*/h) was observed with *A. variabillis* KJ-013, whereas moderate efficiency (28.0–57.0 μM C_2H_4/mg of Chl *a*/h) with *N. commune* KJ-018. Orus and Marco [16] concluded that destabilization of the heterocyst envelope of *Anabaena* sp. 7119 is the first target of herbicide action, inhibiting dinitrogen fixation in 18 and 24 h.

Inhibitory effects of other chemicals on the nitrogen fixation of cyanobacteria were also examined by several investigators. Sardeeshpande and Goyal [22] studied the effect of carbofuran on the nitrogenase activity of the cyanobacteria *Hapalosiphon intricatus*, *H. fontinalis*, *Anabaena iyengarii*, and *Calothrix membranacea*. The insecticide concentrations of 0.5 and 1 ppm were inhibitory to both growth and nitrogen fixation in *H. intricatus* and *H. fontinalis*. However, these processes in *A. iyengarii* and *C. membranacea* were stimulated by 1 ppm of carbofuran. The data shown herein also suggested dual characteristics of bensulfuron-methyl (Fig. 1 and Table 1).

The effect of bensulfuron-methyl was less marked in *N. commune* KJ-018. The fact that bensulfuron-methyl did somewhat affect nitrogenase activity should be considered when this herbicide has to be used on rice. The inhibition of nitrogenase activity in the presence of ammonium (Table 3) was similar to the study reported by Prosperi *et al.* [18]. High ammonium concentration between 0.5 to 1.0 mM can occur in rice fields, when fertilizer is applied, and it should be diluted or consumed in a relatively short time period in order to maintain the beneficial effects of cyanobacteria.

One of the important environmental factors of the rice paddy field is pH. In an effort to examine the influence of pH on the effectiveness of bensulfuron-methyl, pH was found to be an extremely critical factor, not only because the inhibition level was drastically changed by the pH, but also because every metabolic rate in the cell would be a function of pH. Bensulfuron-methyl of 10 ppm was the most toxic at pH 4–6, whereas it was much less malignant at pH 7–10. Differences in the toxicity could be explained by the influence of pH on the persistence and alkaline-catalyzed hydrolysis. Siddaramappa *et al.* [23] reported that 86% of carbofuran was hydrolyzed to carbofuran phenol in 10 days at pH 7.1, whereas 70% of the applied carbofuran remained intact at pH 6 for the same period.

The nitrogen-fixing cyanobacteria play an important role in rice paddy fields as a natural biofertilizer. However, few researches have been carried out on the effect of herbicides on these beneficial cyanobacteria. The influence

of a herbicide, bensulfuron-methyl, on the non-targeted cyanobacteria *Anabaena variabillis* and *Nostoc commune* was investigated. *A. variabillis* was found to be more sensitive to the herbicide than *N. commune*. Both cyanobacteria were able to utilize low concentrations of bensulfuron-methyl, whereas higher concentrations were found to be toxic. Growth inhibition reached over 50% by 8 to 10 ppm of the herbicide. Nitrogenase activity in *A. variabillis* was reduced to 2–6% and to about 15% in *N. commune* after 24 h of treatment with 10 ppm and 20 ppm of bensulfuron-methyl. Ammonium salts also inhibited acetylene reducing activity at as low as 0.05 mM. Toxicity of the herbicide was the highest at acidic pH, whereas that at basic pH was almost benign. Consequently, if bensulfuron-methyl were to be used, the pH of the field, concentration of herbicide, and timing of nitrogen fertilizer application should be considered to minimize any harmful effect of the herbicide on beneficial cyanobacteria.

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References

1. Atri, N. and L. C. Rai. 2003. Differential responses of three cyanobacteria to UV-B and Cd. *J. Microbiol. Biotechnol.* **13:** 544–551.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72:** 248–254.
3. Baek, K.-H., H.-S. Kim, S.-H. Moon, I.-S. Lee, H.-M. Oh, and B.-D. Yoon. 2004. Effects of soil type on the biodegradation of crude oil by *Nocardia* sp. H17-1. *J. Microbiol. Biotechnol.* **14:** 901–905.
4. Brown, H. M. 1990. Mode of action, crop selectivity and soil relations of the sulfonylurea herbicides. *Pestic. Sci.* **29:** 263–281.
5. Dastgheib, F., M. Andrewa, J. D. Morton, and M. F Barnes. 1995. Mode of action of chlorsulfuron in sensitive wheat (*Triticum awativum*) cultivar; primary and secondary effect on nitrogen assimilation. *Ann. Appl. Biol.* **127:** 125–135.
6. Evans, R. D. and J. R. Johansen. 1999. Macrobiotic crusts and ecosystem processes. *Crit. Rev. Plant Sci.* **18:** 183–225.
7. Fernández-Valiente, E., A. Ucha, A. Quesada, F. Leganés, and R. Careres. 2000. Contribution of N_2 fixing cyanobacteria to rice production: Availability of nitrogen from ^{15}N-labelled cyanobacteria and ammonium sulphate to rice. *Plant Soil* **211:** 107–112.
8. Fletcher, J. S., G. P. Thomas, and C. Hllman. 1993. Potential environmental risks associated with the new sulfonylurea herbicides. *Environ. Sci. Technol.* **27:** 2250–2252.
9. Hammamda, S., C. Michelle, and P. C. Jean. 1994. Kinetics and hydrolysis mechanism of chlorsulfuron and metsulfuron-methyl. *Pestic. Sci.* **40:** 71–76.
10. Hammouda, O. 1999. Response of the paddy field cyanobacterium *Anabaena doliolum* to carbofluran. *Ecotoxicol. Environ. Safety* **44:** 215–219.
11. James, V. H. 1990. Chemistry of sulfonylurea herbicides. *Pestic. Sci.* **29:** 247–250.
12. Kim, Y. H., J. Lee, and S.-H. Moon. 2003. Uniqueness of microbial cutinase in hydrolysis of *p*-nitrophenyl esters. *J. Microbiol. Biotechnol.* **13:** 57–63.
13. Kuk, Y. I., H. I. Jung, O. D. Kwon, D. J. Lee, N. R. Burgos, and H. O. Guh. 2003. Sulfonylurea herbicide-resistant *Monochoria vaginalis* in Korean rice culture. *Pest. Manag. Sci.* **59:** 949–961.
14. Kumar, A. and H. D. Kumar. 1998. Nitrogen fixation by blue-green algae, pp. 85–103. *In* S. P. Sen (ed.). *Proceedings of the Plant Physiological Research.* Society for Plant Physiology and Biochemistry, 1st International Congress of Plant Physiology, New Delhi, India.
15. Lee, S.-J., J.-Y. Cho, J.-I. Cho, J.-H. Moon, K.D. Park, Y. J. Lee, and K.-H. Park. 2004. Isolation and characterization of antimicrobial substance macrolactin A produced from *Bacillus amyloliquefaciens* CHO 104 isolated from soil. *J. Microbiol. Biotechnol.* **14:** 525–531
16. Orus, M. I. and E. Marco. 1991. Heterocysts structure alteration and oxygen-mediated inhibition of denitrogen fixation by trichlorfon in *Anabaena* 7119. *J. Exp. Botany* **95:** 1595–1600.
17. Parsons, T. R. and J. D. H. Strickland. 1963. Discussion of spectrophotometric determination of marine-plant pigments, with revised equations for ascertaining chlorophyll *a* and carotenoid. *J. Mar. Res.* **21:** 155–163.
18. Prosperi, C., L. Boluda, C. Luna, and E. Fernández-Valiente. 1992. Environmental factors affecting *in vitro* nitrogenase activity of cyanobacteria isolated from rice-fields. *J. Appl. Phycol.* **4:** 197–204.
19. Ray, T. B. 1984. Site of action of chlorsulfuron; inhibition of valine and isoleucine biosynthesis in plants. *Plant Physiol.* **75:** 827–831.
20. Rippika, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stainier. 1979. Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* **11:** 1–61.
21. Sabater, C., A. Cuesta, and R. Carrasco. 2002. Effects of bensulfuron-methyl and cinosulfuron on growth of four freshwater species of phytoplankton. *Chemosphere* **46:** 953–960.
22. Sardeeshpande, J. S. and S. K. Goyal. 1982. Effect of insecticides on the growth and nitrogen fixation by blue-green algae, pp. 588-605. *In*, IARI (ed.). *Proceedings of National Symposium on Biological N_2-Fixation.* IARI, New Delhi, India.
23. Siddaramappa, R., A. Tirol, C. Seiber, E. A. Herinrichs, and I. Watanabe. 1978. The degradation of carbofuran in paddy water and flooded soil of untreated and retreated rice fields. *J. Environ. Sci. Health* **13:** 369–380.