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Effects of Butachlor on the Growth of Purple Non-sulfur Bacteria

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홍색 비유황광합성 세균에 미치는 제초제 Butachlor의 영향

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ABSTRACT: The effects of a herbicide butachlor[2-chloro-2′,6′-diethyl-N-(butoxymethyl) acetanilide] on the growth of the purple non-sulfur bacteria were investigated. The butachlor inhibited the growth of all species tested by 18-51%, except *Rhodospirillum rubrum* at concentrations of 10^{-3} M, which would be field capacity. The photosynthetic growth rate of the species in the presence of butachlor was influenced by the nitrogen source. Cultures supplied with $(NH_4)_2SO_4$ showed a somewhat higher growth rate than those fixing dinitrogen, but they were more susceptible to butachlor (26-51%). On the contrary, butachlor enhanced the growth rate of *Rhodospirillum rubrum* in nitrogen gas conditions. When the culture was performed in medium with butachlor as the carbon source, the cells of fixing dinitrogen showed a higher exhaustion of butachlor than those supplemented with $(NH_4)_2SO_4$, which exhaustion was examined by a decrease of the major absorbance at 213 nm and 260 nm. The exhaustion of butachlor as the carbon source had relation with the growth of the cells. The alkalization of culture supplemented with nitrogen gas was found in the cells treated with butachlor or untreated. The butachlor affected the carotenoid region but bacteriochlorophyll remained unaffected.

KEY WORDS □ Butachlor, Purple non-sulfur bacteria, Growth rate, Residue, Photosynthetic pigment

The purple non-sulfur bacteria are distributed widely in nature where they carry out anoxygenic photosynthesis and nitrogen fixation. There have been a few studies on the effects of photosynthesis-inhibiting herbicides on the purple non-sulfur bacteria (Yamashita and Kamen, 1968; Maudinas *et al.*, 1973; Gilbert *et al.*, 1985). Recently, the butachlor and atrazine herbicides were shown to inhibit the photosynthetic growth of the purple non-sulfur bacteria(Brown *et al.*, 1984; Stein *et al.*, 1984; Sutton *et al.*, 1984; Lee and Lee, 1989; Brown *et al.*, 1990).

Studies of atrazine resistance in photosynthetic bacteria have been confined to the isolates selected from the culture conditions (Sutton *et al.*, 1984). In *Rhodobacter sphaeroides*, atrazine specially binds the L subunit of the bacterial reaction center (Brown *et al.*, 1984; de Vitry and Diner, 1984; Stein *et al.*, 1984; Williams *et al.*, 1984) and the resistance to atrazine was the result of diminished binding of the herbicide to the L subunit of the bacterial reaction center (Erickson *et al.*, 1985; Paddock *et al.*, 1988). Atrazine-resistant strains of *Rhodopseudomonas acidophila*, *Rp. palustris*, and *Rhodocyclus gelatinosus* were isolated and characterized. These isolates exhibited degree of atrazine resistance which ranged from 1.5 to 4 times greater than that of ATCC reference strains tested (Brown *et al.*, 1990). Differences in atrazine resistance for these isolates probably occurs by undefined mechanisms and not necessarily by changes in the binding of the herbicide to the L subunit of the photosynthetic reaction center (Brown *et al.*, 1990).

On the other hand, butachlor, commercial name 'Machete' is generally applied as a preemergence herbicide in rice fields. Recently, the biological effect of the butachlor on the purple non-sulfur bacteria have been studied (Lee and Lee, 1989a, 1989b). *Rhodospirillum rubrum* could grow in the presence of toxic concentrations of the butachlor which would be about 100-200 μg/ml of soil water at field capacity (Lee and Lee, 1989a). In the presence of 10^{-3} M of the butachlor, the growth of *Rhodospirillum rubrum* was conversely higher than untreated butachlor cultures and its typical vesicular intracytoplasmic membrane disappeared

in nitrogen fixing conditions (Lee and Lee, 1989a). At the recommended dose, *Rhodospirillum rubrum* isolated from the rice fields was more resistant than the other nitrogen fixing organisms in nature (Singh *et al.*, 1978; Singh and Vaishampayan, 1978; Singh *et al.*, 1986; Lee and Lee, 1989a). Furthermore, the alkaline pH range slightly reversed the inhibitory action of butachlor(Lee and Lee, 1989a). Biodegradability of the butachlor in the purple non-sulfur bacteria was detected by decrease of the absorbance for the butachlor(Lee and Lee, 1989b). There was evidence that plasmids could be encoded for the degradation of butachlor in the purple non-sulfur bacteria (Lee and Lee, 1989b).

In this paper, we described the effects of the herbicide butachlor on the growth and photosynthetic pigment of the purple non-sulfur bacteria.

MATERIALS AND METHODS

Organisms

Rhodopseudomonas acidophila(ATCC 25092) and *Rhodobacter sphaeroides*(ATCC 17023) were obtained from the American Type Culture Collection and *Rp. viridis* (DSM 133) from the Deutsche Sammulung für Mikroorganismen. *Rhodospirillum rubrum* (KS-301) was isolated and maintained in our laboratory (Lee and Lee, 1982).

Media and Growth conditions

Cultures were grown on a mineral salt medium (Ormerod, 1961) supplemented with sodium pyruvate as a carbon source and either $(NH_4)_2SO_4$ or N_2(100% in gas phase) as a nitrogen supply. For growth on N_2, Ormerod medium was modified by the omission of $(NH_4)_2SO_4$ and yeast extract, were replaced by biotin(8 μg/ml) and a flow of dinitrogen gas (50 ml/min). Anaerobic photosynthetic growth was achieved in 10 ml culture tubes completely filled with media, fitted with screw caps and illuminated with 60W incandescent lamps (ca. 3,000 Lux, 28±2℃). Cells fixing dinitrogen were grown in suction flasks (100 ml) containing 50 ml of culture solution. The flasks fitted with rubber septa were throughly gassed with nitrogen gas via entry and exit hypodermic needles and then incubated at 28±2℃ under the incandescent lamps (ca. 10,000 Lux). The growth was monitored by measuring the increase in the optical density at 660 nm. The specific growth rate was calculated according to the equation of Schlegel (1976). To determine the changes of pH, the sterilized N-free media were adjusted to pH 7.0 by adding either 1N NaOH or 1N HCl. Addition of herbicide did not affect the pH of the media.

Butachlor residues in media

Cultures containing the butachlor were grown photosynthetically in modified Ormerod medium, without carbon source. To determine the exhaustion of the butachlor, the culture media were scanned from 190 to 300 nm by using a Shimazdu 240 spectrophotometer. The absorbances of butachlor were examined to determine the disappearance of the parent compound. After 5 days of the cultivation, butachlor in media was extracted with chloroform by Soxhlet apparatus. Chloroform and water were removed under vaccum and washed out with benzene. It was then dried over anhydrous sodium sulfate, diluted to appropriate concentration and the remaining butachlor was determined by UV-scanning spectrophotometer (Chen and Chen, 1979). Nutrient broth containing a 10^{-3} M of butachlor without inoculation was used as a control.

Absorption spectra

Cells were broken by cavitation at 90W for 2 min. with a Ultrasonic 2000 sonifier (four periods of 30s cavitation, each followed by a 20s pause). It was then centrifugated at 5,000 g for 10 min. and absorption spectra of supernatant with 60% sucrose was determined from 300 to 900 nm by Shimazdu 240 spectrophotometer.

Herbicide, Chemicals and Gas

Butachlor of 98.7% purity was obtained from Hankuk Nongyak Company, Korea. All other chemicals were of the highest purity. Dinitrogen gas (99.9% v/v) was purchased from Dongjin Gas Company, Korea.

RESULTS

Effect of butachlor on the photosynthetic growth

Except for *Rhodospirillum rubrum*, photosynthetic growth rates of the species in the presence of butachlor were similar in cultures supplied with $(NH_4)_2SO_4$ or N_2 as nitrogen source in all conditions (Table 1). The butachlor (10^{-3} M) inhibited the growth by 18-15%. The inhibition was dependent on the nitrogen supply in culture conditions. Cells grown on dinitrogen were inhibited 18-44% and those supplied with $(NH_4)_2SO_4$ by 26-51% under the treatment of butachlor. The highest growth rate was seen in *Rhodospirillum rubrum*, which was about 1.3-2 times higher than the other species in all conditions tested and even the growth was enhanced a little in culture with butachlor at nitrogen fixing conditions. The control growth of *Rhodobacter sphaeroides* was next to that of *Rhodospirillum rubrum*, but the growth rate of *Rhdopseudomonas viridis* was higher than that of *Rhodobacter sphaeroides* when the culture treated with butachlor. *Rhodopseudomonas acidophila* showed the lowest growth in the species tested; growth was 49% and 56% of controls for $(NH_4)_2SO_4$ and N_2 containing cultures, respectively.

Effect of butachlor on pH of the medium

According to the culture conditions, the changes of pH value with the growth were detected as seen in Table 2. Regardless of the treatment of the

Table 1. *Effect of butachlor on the growth rate for the purple non-sulfur bacteria grown photosynthetically with $(NH_4)_2SO_4$ and N_2 as nitrogen source in culture solution.*

Bacterial strains	Growth rate (μ)			
	$(NH_4)_2SO_4$		N_2	
	Control	Butachlor	Control	Butachlor
Rhodospirillum rubrum	0.140±0.009*	0.103±0.005	0.110±0.002	0.114±0.007
Rhodobacter sphaeroides	0.136±0.003	0.077±0.004	0.087±0.004	0.062±0.006
Rhodopseudomonas viridis	0.112±0.010	0.079±0.005	0.082±0.009	0.067±0.010
Rhodopseudomonas acidophila	0.110±0.0015	0.054±0.004	0.085±0.007	0.048±0.004

*Number±S.E. are the mean of 5 replicates

Table 2. *Effect of butachlor on the changes of pH during photosynthetic growth of the purple non-sulfur bacteria with $(NH_4)_2SO_4$ and N_2 as nitrogen source.*

Bacterial strains	Ghanges rate pH			
	$(NH_4)_2SO_4$		N_2	
	Control	Butachlor	Control	Butachlor
Rhodospirillum rubrum	6.8	6.2	8.4	8.2
Rhodobacter sphaeroides	6.6	6.4	8.2	8.2
Rhodopseudomonas viridis	6.6	6.4	8.2	8.2
Rhodopseudomonas acidophila	6.6	6.8	8.2	7.8

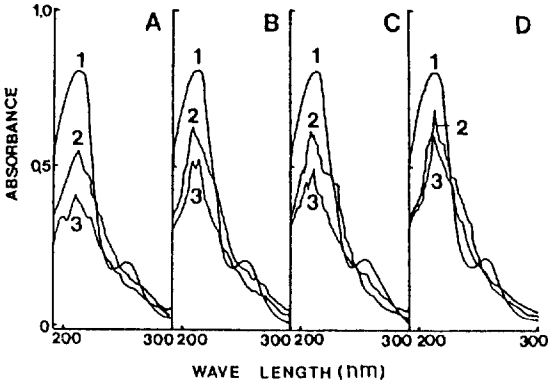

Fig. 1. *UV-scanning spectra of butachlor residues in the medium. The cells were grown in the Ormerod medium supplemented with butachlor as a carbon source and either $(NH_4)SO_4$ or N_2 as a nitrogen sources.*
1. Medium without inoculation; 2. $(NH_4)_2SO_4$ medium; 3. N_2 medium
A. *Rhodospirillum rubrum*; B. *Rhodobacter sphaeroides*; C. *Rhodopseudomonas viridis*; D. *Rhodopseudomonas acidophila*.

butachlor, initial pH of culture was adjusted to 7.0 except *Rhodopseudomonas acidophila* (pH 5.6). The addition of the butachlor had no effect on the pH of the media. The rate of change of pH in cultures supplied with $(NH_4)_2SO_4$ or N_2 as nitrogen source were different(Table 2). In the growth with $(NH_4)_2SO_4$, the pH value of cells changed from 6.2 to 6.8, whereas 7.8 to 8.4 under nitrogen gas after 5 days of growth period. The alkaliza tion of the culture was observed when dinitrogen gas was used as nitrogen source regardless of whether the cells treated with butachlor. For *Rhodopseudomonas acidophila*, the pH value with $(NH_4)_2SO_4$ was slightly higher than others while it showed lower value than others in nitrogen fixing conditions (Table 2).

Butachlor residues in media

Preliminary findings in this study indicated that the butachlor was less toxic at nitrogen fixing conditions(Table 1). Thus, to examine the relation between the growth rate and the exhaustion of butachlor, residues of butachlor in media after 5 days of growth was determined by the scanning of absorption spectra of the medium 190 to 300 nm (Fig. 1.). The culture containing butachlor without inoculation showed a absorption peak at 213 nm and 260 nm (Fig. 2A), which were due to the typical peaks in pure butachlor (Fig. 2B). Thus, the exhaustion of butachlor was characterized by a decrease in absorbance at 213 nm and 260 nm. When the culture was performed in Ormerod medium with butachlor as the carbon source, initial absorbance of butachlor decreased at 260 nm and showed a lower absorbance at 213 nm(Fig. 1,A,B,C,D). The butachlor residues in media were detected a higher absorbance in cells supplied with $(NH_4)_2SO_4$. Thus, butachlor seemed to be utilized in cultures supplied with N_2 as nitrogen source. *Rhodospirillum rubrum* showing the highest growth rate to butachlor was also determined to have the highest exhaustion of butachlor (Fig. 1. A) whereas *Rhodopseudomonas acidophila* showing the lowest

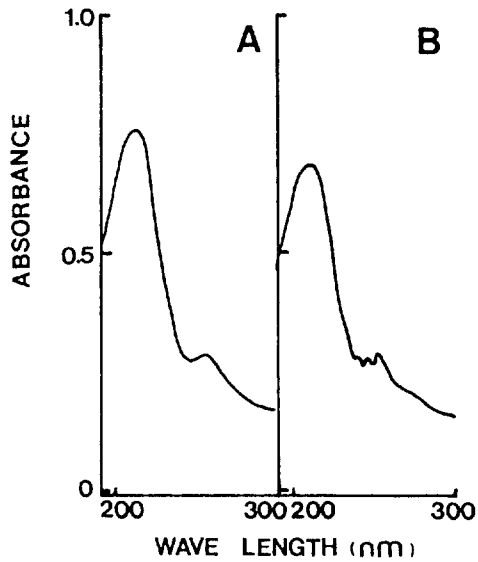

Fig. 2. *UV-scanning spectra of butachlor*
A, The culture medium with butachlor (10^{-3} M), B, Pure butachlor.

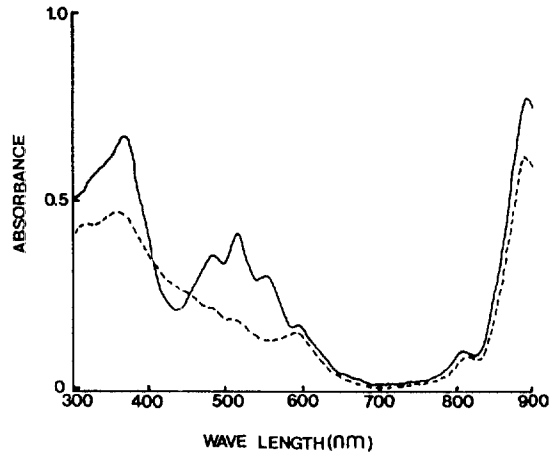

Fig. 3. *Absorption spectra of photopigment in Rhodospirillum rubrum at nitrogen fixing condition.*
The cells were grown in the absence of an inhibitor (——) and in the presence of butachlor 10^{-3} M (------).

growth rate had the lowest exhaustion of butachlor (Fig. 1,D).

Absorption spectra

In case of an addition of butachlor to the culture, *Rhodospirillum rubrum* showed a slightly higher growth than the control in N_2 medium (Table 1) and a higher exhaustion of the butachlor than the other species (Fig. 1, A, B, C, D). To investigate the effect of butachlor on the photosynthetic pigment, it was preferentially detected in absorption spectra of *Rhodospirillum rubrum* (Fig. 3). The spectra showed the major absorbance peaks at the 807 nm and 882 nm region and between 400 and 600 nm. The spectrum of the butachlor treated cells showed a considerable absorption in the 807 nm and 882 nm, which were typical absorption peaks of the bacteriochlorophylls in *Rhodospirillum rubrum*. But, absorption of the carotenoids decreased in the typical peaks of 515 nm and 550 nm to the control. As a result, the butachlor affected considerably the carotenoid region, but did not influence the bacteriochlorophylls.

DISCUSSIONS

The biological effects of the amide herbicide butachlor, currently used extensively in weed control of rice fields, have not been reported previously for any photosynthetic bacteria. More recently, the butachlor was shown to inhibit the photosynthetic growth and intracytoplasmic membrane formation of *Rhodospirillum rubrum* (Lee and Lee, 1989a). Our studies showed that the herbicide butachlor caused 18-51% inhibition of the growth in the purple non-sulfur bacteria (except *Rhodospirillum rubrum*) at concentrations of 10^{-3} M, which would be field capacity. Cultures supplied with $(NH_4)_2SO_4$ showed a somewhat higher growth rate than those with dinitrogen gas, as previously reported (Yoch, 1978; Nordulund and Baltscheffsky, 1983), but they were more susceptible to inhibition with the same concentrations of butachlor (26-51% inhibition) (Table 1). The growth of *Rhodospirillum rubrum* was especially of interest, because it showed a little increase in nitrogen fixing conditions with the butachlor. These results suggested that resistance to the butachlor might exist in nitrogen fixing conditions.

Sutton *et al.*, (1984) reported that the photosynthetic growth of the purple non-sulfur bacteria was strongly inhibited by atrazine and atrazine-resistant strains of *Rhodobacter sphaeroides* selected in the presence of 100 μM atrazine. Because of resistance to atrazine, *Rhodobacter sphaeroides* has been used as a model system to characterize how the atrazine affect photosynthetic electron transport(Brown *et al.*, 1984; Stein *et al.*, 1984). In *Rhodobacter sphaeroides*, the L subunit of the bacterial reaction center binds atrazine(Brown *et al.*, 1984; de Vitry and Diner, 1984; Stein *et al.*, 1984; Brown *et al.*, 1990). Resistance to this class of herbicides involves various amino acid substitutions in the respective binding protein, resulting in a diminished affinity of atrazine for the L subunit (Erickson *et al.*, 1985; Paddock *et al.*, 1988). The mode of action of the herbicide and the nature of resistance in the bacterial system are functionally

identical to the effects of atrazine on photosystem II in higher plants(Brown *et al.*, 1984; Stein *et al.*, 1984). *Rhodopseudomonas acidophila, Rp. palustris* and *Rhodocyclus gelatinosus* from an ecosystem exhibited degrees of atrazine resistance which ranged from 1.5 to 4 times greater than that of ATCC reference strains tested(Brown *et al.*, 1990). Resistance to the herbicide in these species was not the result of diminished binding of the herbicide to the L subunit of the bacterial reaction center, it may occur because of undefinded mechanisms. In the present study, we do not understand the mechanisms of butachlor resistance in the purple non-sulfur bacteria, as mechanism of atrazine resistance described for *Rhodobacter sphaeroides*. Therefore, to determine the nature of resistance to the butachlor, it can be studied the binding mechanism with isolated membranes and the photoaffinity label method(Brown *et al.*, 1984; de Vitry and Diner, 1984).

On the other hand, the toxicity of the herbicide propanil was reduced at the elevated pH value in *Nostoc calcicola* (Pandey *et al.*, 1984). Also, *Rhodospirillum rubrum* has shown a diminution of inhibitory action of butachlor at alkaline pH ranges(Lee and Lee, 1989a). As seen in Table 2, alkalization of culture in nitrogen fixing condition suggests that the purple non-sulfur bacteria may have an increase of resistance to the butachlor. Pandey *et al.*, (1984) noted that the increased herbicide toxicity at low pH values may be due to the increased concentration of H^+ ions, as compared to herbicide molecules available at higher pH levels. Thus, the bioavailability of the herbicide may be mostly governed by the pH.

The utilization of butachlor as one carbon source was detected in *Rhodospirillum rubrum, Rhodopseudomonas acidophila, Rhodobacter sphaeroides* and *Rp. viridis* (Lee and Lee, 1989b). There was also evidence for plasmids that could encode the degradation of butachlor in these species (Lee and Lee, 1989b). In this study, the cells fixing dinitrogen showed a higher exhaustion of butachlor than those supplied with $(NH_4)_2SO_4$, which exhaustion as the carbon source had relation with the growth of the cells (Table 1, Fig. 1). For *Rhodopseudomonas viridis* containing the bacteriochlorophyll b, the butachlor was highly utilized(Lee and Lee, 1989b) and was more resistant than the other species to atrazine(Sutton *et al.*, 1984) and butachlor(Table 1). Further elucidation of the coupling of reaction center protein and herbicide will require the development of a reliable binding assay.

Rhodopseudomonas acidophila contains two quinone moieties, MK-10 and ubiquinone, the presence of ubiquinone might influence the binding of the quinone antagonists such as atrazine (Hiraishi and Hoshino, 1984; Kato *et al.*, 1985). This organism was species seven times more resistant to atrazine(Brown *et al.*, 1990), but it turn out to be the lower resistant species to the butachlor in our data(Table 1, Fig. 1, D).

Since it has been reported that the bacterial reaction center binds the herbicide in the purple non-sulfur bacteria(Brown *et al.*, 1984; Stein *et al.*, 1984; Williams *et al.*, 1984), we preferentially examined the effect of butachlor on the photosynthetic pigment of *Rhodospirillum rubrum*(Fig. 3.). The butachlor affected the carotenoid region but bacteriochlorophyll was unaffected, as has been investigated for *Rhodospirillum rubrum* by 2-hydroxybiphenyl (Maudinas *et al.*, 1973).

Consequently, more information and research are needed on how the herbicide interacts with membranes. Studies of these mechanisms will undoubtedly contribute to our understanding of photosynthesis and the physiology of the purple non-sulfur bacteria.

적 요

논 제초제로 널리 쓰이는 butachlor가 홍색 비유황 광합성세균의 생장에 미치는 영향에 관하여 조사하였다. Butachlor의 실제 사용범위인 10^{-3} M로 첨가하면 *Rhodospirillum rubrum*을 제외한 *Rhodobacter sphaeroides, Rhodopseudomonas viridis, Rhodopseudomonas acidophila*등의 생장이 18-51% 저해되는것으로 나타났다. Butachlor를 첨가하지 않고 $(NH_4)_2SO_4$를 질소원으로 하여 배양한 균의 생장률은 N_2를 질소원으로 할때보다 높았으나, butachlor를 첨가하면 생장이 N_2 배지에서 보다 (18-44%) 더 저해 되었다.(26-51%). 따라서 질소원을 N_2로 하였을때 butachlor에 대한 저항성이 높은 것으로 추측된다. 한편, butachlor를 탄소원으로 배양시킨후에 배지내의 butachlor소모량을 측정하여 생장에 대한 이용도를 조사하였다. 배지내의 butachlor잔여량은 $(NH_4)_2SO_4$를 질소원으로 하여 배양할때 적게 측정되어, 이는 생장에 butachlor가 효과적으로 이용되는것을 의미한다. 특히, 측정세균중 N_2를 질소원으로 할때 *Rhodospirillum rubrum*은 butachlor를 첨가하면 생장이 오히려 증가되었으며, 단일 탄소원으로의 이용도도 다른종에 비하여 높게 나타났다. Butachlor를 첨가하지 않았을때 *Rhodobacter sphaeroides*보다 생장률이 낮은 *Rhodopseudomonas viridis*는 butachlor를 첨가하면 다른 종보다 생장에 대한 저해를 덜 받았다. *Rhodopseudomonas acidophila*는 butachlor에 의해 생장이 크게 저해받았고, 배지에서의 소모량도 낮았다. 또한 butachlor는 광합성색소중 carotenoid에 대하여 큰 저해를보이고 bacteriochlorophyll에는 영향을 거의 주지 않는 것으로 나타났다.

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