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Effects of herbicide butachlor on *Rhodospirillum rubrum* KS-301

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Rhodospirillum rubrum KS-301에 미치는 제초제 butachlor의 영향

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ABSTRACT : The biological effect of the preemergence rice field herbicide, butachlor(commercial name, Machete) on purple nonsulfur photosynthetic bacterium *Rhodospirillum rubrum* KS-301 has been studied under cultural conditions. Bacterial growth showed a tendency to decline according to the degree of the concentration of butachlor until 10^{-3} M and almost stopped at 10^{-2} M. The growth inhibitory action at 10^{-3} M of butachlor was evident (4.2-18.7% inhibition of growth rate) but had little effect in nitrogen fixation. Conversely, there was a little enhancement effect(1%) in pyruvate, dinitrogen gas growing cultures. At concentration of 10^{-3} M, instead of spiral form, rod shapes were observed through phase contrast microscope and instead of vesicular intracytoplasmic membrane, irregular tubular forms were observed through electron microscope. Alkaline pH value slightly reversed the inhibitory action of butachlor.

KEY WORDS □ Butachlor, *Rhodospirillum rubrum*, Growth rate, Intracytoplasmic membrane, pH value

Herbicides are an important and highly sucessful tool for weed control, but extensive use of herbicides in agriculture has given rise to the problem of their deleterious effect on beneficial microorganisms, such as nitrogen fixing bacteria and cyanobacteria.

Photosynthetic bacteria are the one of the most beneficial nitrogen fixing organisms in the rice paddy. It is, therefore, desirable to examine the effect of rice-field herbicides on their survival and growth. There are only a few reports on the tolerance of purple nonsulfur photosynthetic bacteria against these chemicals under defined conditions (Habte and Alexander, 1980 ; Lee and Lee, 1982a ; Lee, 1984).

The herbicide butachlor(2-chloro-2′ ,6′-diethyl-N(butoxymethyl)-acetanilide) is known as a preemergence herbicide in rice-fields, due to its excellent selectivity between the weeds and crops. Chemically it is an α-chloroacetamide herbicide, together with alachlor and propachlor. They exert their biological effects primarily by inhibiting protein synthesis in weed plants and cyanobacteria (Dodge, 1975 ; Hawxy, *et al.*,1977). The fate and behavior of butachlor in the environment have been studied mainly by Y. L. Chen and his co-workers(1978a, b, 1979, 1982). For the study of nitrogen fixing organisms, butachlor has recently been shown to be strongly growth inhibiting and to provoke mutagenic action in various strains of *Nostoc muscorum* at the concentration of 10μg/ml. (Singh and Vaishampayan, 1978 ; Singh, *et al.*, 1979). And at the same concentration it has little inhibitory effect on growth, respiration and nitrogenase activity in *Gloeocapsa* sp. (Singh, *et al.*, 1986). The *Rhizobium* tolerated ten to fifty fold higher concentrations of butachlor(100μg/ml) than the cyanobacteria (2 or 10μg/ml). At the low concentrations of butachlor (0.5-2μg/ml), *Anabaena doliolum* has shown a significant increase of heterocyst (Singh, *et al.*, 1978). The biological effects of herbicide butachlor, currently used extensively in weed control in rice-fields, have not been reported previously for any purple nonsulfur photosynthetic bacteria.

This investigation is an attempt to initiate to determine the effect of the herbicide butachlor on growth and membrane of purple nonsulfur photosyn-

thetic bacterium *Rhodospirillum rubrum* which isolated from Korean paddy soil.

Materials and Methods

Bacterial strain

The strain used in this study was *Rhodospirillum rubrum* KS-301, which was isolated from Korean rice paddy and maintained in our laboratory (Lee and Lee, 1982b).

Culture conditions

The culture was grown photosynthetically (ca. 3,000Lux) in screw-capped bottles on Ormerod medium (Ormerod, 1961) at 28±2℃ under anaerobic conditions. When treated for growth in dinitrogen gas, $(NH_4)_2SO_4$ and yeast extract in Ormerod medium were replaced by biotin (8㎍/ml) and 50ml/min. flow of nitrogen gas. Cells fixing dinitrogen were grown in suction flask (100ml) containing 50ml of culture solution. The flask with rubber septa were throughly gassed with dinitrogen gas via entry and exit hypodermic needles and then incubated in culture room at 28±2℃ in the light (ca. 10,000Lux). Culture were in a medium supplemented with sodium pyruvate, DL-malate and D-ribose as a carbon sources. To study the effect of pH, the sterilized N-free media were adjusted to different pH values by adding either 1 N HCl or 1 N NaOH. Addition of the herbicide did not affect the pH of the media.

Herbicide

The herbicide, butachlor, used in this study was 98.7% of purification, which obtained from Hankuk Nongyak Company, Korea. Herbicide was prepared by appropriate dilution with sterile distilled water which was then added to the autoclaved growth medium.

Cell growth determination

Growth was determined by measuring the optical density at 660nm(Shimazdu spectrophotometer UV-240). The specific growth rate was calculated by the equation of Schlegel(1976).

Protein concentration determination

Protein concentration was measured by the method of Lowry, *et al.* (1951) after cell digestion by 1 N NaOH for 1h at 90℃, using bovine serum albumine as standard.

Cell observation

The cell shapes were observed with the phase contrast microscope (Olympus, BH).

Electron microscopy

Normal and butachlor treated cells were harvested by centrifugation and then cells were fixed with 2.5%(v/v) glutaraldehyde in 0.1M phosphate buffer (pH 7.0), washed and postfixed in 1% (w/v) osmium tetroxide in same buffer, dehydrated in a graded series of ethanol(30%-absolute) and embeded in Epon. Section was made a microtome (Sorvall porter-Blum MT-2) and stained with uranyl acetate. All samples were examined in a JEOL, 120 CX-II electron microscope(80 kv).

Results

Effective concentration of butachlor

In order to determine the effective concentration of butachlor, *Rhodospirillum rubrum* was grown in basal medium(Ormerod, 1961) with pyruvate, malate and ribose as carbon sources and treated with butachlor at the graded concentration of $10^{-6}-10^{-1}$M. The growth of *Rhodospirillum rubrum* was measured concerning protein concentration(mg/ml). And the results are represented in Fig. 1.

The growth of *Rhodospirillum rubrum* showed a tendency to decline according to the concentration of butachlor until 10^{-3}M and it almost stopped at 10^{-2}M. Experiment was done at 10^{-3}M concentration of butachlor in pyruvate and malate as carbon sources except ribose, which was detected the poor growth(Fig. 1).

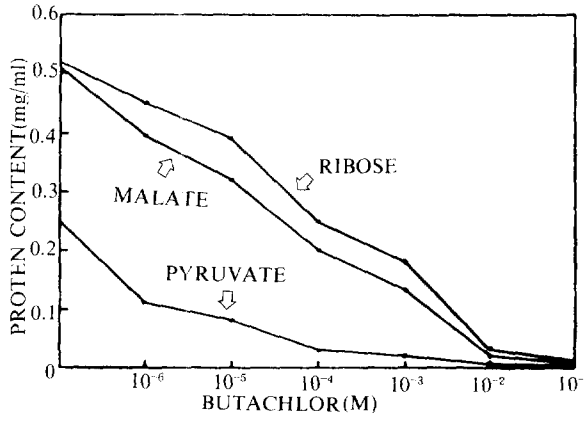

Fig. 1. *The effect of different butachlor concentration on the growth of Rhodospirillum rubrum KS-301. Cells were grown in the basal medium which was the same as described in Materials and Methods with pyruvate, malate and ribose as carbon sources.*

Effect of butachlor on the growth of *Rhodospirillum rubrum*

With cells grown $(NH_4)_2SO_4$ as nitrogen source, the control (untreated with butachlor) grew 1.36−1.4 times better than that treated with butachlor(Table 1).

Cells on a cultured nitrogen fixing conditions, N_2, showed a lower growth rate than that with (NH

Table 1. *Effect of butachlor on the growth rate of Rhodospirillum rubrum KS-301 in the Ormerod medium with $(NH_4)_2SO_4$ and N_2 as nitrogen sources*

Treatment	Growth rate			
	$(NH_4)_2SO_4$		N_2	
	Pyruvate	Malate	Pyruvate	Malate
Control	0.140±0.009*	0.143±0.005	0.110±0.002	0.096±0.006
Butachlor (10^{-3}M)	0.103±0.005	0.102±0.008	0.114±0.007	0.092±0.010

* Numbers±SE are the mean of 5 replicates

$_4)_2SO_4$ as nitrogen source. The cells cultured in pyruvate medium, which treated with butachlor, always showed a better growth than those cultured in malate. The growth inhibitory at 10^{-3}M of butachlor was evident(4.2-18.7% inhibition of growth rate) but had little effect in dinitrogen fixing conditions. Conversely, there was a little enhancement effect (1%) in pyruvate-dinitrogen gas growing cultures.

Effect of butachlor on membranes of *Rhodospirillum rubrum*

To verify the effect of butachlor on bacterial growth, its effects on intracytoplasmic membrane variation in culture grown with pyruvate and dinitrogen gas were investigated(Fig. 2).

Fig. 2 compares representative cells of *Rhodospirillum rubrum* grown in the presence and in absence of butachlor. In the former case, instead of spiral shape, rod shape was observed through the phase contrast microscope(Fig. 2, A and C) and the vesicular intracytoplasmic membrane system of *Rhodospirillum rubrum* disappeared and irregular tubular form showed(Fig. 2, B and D).

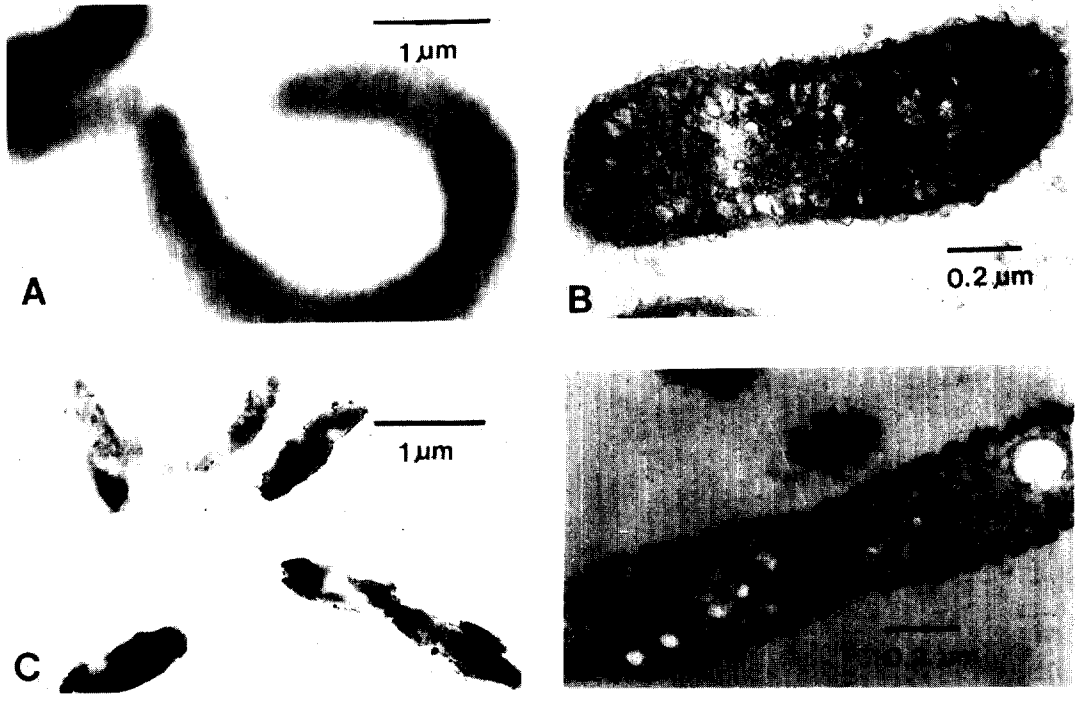

Fig. 2. *Electron micrographs of Rhodospirillum rubrum KS-301 in the outer shapes(A, C) and the inner shapes (B, D). Cells cultivated in the absence(A, B) or in the presence(C, D) of butachlor(10^{-3}M) with pyruvate as carbon source in nitrogen fixing conditions.*

Effect of pH on the growth of *Rhodospirillum rubrum*

The changes of pH value in cultures after 5 days of growth was determined in Table 2. In spite of either the cells treated with butachlor or not, the initial pH(pH 7.0) was decreased in cells cultured on $(NH_4)_2SO_4$ as nitrogen source. But in cells cultured in nitrogen fixing condition, it was increased.

In order to examine the reduction of toxic effect on growth, dinitrogen media were adjusted to different pH values at the different concentrations of herbicide. The results are presented in Fig. 3. Maximum growth of *Rhodospirillum rubrum* was obtained at pH value 7.0 regardless of the dosages of the herbicide. The present observation demonstrated

Table 2. *Effect of butachlor on the changes of pH in the basal medium and in the same medium with N_2 gas as nitrogen soures cultivated for 5 days Rhodospirillum rubrum KS-301.*

Treatment	Changes of pH*			
	$(NH_4)_2SO_4$		N_2	
	Pyruvate	Malate	Pyruvate	Malate
Control	6.8	7.4	8.4	8.2
Butachlor, 10^{-3}M	6.2	6.6	8.2	8.4

* The initial pH value is 7.0

increased pH values up to 9 invariably stimulated bacterial growth and the herbicide was less toxic at these conditions(Fig. 3). Thus, it is concluded that alkaline pH value protects the bacteria from herbicide toxicity.

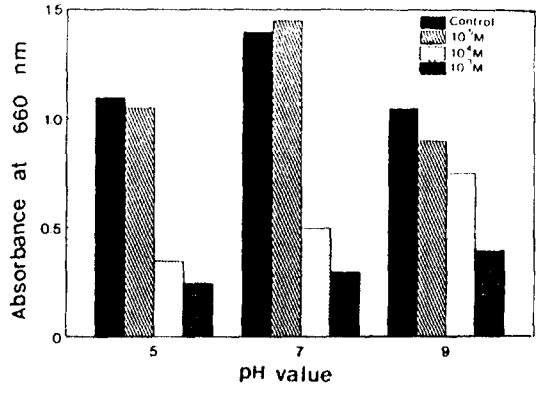

Fig. 3. *Effect of butachlor on the grwoth of Rhodospirillum rubrum KS-301 in nitrogen fixing condition with pyruvate as carbon source at different pH values.*

Discussion

Butachlor is applied at a concentration of 2.5g/L, which would be about 100~200μg/ml of soil water at field capacity. Growth of the cyanobacteria was inhibited at a low concentration of the butachlor (2~10μg/ml), at which the butachlor was apparantly mutagenic to cyanobacteria(Singh, *et al.* 1978 ; Singh and Vaishampayan, 1978). The *Rhizobium* sp. and *Gloeocapsa* sp. tolerated ten to fifty fold higher concentrations of butachlor(100μg/ml) than the cyanobacteria(Singh, *et al.*, 1978 ; Singh, *et al.*, 1986).

In the present study, 10^{-3}M butachlor(ca. 156μg/ml) caused a slow down of bacterial growth and at a concentration of 10^{-2}M butachlor(ca. 1.56mg/ml), the growth was stopped completely. Thus, it is obvious that at the recommended dose, 10^{-3}M, *Rhodospirillum rubrum* isolated from the rice-field could endure and survive better in nature(100~200 μg/ml) than other nitrogen fixing organisms.

Singh, *et al.* (1978) have postulated the sensitivity of an organisms to butachlor associated with the degree of its evolution. As compared with his results, *Rhodospirillum rubrum* was more resistant than rhizobia, which in turn was more tolerant than cyanobacteria. And the cyanobacteria seems to be more resistant than highly evolved crop plants and weeds.

Cells cultured on $(NH_4)_2SO_4$ compared to those grown in nitrogen gas as nitrogen source showed a 1.36~1.4 times higher growth rate, Yoch(1978) also indicated this results. Conversely, *Rhodospirillum rubrum* had a better increasing growth rate by 1%, when treated with butachlor than when untreated in dinitrogen gas-pyruvate medium. It could be said that more ATP effect occured, but it needs more study.

In the electron micrograph with *Rhodospirillum rubrum* treated with butachlor(10^{-3}M) in nitrogen fixation(Fig. 2), the typical vesicular intracytoplasmic membrane system of *Rhodospirillum rubrum* disappeared and showed on irregular tubular form. Also, the typical spiral shapes of *Rhodospirillum rubrum* changed into rod shape. It could be supposed that butachlor would be decomposed and utilized by *Rhodospirillum rubrum*. According to Maudinas, *et al.* (1973), the effects of 2-hydroxybiphenyl upon

intracytoplasmic membrane of *Rhodospirillum rubrum* was investigated. At concentration of 330μM, instead of vesicular intracytoplasmic membranes concentric membraneous layers were found in electronmicrographs of whole cells. This changes of other inhibitors has also been known to occur in microorganisms (Raju and Rangaswani, 1971 ; Brar and Sethi, 1972 ; Singh, *et al.*, 1978).

In the microbial hydroxylation of pesticides, it is understood that the reaction take place only in the presence of oxygen and NADPH(Daly, 1971 ; Beestman and Deming, 1974 ; Chen and Chen, 1979). However, *Rhodospirillum rubrum* was able to degrade in anaerobic conditions in our investigation.

Pandey, *et al.* (1984) reported that cyanobacteria treated with propanil grown in alkali medium(pH 9-13) exhibited less toxicity than those grow in acidic medium. We determined better linear growth according to an increase of pH value in higher concentration of butachlor(Fig. 3), Singh, *et al.* (1978) also indicated this results.

The effectiveness of this herbicide in other purple nonsulfur photosynthetic bacteria warrants further investigation.

적 요

한국에서 분리한 비유황 광합성 세균 *Rhodospirillum rubrum* KS-301의 생장에 대하여, 논 잡초제로 널리 쓰이는 butachlor(제품명 : Machete)의 영향을 본 것이다.

실제 논에서 사용되고 있는 농도인 10^{-3}M에서도 균의 생장은 이루어졌고 10^{-1}M의 농도에서 억제되어 다른 생물체보다 높은 저항력을 가지는 것으로 나타났다. 더욱이 pyruvate를 탄소원으로 하여 질소고정 상태로 배양했을 때 butachlor를 처리한 실험군에서 미약하나마 오히려 생장이 촉진되었다(1%).

전자현미경으로 내부막조직을 관찰한 결과 *Rhodospirillum rubrum*의 전형적인 vesicular type이 tubular type으로 변형된 것으로 보아 제초제의 흡수가 간접 확인되었다.

또한 pH가 알칼리 조건일 때 질소고정상태에서 균생장에 대한 제초제의 저해력이 떨어지는 것으로 나타났다.

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