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Degradation of the Herbicide, Alachlor, by Soil Microorganisms

Part I. Degradation in the flooded paddy soils

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제초제 Alachlor의 토양미생물에 의한 분해

第一報. 담수답토양에서의 분해

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抄　錄

담수답 토양중에서 배양된 Alachlor는 그 분해산물로 1-formyl-2, 3-dihydro-7-ethyl-indole, 2, 6-diethylaniline, 2, 6-diethylacetanilide, 2, 6-diethyl-N-(methoxymethyl) acetanilide, 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide, 그리고 三種의 미확인 화합물을 생성하였다. 環-^{14}C 標識 Alachlor의 사용으로 입증된 바와 같이 토양 현탁액중에서 Alachlor의 수용성 분해산물은 배양기간이 증가함에 따라 증가하였고 *Rhizoctonia solani*의 배양에서도 유사한 결과를 얻었다. *Streptomyces lavendulae* Ru3340-8은 주요 분해산물로 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide를 최고 25%까지 생성하는 반면 *Bacillus brevis* IFO 3331, *Bacillus cruciviae*, 그리고 *Pseudomonas putida*는 그것을 생성하지 않았다.

Introduction

Alachlor, 2-chloro-2′, 6′-diethyl-N-(methoxymethyl) acetanilide, is a preemergence α-chloroacetanilide herbicide used for weed control in corn, soybeans, and many other crops.

Hargrove and Merkle[1] studied the effects of temperature and relative humidity on the degradation and volatilization of Alachlor in soil. They identified 2-chloro-2′, 6′-diethylacetanilide as the degradation product at 0% relative humidity (38°C or 46°C). This product was concluded to result from the acid-catalyzed hydrolysis of Alachlor on mineral surfaces.

In the investigation on the interaction of Alachlor and an insecticide, Carbofuran, Hamill and Penner[2] separated some metabolites from barley and corn plants treated with Alachlor, but did not identify them.

Armstrong et al.[3] reported that Alachlor was metabolized rapidly in yellow nutsedge, a perennial weed, to at least one water-soluble metabolite.

The research of Kaufman and Blake[4] showed that Alachlor was dechlorinated in the pure-culture of *Fusarium oxysporum*, but the aniline moiety was not detected.

According to Beestman and Deming[5], the half-life of Alachlor in soil was quite short and the major route of dissipation from soil turned out to be microbial decomposition.

Yu et al.[6] studied the fate of Alachlor in a model ecosystem designed by Metcalf and his associates[7] by means of the ring-^{14}C-labeled one. They reported that at least eight Alachlor degradation products were found in the water, the intact parent compound constituting 1.8% of the radicactivity in the water at the end of the 33 day experimental period.

Smith and Phillips[8] conducted research to examine the effect of Alachlor on the growth and development of *Rhizoctonia solani* Kuehn and to determine the rate of Alachlor degradation by this fungus in nutrient media containing different concentrations of sucrose.

Tiedje and Hagedorn[9] showed that degradation of Alachlor by a common soil fungus, *Chaetomium globosum* produced four identifiable organic metabolites besides chloride.

In the present investigation, Alachlor was incubated somewhat anaerobically in the flooded paddy soils of two types for 2, 4, and 6 months, respectively, in order to elucidate the formation and structure of the degradation products, since no research has been undertaken under this condition.

In addition, by means of the ring-^{14}C-labeled Alachlor, the partition of the organic-and water-soluble degradation products as a function of incubation periods in soil suspensions and in the pure culture of *Rhizoctonia solani* was followed in an attempt to verify the formation of water-soluble products.

Finally, the Alachlor degradability of some selected microorganisms was examined and the major degradation product was elucidated.

Materials and Methods

Soils used

The same soils as those adopted in the previous experiments[10] were used. Their physico-chemical characteristics include the following. Soil A: Total sand, 43.1%; Silt, 41.4%; Clay, 15.4%; Textural class, Silty loam; pH(1 : 1, H_2O), 4.83; Organic matter, 2.45%; C.E.C., 7.7me/100g. Soil B: Total sand, 30.5%; Silt, 53.1%; Clay, 16.5%; Textural class, Clay loam; pH(1 : 1, H_2O), 6.43; Organic matter, 2.9%; C.E.C., 14.14me/100g.

Incubation of soils treated with Alchlor

100g of soil A and B, respectively, was put in 500m*l* Erlenmeyer flasks and 100m*l* of distilled water added to each soil. Alachlor dissolved in a small amount of acetone was added to each flask to give a final concentration of 500 ppm and the soil mixture shaken evenly. The water layer above soil surface was about 0.5 cm thick and the incubation continued at 28°C for 2, 4, and 6 months respectively. As the controls, 100g of soil A and B, respectively, was autoclaved at 121°C for 30min., the same procedure being repeated two more times, and 100m*l* of sterile distilled water was added to each soil. The incubation was done in the same manner as the above.

Extraction of the soils

In order to get the degradation products from the soils after the given periods of incubation, water was separated from soil by centrifugation. The soils were then extracted with four 100m*l*-portions of methanol and two 100m*l*-portions of acetone by shaking for 30min. at 180r.p.m. on a rotary shaker. The soils shaken with organic solvents were centrifuged at 4,000r.p.m. for five min. in each extraction. The supernatants collected and combined after filtration were concentrated to a small volume for analyses.

Gas-liquid chromatography

The analyses were performed with an instru-

ment of Shimadzu, Gas Chromatograph GC-4C (PTF) equipped with a flame ionization detector. The column was a Pyrex glass of 6mm(OD)× 8ft packed with 5% Silicone GE SE-30 on 60~ 80mesh Shimalite W(AW-DMCS). Operating parameters were as follows: helium carrier flow, 50m*l*/min.; air, 0.8kg/cm²; hydrogen, 0.7 kg/cm²; injection port temp., 200°C; detector temp., 230°C; column temp., 150°C.

Thin-layer chromatography

The precoated analytical plates of silica gel HF-254 with a fluorescent indicator were used for the identification of the degradation products. The developing solvent was a mixture of benzene and methanol (85 : 15, V/V). The locations of the spots were detected under a U.V. lamp with a wavelength of 254nm.

Mass spectrometry.

Mass spectra by GLC-MS and direct inlet probe were obtained with a Finnigan 3200 Gas chromatograph-Mass spectrometer. The electron ionization potential was 70eV.

Measurement of radioactivity

The radioactivity was measured with an Isocap/300 Liquid Scintillation System, Nuclear Chicago. Each sample was put in the scintillation vials containing 10μl of Bray's solution.

Soil suspension experiments with the ring-^{14}C-labeled Alachlor

95m*l* of the glucose-inorganic salt medium[20] and 5g of the two soils, respectively, were put in a 250m*l* erlenmeyer flask and mixed well. The ring-^{14}C-labeled Alachlor stock solution was added to these soil suspensions to give a concentration of 40ppm, the radioactivity per flask being 25,000dpm. The soil suspensions were shake-cultured at 30°C as long as uo to 20 days. At the intervals of 0, 6, 13, and 20 day's incubation, the radioactivities of the aqueous and benzene layers were measured. For the measurement of radioactivities, 3m*l* or supernatants and 3m*l* of benzene were agitated on a sonicator and after standing for a few minutes, 0.5m*l* from aqueous and benzene layers, respectively, was taken out into a vial containing 10m*l* of Bray's solution and measured. For the total radioactivity, 0.5m*l* of the incubation mixture was measured in the same manner.

Degradation of ring-^{14}C-labeled Alachlor by *Rhizoctonia solani*

R. solani was grown in 100m*l* of potato dextrose broth medium[11] for 5 days. To this culture medium' 0.25m*l* or ring-^{14}C- labeld Alachlor stock ethanol solution was added aseptically to give a concentration of 25ppm. The radioactivity was 44,270dpm. The fungus was allowed to grow for 8 more days. Two replicates were placed, in addition to the control which is the meidium containing the same amount of ring-^{14}C-labeled Alachlor, without inoculation. At the end of the incubation, the pellets of fungal mycelia were removed by centrifugation. The supernatant was extracted with two 40m*l*-portions of chloroform to partition the water and organic solvent-soluble degradation products. After adjusting the volumes of the aqueous and chloroform layers to be equal, the radioac tivity of 0.5m*l* of each layer was measured in a vial containing 10m*l* of Bray's solution to compare the partition of the products between the two phases.

Alachlor-degradability of microorganisms

Some selected microorganisms used for the Alachlor-degradability test include 12 strains: An isolate from soil B in with Alachlor had been incubated, *Bacillus brevis* IFO 3331, *Bacillus subtilus* W-23, *Bacillus megaterium*, *Bacillus cruciviae*, *Streptomyces erythreus* like 549, *Streptomyces lavendulae* Ru 3340-8, *Streptomyces coelicolor* 1023, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Pseudomonas denitrificans*, and *Arthrobacter suiflex*. For the culture of the above microorganisms, Bacto Nutrient Broth (Difco Laboratories, Detroit I, Michigan, U.S. A.) was used. 8g of the medium was dissolved in 1,000m*l* of distilled water and the pH was adjusted to 7.0 with 0.1N-NaOH solution 20m*l* of the medium was put in large test tubes and autoclaved at 121°C for 15min. The Alachlor

stock solution dissolved in ethanol was added aseptically to the medium to give a concentration of 40ppm and each microorganism was incubated at 30~32°C for 10 days. As the controls, the medium inoculated with each microorganism without Alaclhor and the medium containing 40ppm of Alachlor without inoculation were incubated at the same time. At the completion of the incubation, the mixture was extracted with two 10m*l*-portions of chloroform and the extracts were dried over anhydrous sodium sulfate, concentrated, and subjected to GLC.

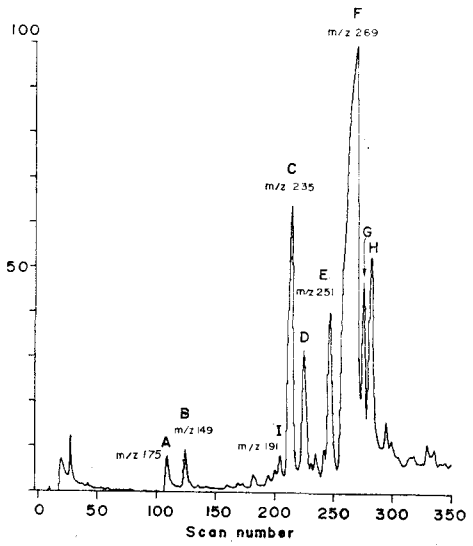

Fig. 1. Gas-liquid chromatogram of the degradation products of Alachlor in the flooded paddy soil B

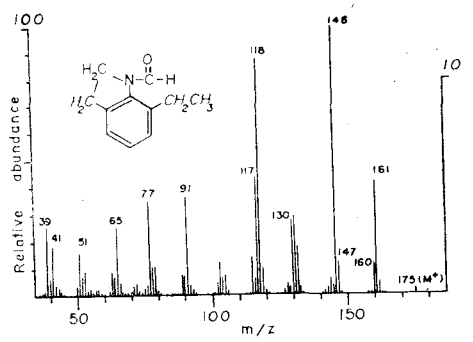

Fig. 2. Mass spectrum of the product A, 1-formyl-2, 3-dihydro-7-ethylindole

Results and Discussion

Identification of the degradation products

When soil A and B which had been incubated in flooded conditions for 2, 4, and 6 months were extracted with organic solvents and the extracts subjected to GLC analyses, almost the same degradation products were obtained from all the incubation periods in the two soils. Fig. 1 shows the GLC chromatogram of the extract fromsoil B which had been incubated for 4 months as an example. For the structural elucidation of the degradation products corresponding to the peaks on this chromatogram, GLC-MS was performed.

Product A. The mass spectrums of the product A is shown in Fig. 2. In this pectrum, the molecular ion occurs at m/z 175 as a weak peak. The fragment peaks at m/z 161 and 160 correspond to M-CH_2 and M-CH_3, respectively. The base peak occurring at m/z 146 is thought to be due to the loss of CHO from the molecular ion. The fragment peak at m/z 147 corresponds to M-CO. The most prominent peak at m/z 118 next to the base peak corresponds to M-CHO, C_2H_4, the fragment peak at m/z 117 being M-CHO, CH_2CH_3. It is believed that the fragmentation of this kind of formamide is different from that of an aldehyde, since the characteristic M-1 peak in an aldehyde is not reco-

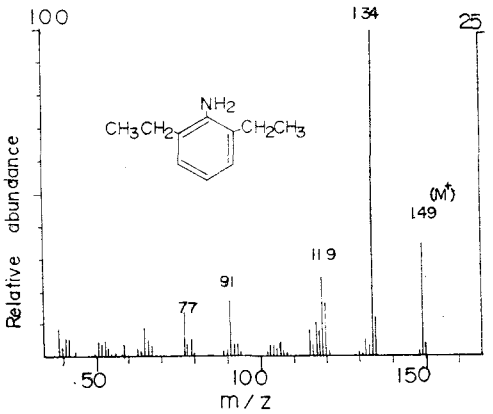

Fig. 3. Mass spectrum of the product B, 2,6-diethylaniline

gnized. The reason for the absence of M-1 peak is not clear, because the molecular ion itself is very weak in intensity.

Product B. As can be seen in Fig 3, the molecular ion occurs at m/z 149. The base peak at m/z 134 corresponds to M-CH_3 and the fragment peak at m/z 119 is M-CH_3, CH_3. The fragment peak at m/z 91 is consistent with M-CH_2CH_3, CH_2CH_3.

Product C. The mass spectrum is shown in Fig. 4. In this spectrum, the molecular ion occurs at m/z 235. The fragment peaks at m/z

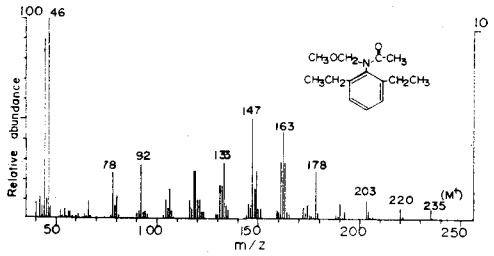

Fig. 4. Mass spectrum of the product C, 2,6-diethyl-N-(methoxymethyl)

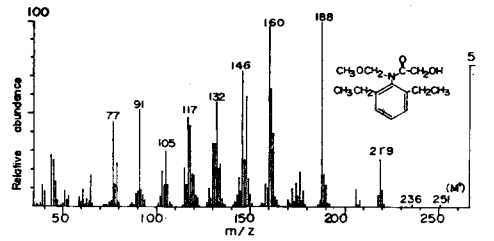

Fig. 5. Mass spectrum of the product E, 2-hydroxy-2′, 6′-diethyl-N-(methoxymethyl) acetanilide

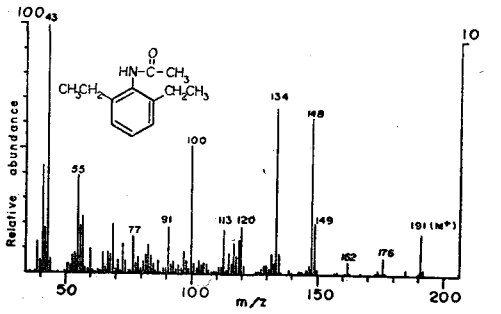

Fig. 6. Mass spectrum of the product I, 2,6-diethylacetanilide

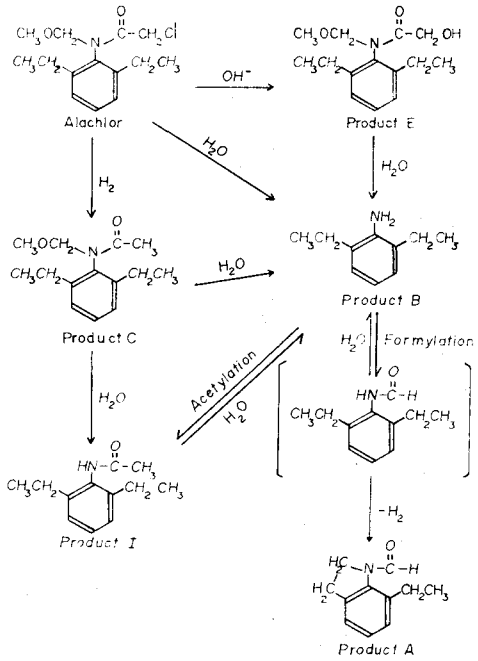

Fig. 7. Possible pathways for the formation of the degradation products of Alachlor in the flooded paddy soils

220, 204, 203, 192, and 190 are consistent with M-CH_3, M-CH_3O, M-CH_3OH, M-$COCH_3$, and MCH_3OCH_2, respectively. The major fragment speaks at m/z 178, 163, 162, and 147 are thought to correspond to M-CH_2=C=O, CH_3 M-$COCH_3$, CH_2CH_3, M-CH_2=C=O, CH_3O, and M-$COCH_3$, CH_2CH_3, CH_3, respectively.

Product E. The mass spectrum is shown in Fig. 5. In this spectrum the molecular ion occurs at m/z 251 as a weak peak. The major fragment peaks at m/z 236, 220, 219, and 206 correspond to M-CH_3, M-OCH_3, M-CH_3OH, and M-CH_3OCH_2, respectively. The base peak at m/z 188 is consistent with either M-CH_3OH, CH_2OH or M-CH_3OCH_2, H_2O. The prominent fragment peaks at m/z 160 and 146 correspond to M-CH_3OCH_2, CH_2OH, CH_3 and M-CH_3OCH_2, CH_2OH, CH_2OH_3 CH_2CH_3, respectively. The other fragment peaks were not followed.

Product I. The mass spectrum is shown in Fig. 6. As can be seen in this spectrum, the molecular ion occurs at m/z 191. The major

fragment peaks at m/z 176, 162, 149, 148, and 134 correspond to M-CH_3, M-CH_2CH_3, M-CH_2 =C=O, M-$COCH_3$, and M-$NCOCH_3$, respectively. This fragmentation pattern is in good agreement with what is expected of an acetanilide. The base peak at m/z 43 is suggestive of the presence of the CH_3CO group in the structure.

Other products. The product F is the intact Alachlor. The other products D, G, and H could not be elucidated at the present time, due to the unclear mass spectra. In order to illustrate the formation of the Alachlor degradation products, Fig. 7. is presented. In these pathways, product C can be readily formed by dechlorination of Alachlor, followed by hydrogenation under the anaerobic soil conditions. The fact that product C is the major degradation product of Alachlor incubated in the flooded paddy soils bears out this proposition. Product E can be readily formed by dechlorination followed by hydroxylation of Alachlor. This transformation was also confirmed in the subsequent pure culture experiments of some selected microorganisms. Product B can be for med from Alachlor, product C, E, I, and 2,6-diethylformamide which is a hypothetical intermediate leading to the formation of product A. Product I can be formed by acetylation of product B and hydrolysis of product C. Product A can be formed from 2,6-diethylaniline via 2,6-diethylformamide which could not be detected in the GLC M-S analyses Tiedje T. and Hagedorn[9] reported a cyclic product, 1-chloroacetyl-2, 3-dihydro-7-ethylindole as one of the degradation products of Alachlor by a soil fungus, *C. globosum*. In their investigation, the cyclization was shown to result from 2-chloro-2, 6/-diethylacetanilide via an intermediate which was not observed. Likewise, in the present investigation, the possibility of dehydrogenation of the ethyl group in an intermediate, 2,6-diethylformamide, prior to cyclization may not be ruled out.

Comparison of water and organic soluble degradation products in soil suspensions

In order to verify the progressive formation of water-soluble products in the incubation of Alachlor in soil suspensions, ring-^{14}C-labeled Alachlor was used and the results are presented in Table 1. In Table 1, in the control where only sterile glucose-inorganic salt medium with out soils was used, more than 98% of the radioactivity was in the benzene phase. Considering the fact that the water solubility of Alachlor at 24°C is only 240ppm[5], it is readily recognized that Alachlor remains intact up to 13 days of incubation in the medium without soil microorgnaisms. At 20 days of incubation, the med ium of the control was contaminated by micro-

Table 1. Distribution of radioactivity between aqueous and organic phases as a function of incubation periods of ring-^{14} C-labeled Alachlor in soil suspensions

Treatment	Phase	Radioactivity (dpm) at each incubation period (days)			
		0	6	13	20
Control	Total	378.98	439.92	425.9	
	Water	4.24(1.12%)	6.67(1.52%)	3.38(0.79%)	
	Benzene	374.74(98.88%)	433.25(98.48%)	422.52(99.21%)	
Soil A	Total	370.08	323.97	327.62	323.26
	Water	3.14(0.85%)	16.06(4.96%)	22.17(6.77%)	52.13(16.13%)
	Benzene	366.94(99.15%)	307.91(95.04%)	305.45(93.23%)	271.13(83.87%)
Soil B	Total	351.05	305.71	286.1	326.67
	Water	1.18(0.34%)	9.22(3.02%)	20.38(7.12%)	46.67(14.29%)
	Benzene	349.87(99.66%)	296.49(96.98%)	265.72(92.88%)	280.00(85.71%)

Table 2. Distribution of the aqueous-and organic-soluble degradation products of ring-^{14}C-labeled Alachlor in the pure culture of *R. solani*

Treatment	Radioactivity(dpm)				
	Initial	Supernatant	Aqueous layer	Chloroform layer	Other losses
Control (without inoculation)	44,270	42,718(96.49%)	793(1.79%)	45,302(102.33%)	1,552(3.51%)
1	44,270	36,595(82.66%)	19,101(43.15%)	19,041(43.91%)	7,675(17.34%)
2	44,270	39,465(89.15%)	30,465(68.74%)	30,429(14.60%)	6,463(10.85%)
Mean	4,805	44,270(85.91%)	38,030(55.95%)	24,765(28.81%)	12,752(14.10%)

Table 3. Comparison of the degradation product, 2-hydroxy-2,'6'-diethyl-N-(methoxy methyl) acetanilide, produced by some selected microorganisms

Microorganisms	Alachlor(%)	Degradation product(%)
An isolate*	95.4	4.6
Bacillus brevis IFO 3331	100	0
Baciilus subtilis W-23	89.8	10.2
Bacillus megaterium	95.5	4.5
Bacillus cruciviae	100	0
Streptomyces erythreus like 549	96.4	5.4
Streptomyces lavendulae Ru 3340-8	75	25
Streptomyces coelicolor 1023	91.4	8.6
Pseudomonas aeruginosa	99.3	0.7
Pseudomonas putida	100	0
Pseudomonas denitrificans	98.4	1.6
Arthrobacter suiflex	86	14

* A bacterium isolated from soil B which had been incubated with Alachlor

organisms, so the radioactivities were not measured. Meanwhile, in soil A and B suspensions, as the incubation proceeds for 20 days, the radioactivities in the aqueous phases increased up to 16.13% and 14.29% in soil A and B, respectively. In the light of the fact that the radioac tivities of the aqueous layers increased with the incubation periods, it is readily understood that Alachlor can be transformed into some types of water-soluble products, among which the glutathione-conjugate can be included, by virtue of soil microorganisms. It is well-known that glutathione occurs in bacteria, plants, and animal tissues as a characteristic component of nearly all living cells.[12] The glutathione conjugate of propachlor (2-chloro-N-isopropylaceta nilide) which is an Alachlor-analogue has been isolated from corn and a nonenzymatic glutathione-propachlor conjugation reaction described by Lamoureux et al.[13] Leavitt and Penner[14] reported that the nonenzymatic reaction in vitro of glutathione with Alachlor and some other herbicides formed glutathione-herbicide conjugates. They also showed that Alachlor conjugated with the thiol-containing compounds, cystein, dithiothreitol, and coenzyme A. Based on the fact that the Alachlor-glutathione conjugation reaction yielded more product with increased pH(over pH 6.0). it was indicated that the in vitro reaction proceeds by way of the GS ion.

Degrada tion of ring-^{14}C-labeled Alachlor by *R. solani*.

The results of the incubation of ring-^{14}C-labeled Alachlor in pure culture of *R. solani* are presented in Table 2. As seen in Table 2, when ring-^{14}C-labeled Alachlor was incubated in the potato dextrose broth medium without inoculation, almost no water-soluble degradation products were formed. However, when the medium was incubated with *R. solani* in the presence of Alachlor, an average of 55.95% of the initial radioactivity was found in the aqueous layer, whereas only 28.81% found in the chloroform layer. Accordingly, it is well understood that in the study of degradation products, the organic-soluble products which are subjected to GLC analysis account for only part of the total products. For the water-soluble products, some other techniques including HPLC would be needed.

Degradation of Alachlor by microorganisms in pure culture

By the Alachlor-degradability test of some selected microorganisms, it was found that Alachlor was largely degraded into 2-hydroxy-2, '6'-diethyl-N-(methoxymethyl) acetanilide. The chemical structure of this degradation product was elucidated by GLC-MS and the comparison of the retention time (4.4 min.) on GLC and the Rf value (0.56) on TLC with those of the authentic compound which was synthesized. The relative amonuts of the product formed by each microorganism are presented in Table 3. As can be seen in Table 3, *Bacillus brevis* IFO 3331, *Bacillus cruciviae*, and *Pseudomonas putida* did not at all produce 2-hydroxy-2, '6'-diethyl-N-(methoxymethyl) acetanilide as the degradation product, whereas *Streptomyces lavendual* Ru 3340-8 produced the product at the maximum of 25%. Next to this microorganism, *Arthrobacter suliflex* and *Bacillus subtilis* W-23 produced 14% and 10.2%, respectively. Previously, Kaufman and Blake [4] reported that the degradation of Alachlor by *F. oxysporum* in 20-day incubation period resulated in 22% of chloride ion and 0% of aniline moiety. Even though they did not clarify the formation of the hydroxylation product, 2-hydroxy-2, '6'-diethyl-N-(methoxymethyl) acetanilide following the hydroxylation can be easily supposed by the microbial degradation of propachlor[4,15] which is structurally analogous to Alachlor. Based on the present investigation, it is obvious that many microorganisms other than *F. oxysporum* could bring about dichlorination followed by hydroxylation.

Abstract

Alachlor, 2-chloro-2, '6'-diethyl-N-(methoxymethyl) acetanilide, which had been incubated in the flooded paddy soils yielded 1-formyl-2,3-dihydro-7-ethylindole, 2,6-diethylaniline, 2,6-diethylacetanilide, 2,6-diethyl-N-(methoxymethyl) acetanilide, 2-hydroxy-2, '6'-diethyl-N-(methoxymethyl) acetanilide, and three unidentifiable compounds as its degradation products. The water-soluble products of Alachlor in soil suspensions increased with incubation periods and similar results were obtained from the incubation of *Rhizoctonia solani*, as verified by use of the ring -^{14}C- labeled Alachlor. *Streptomyces lavendulae* Ru 3340-8 produced 2-hydroxy-2, '6'-diethyl-N-(methoxymethyl) acetanilide as the major degradation product as much as 25%, whereas *Bacillus brevis* IFO 3331, *Bacillus cruciviae*, and *Pseudomonas putida* did not produce it.

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