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Degradation of Oxyfluorfen by *Azotobacter chroococcum* (Beijerink)

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Oxyfluorfen [2-Chloro-1- (3-ethoxy- 4-nitrophenoxy)- 4-(trifluoromethyl) benzenel, a member of nitrodiphenyl ether class of herbicides, showed a high degree of weed control and a great vercatility of application (Yin and Swithenbank, 1975). Nitrodiphenyl ethers have been established as commercial herbicides since the introduction of nitrofen and fluorodifen in the mid 1960's.

Nitrodipheyl ether herbicides require light to disrupt membrane permeability, elicit biochemical changes and kill plant cell (Matsunaka, 1969; Orr and Hess, 1982), possibly involving light induced reduction (Kunert and Boger, 1981) for herbicidal activity. Nitrodiphenyl ether herbicides are also activated as bacterial mutagens on chemical and photochemical reduction to the nitroso aryl and hydroxyl amino derivatives (Draper and Casida 1983 a, b). 4-Nitro diphenyl ethers and 4- aminodiphenyl ethers with no 3-substitution are mutagens but only on metabolic activation. Where as with 3- substituted 4-nitro dipheylether herbicide like oxyfluorfen and their amino derivatives are not mutagens. However, nitroso-oxyfluorfen is direct acting mutagens. Rat reduced orally administrated oxyfluorfen, presumaly via nitroso and hydroxy amino intermediate to their amino compounds (Draper and Casida, 1983b).

A review of degradation of several diphenyl ether herbicides in soil has also been published (Kuwatsuka 1972). Adler *et al.* (1977) studied the metabolism of oxyfluorfen in rats. A few systematic studied have so far been carried out on metabolism and degradation of nitro diphenyl ether herbicides (Gutenman and Lisk, 1967; Nakagawa and Crosby, 1974a, b; Ruzo *et al.*, 1980 Pusino and Gessa 1991). The present study has focused on the microbial degradation of oxyfluorfen by a soil borne bacteria, *Azotobacter chroococcum* (Beijerink) with an objective to identify the degradative product thus formed. The information provided by the present study could be of aid in the understanding of the environmental behaviour of the herbicide.

MATERIALS AND METHODS

Technical grade oxyfluorfen was provided by M/s. Indofil Chemicals Ltd., India, which was further purified by repreated crystallization from chloroform followed

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by crystallization from ethanol (99.2%, m.p. 84-85^{0}C).

Pure culture of *Azotobacter chroococcum* (Beijerink) was supplied by the Department of Plant Pathology, Bidan Chandra Krishi Viswavidyalaya. The bacterial culture was maintained by aseptic transfer in a sterilized mineral salt solution consisted of 0.5g of K_2HP_4 ; 0.2g of $MgSO_4$.$7H_2O$; 0.2g of PO_4 NaCl; 0.005g of $NaMoO_4$, 0.005g of $MnSO_4$. $4H_2O$; 0.005g of $FeCl_3$ and 10.00g of manntol per 1L of distilled water. During the study of microbial (bacterial) degradability of oxyfluorfen in sterilized nutrient salt (reaction) solution, manintol was substituted by pure oxyfluorfen (99.2% pure) where the final concentration of oxyfluorfen in reaction solution was 250 ppm. The sterilized nutrient solution containing oxyfluorfen was taken in ten 500 mL Erlynmayer flask each containing 200 ml solution. Each flask was then inoculated by the cells *chroococcum* and incubated at 28±1^{0}C for 7 days. Before inoculation the bacterial cells was washed six times with 0.1M phosphate buffer (pH 7.2) to make cells free from residual carbon sources. Two sets of control flasks containing – (I) bacterial cells and nutrient salt solution with oxyflurofen and (ii) nutrient solution and oxyflurofen without bactrial cells, were also incubated at 28±1^{0}C for 7 days.

For rate study, samples (20 mL each) were drawn periodically (every 24 hrs.) from reaction mixture as well as controls under aseptic condition, extracted with ether and analyzed by GC. After 7 days of incubation the reaction (nutrient) mixture of all the ten flask were combined, filtered through celite pad and aqueous filtrate was collected. The celite pad-containing residue was stirred for 5 hr. in 1 L ether which was further filtered through whatman No. 42 filter paper. The ether extract was then concentrated to 10 mL with a rotary vacuum evaporator at 45^{0}C and mixed with aqueous filtrate. The combined aqueous filtrate was then extracted with ether according to Nakagawa and Crosby (1974) and obtained three ether fractions – Neutral, Basic and Acid. The degradative products were then isolated and identified from each fraction by spectroscopic method.

Routine samples were analyzed on a Hewlett-Packard 5890 GC equipped with FID coupled with HP3392A integrator and 1.8m x 2 mm i.d. glass column packed with 5% DC – 200 on chromosorb WAW HP (100 – 120 mesh) was used. The injector and detector temperature were 210 and 250^{0}C, respectively and the oven temperature programmed from 150 – 230^{0}C at the rate of 8^{0}C / min with initial temperature at 150^{0}C for 2 min. Nitrogen 40mL / min was used as carrier gas.

GC-MS used for structural assignment of the isolated products was performed on Hewlett Packard 39928 instrument equipped with HP capillary column SE-52 coated with 5% phenyl methyl silicon (25m x 0.33 mm i.d.). The temperature was programmed from 80^{0}C to 250^{0}C at 4^{0}C / min. Injector temperature 225^{0}C, carrier gas He at the rate of 2 mL / min. An ionisation potential of 70 eV was used for EI-MS spectra.

^{1}H NMR spectra of the products in $CDCl_3$ were recorded by using Bruker WM-400 instrument equipped with a Fourier transform system.

RESULTS AND DISCUSSION

The routine GC analysis of the reaction solution showed that *A. chroococcum* degraded more than 60% of the added oxyfluorfen in 7 days (Table 1). After 7 days of incubation the oxyfluorfen concentration gradually reduced from 240 ppm (0 days) to 96.22 ppm. The bacteria utilized oxyfluorfen as a sole carbon source.

A. chroococcum formed two major oxyfluorfen metabolites (**I & II**) during microbial decomposition. Metabolite **II** [4-[2-chloro-4-(trifluoromethyl) phenoxy] – 2-ethoxy benzene amine] whose concentration initially increased and maximum concentration was observed on the 4th day (42.33 ppm) which subsequently decreased upto 25.49 ppm on incubation, as it presumably degraded to other metabolites. The metabolite **I** [N-[4-{-2-chloro-4-(trifluoromethyl)-phenoxy}-2-ethoxyphenyl] acetamide], maximum concentration attained on 5th day of incubation (19.32) which further reduced to 11.95 ppm on 7th day (Table 1).

Table 1. Recovery of oxyfluorfen and its major metabolites (**I & II**) during the degradation of oxyfluorfen by *A. chroococcum*

Incubation (days)	Recovery (ppm)		
	Oxyfluorfen	Metabolite I	Metabolite II
0	241.51	0	0
1	211.73	1.11	2.35
2	182.12	1.89	4.98
3	169.36	6.93	14.76
4	144.82	14.73	42.33
5	122.87	19.32	30.57
6	108.65	13.53	27.98
7	96.22	11.95	25.49

Total seven degradative products (metabolites) of oxyfluorfen have been isolated from the different fraction (**neutral, basic** and **acid**). The GC-MS retention time (R1) and the mass fragmentation pattern of the corresponding metabolites are presented in Table 2.

1H NMR data of major two metabolites (**I** and **II**) are as follows :

Metabolite **I** : □ : 1.45[t, 3H, -CH_3], 2.19[s, 3H, -$COCH_3$], 4.06[q,2H, OCH_2], 6.56[d(J=2.4Hz), 1H, Ar-H], 6.60[m, 1H, Ar-H], 6.89[d(J=8.66) 1H, Ar-H]; 7.39[dd(J=2.2, 8.5Hz), 1H, Ar-H]; 7.63[s, 1H, NH (D_2Oexchanged)]; 7.69[d(J=2.4Hz), 1H, Ar-H]; 8.34[d(J=8.5Hz), 1H, Ar-H].

Table 2. GC-MS retention time (R_1), molecular ion and mass fragmentation pattern of the identified degradative products of oxyfluorfen

Product Identified	GC-MS Rt (min.)	Mass Spectrum (m/z)	Molecular Formula
		NEUTRAL FRACTION	
I	28.3	373[M^+, 4.5%; 331[(M^+-$COCH_3$ + H) 6.0%]; 302[331-C_2H_5) 6.6%]; 274[(302-CO)3.8%];269[10.9%]; 229[40%];138[21.3%)]; 114[10.0%]	$C_{17}H_{15}NO_3ClF_3$
Oxyfluorfen	37.6	361 [M^+, 19.7%]; 342 [(M^+-F), 4.3%]; 300 [(M^+-CH_3-NO_2), 23.0%]; 252 [(M^+-NO_2 – C_2H_5Cl+H), 100%]; 223 (16.5%); 195 [$C_7H_3Cl\,F_3O)^+$, 10.1%]; 183 [$(C_8H_9O_4M)^+$, 6.0%]; 17.9[10.6%]; 69 [$(CF_3)^+$, 45.3%)	$C_{15}H_{11}NO_4ClF_3$
		BASIC FRACTION	
II	21.9	331[M^+, 100%]; 302[(M^+-C_2H_5), 73.2%]; 274 [(302-CO), 71.6%]; 195 [($C_7H_4ClOF_3^+$), 1.9%]; 124 [($C_7H_{10}NO^+$) 60.1%]	$C_{15}H_{13}NO_2ClF_3$
		ACID FRACTION	
III	26.4	183[M^+, 27.7%]; 155[(M^+-C_2H_5+H), 10.9%]; 153[(M^+-NO), 7.6%]; 127 [(155-CO), 11.8%]; 99[127-CO)21.0%]	$C_8H_9NO_3$
IV	27.4	195 [M^+, 13.7%]; 167[(M^+-CO), 43.8%]; 166 [(M^+-C_2H_5), 11.2%]; 152 [(M^+-$COCH_3$), 100%]; 137[33.8%]; 124 [(152-HCNH), 38.7%]; 96 [(152-C_2H_5-CO+H), 17.3%]	$C_{10}H_{13}NO_4$
V	28.7	155[M^+, 14.3%]; 128 [(M^+-CO+H), 11.7%]; 109[M^+-NO_2), 18.2%]; 100 [(128-CO), 9.1%]	$C_6H_5NO_4$
VI	31.6	311[M^+; 3.3%]; 240 [(M^+-CO-$COCH_3$), 11.0%), 210 [(M^+-CHO-$COCH_3$-HCN), 13.3%]; 161 [(M^+-$C_8H_8NO_2$), 100%]; 150 [$(C_8H_8NO_2)^+$, 5.6%]; 69[$(CF_3)^+$, 11.3%)	$C_{15}H_{12}NO_3F_3$
VII	32.2	196(M^+, 100%); 177(M^+-F), 37.1%]; 161 [M^+-Cl), 18.8%]; 148[177-CO+H), 12.2%]; 132(161-CO+H), 21.8%]; 113[(132-F), 12.7%]; 69[$(CF_3)^+$, 4.8%]	$C_7H_4OClF_3$

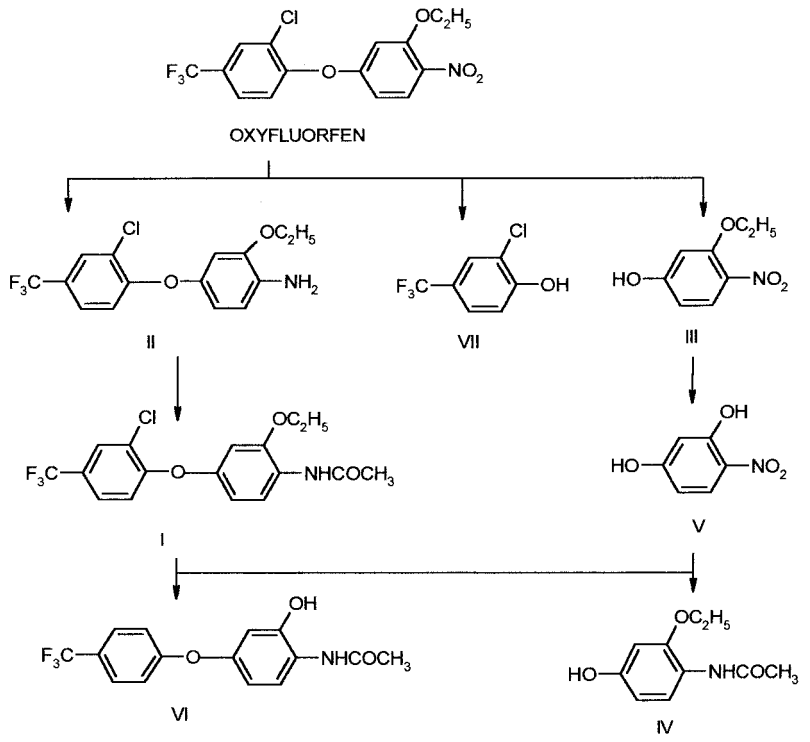

Figure1. Plausible metabolic pathways of oxyfluorfen by Azatobacter chroococcum (Beijerink)

Metabolite **II** : □ : 1.39[t,3H, -CH_3], 4.06[q.2H, OCH_2], 4.47[br s, 2H, NH_2(D_2Oexchanged)]; 6.52[dd(J=2.4, 9.0Hz), 1H, Ar-H], 6.69[d,(J=2.5Hz), 1H, Ar-H], 6.77[d(J=9Hz) 1H, Ar-H]; 6.95[d(J=9Hz), 1H, Ar-H]; 7.61 [dd(J=2.5, 9.0Hz), 1H, Ar-H]; 7.81[d(J=2.3Hz), 1

On the basis of metabolites of oxyfluorfen so far isolated and characterized in the study, a plausible metabolic pathway of oxyfluorfen has been proposed in Fig. 1.

From Fig. 1. It is clear that the main degradative pathways of oxyfluorfen by *A. chroococcum* were hydrolysis of ether linkage into two corresponding phenols, reduction of nitro group to amino compound, further acetylation of amino derivative, O-dealkylation and dechlorination.

Hydrolysis of ether linkage between the two aromatic moieties yielded metabolites III and VII, whereas further hydrolysis of II provided metabolite V through O-dealkylation.

Again oxyfluorfen upon nitro group reduction resulted a major metabolite II, which subsequently acetylated and provided acetyl amino derivative (I), one of the major metabolite of oxyfluorfen. Furthermore metabolite I, on dechlorination and O-dealkylation resulted VI. Metabolite IV formed during hydrolysis of ether linkage of metabolite I.

Niki and Kuwatsuka (1976a) reported that in soil, appreciable portions of several diphenyl ether herbicides were converted into their amino derivatives. Again during the study of degradation of chloromethoxynil (X-52) in soil, Niki and Kuwatsuka (1976b) also detected the acyl amino derivative, hydrolysis of ether linkage to phenol as well as dechlorination product of chloromethoxynil in flooded soil.

Oxyfluorfen metabolism in rat Adler *et al.* (1977) also identified amino as well as acylamino derivatives of oxyfluorfen.

Therefore, from the preceding discussion it may be generalized that in soil, most of the diphenl ether herbicides including oxyfluorfen might be converted into their amino as well as acylamino derivatives through microbial activity.

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REFERENCES

Adler IL, Jones BM, Wargo Jr JP (1977) Fate of 2-chloro-1- (3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene (oxyfluorfen) in rats. J Agric Food Chem 25: 1339-1341.

Draper WM, Casida JE (1983a) Diphenyl ether herbicides:mutagenic metabolites and photoproducts of nitrofen. J Agric Food Chem 31: 227-231.

Draper WM, Casida JE (1983b) Diphenyl ether herbicides and related compounds :structure-activity relationships as bacterial mutagens. J Agric Food Chem 31: 1201-1207.

Gutenmann WH, Lisk DJ (1967) Metabolism of TOKE herbicide in the dairy cow. J Dairy Sci 50: 1516-1518.

Kunert KJ, Boger P (1981) The bleaching effect of the diphenyl ether oxyfluorfen. Weed Sci 29: 169-173.

Kuwatsuka S (1972) Degradation of several hebicides in soil under different conditions. In ; Matsumura E, Boush GM and Misato J. (ed) Environmental Toxicology of Pesticides. Academic Press Inc, New York p. 385-400.

Matsunaka S (1976) Acceptor of light energy in photoactivation of diphenylether herbicide. J Agric Food Chem 17: 171-175.
Nakagawa M, Crosby DG (1974a) Photodecomposition of nitrofen. J Agric Food Chem 22: 849-53.
Nakagawa M, Crosby DG (1974b) Photonucleophilic reaction of nitrofen. J Agric Food Chem 22: 930-933.
Niki Y, Kuwatsuka S (1976a) Degradation products of chlomethoxynil (X-52) in soil. Soil Sci Plant Nutr 22: 233-245.
Niki Y, Kuwatsuka S (1976b) Degradation of diphenyl ether herbicides in *Soil.* Soil Sci Plant Nutr 22: 223-232.
Orr GL, Hess FD (1982) Mechanism of the action of the diphenyl ether herbicide aciflurfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons. Plant Physiol 69: 502-507.
Pusino A, Gessa C (1991) Photolysis of acifluorfen in aqueous solution.Pestic Sci 31: 1-5.
Ruzo Lo, Lee JK, Zabik MJ (1985) Solution phase photodecomposition of several substituted diphenyl ether herbicides. J Agric Food Chem 28 : 1289-1292.
Yih RY, Swithenbank C (1975) New potent diphenyl ether herbicides J Agric Food Chem 23: 592-593.