MICROBIAL POPULATION IN SOIL IN RELATION TO DEFLUORINATION OF ORGANIC FLUORINE COMPOUNDS

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INTRODUCTION

Monofluoroacetate (FA) and its amide (FAA) have been extensively used for the erradiation of insects and rodents in fields. In the light of recent trends in pesticide research, the application of these chemicals to crops may give rise to a problem by virtue of their molecular recalcitrance. The finding of seasonal change of FA in gifblaar, *Dichapetalum cymosum*, however, ignited searches for the presence of an enzyme which catalyzes the splitting of C-F bond of this toxic compound. The enzyme has indeed been found in bacteria belonging to *Pseudomonas* (HORIUCHI, 1961, 1962; TONOMURA et al., 1965).

The defluorinating bacteria can be isolated from soils, especially from soils heavily infested with FA, suggesting that these bacteria would possibly be selected in the infested soils. Since FAA has been sprayed in citrus orchards for the past several years, it is worth-while to see if there is any change in microbial flora in these orchard soils. Furthermore the detection of microorganisms capable of splitting the C-F bond is an important task from the aspect of residue degradation (ALEXANDER, 1965).

The present communication describes the microbial population in orchard soils sprayed with FAA and also the dynamic change of bacterial population in soils added with relatively large amount of the pesticide ingredients.

MATERIAL AND METHOD

Pesticide ingredients: The ingredients used in this experiment were monofluoroacetate (FA), monofluoroacetamide (FAA), and p-bromofluoroacetanilide (BFAA). All these were supplied by the Daikin Industrial Company, Ltd., Osaka, Japan.

Soil sampling: Soils for the analysis of microbial population were collected from citrus orchards in five prefectures in the western part of Japan, i.e., Kanagawa, Shizuoka, Kyoto, Osaka, and Tottori. A reference soil was obtained from the factory where these organic fluorine compounds have been produced. For quantifying the changes in bacterial population, 1g of either FA, FAA or BFAA

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was dissolved in 50ml of water and sprayed on soil in a pot, 15cm in diameter, and soils at different depths were harvested from each pot respectively on 2, 5, 10, 15, and 30 days after spraying and subjected to the population analysis.

Isolation media: Martin's and Jensen's media were employed as basal media for the isolation of fungi and actinomycetes respectively (ALLEN, 1959). The basal medium for the isolation of bacteria contained the following components: K_2HPO_4 0.5g, KCl 0.25g, $NaNO_3$ 1.0g, $MgSO_4 \cdot 7H_2O$ 0.25g, $FeSO_4 \cdot 7\ H_2O$ 0.02g, meat extract 2.0g in 1l of deionized water (Cme medium). For the isolation of ingredient resistant bacteria 5g of FA or FAA, or 2.5g of BFAA was added to 1l of these basal media. As BFAA is scarcely soluble in water, it is crystalized in the medium after cooling. For enumerating microbes capable of utilizing FA, these basal media were respectively deprived of carbon sources and supplemented with 7.8g of FA (CF medium).

Isolation: For direct isolation, soil samples were suspended in 10ml of sterile deionized water and vigorously shaken for 15 min and the suspensions were centrifuged at 2,000 rpm for 10min. The supernatants were then subjected to serial dilution and plating. For indirect isolation, the supernatant was diluted to 10^{-2} and 0.1ml was plated on Cme medium. The plates were incubated at 25°C for 24-48 hrs and colonies appeared were replica-plated on CF medium. Only the colonies appeared on the latter plate were taken as test bacteria.

Defluorination in soil: To 10g of sample soil was added 20ml of conc. H_2SO_4 and 10ml of water in a constant temperature steam-distillation apparatus reported by HUCKABAY et al. (1947). Distillation was continued until the distillate filled to a 50ml volumetric flask. Fluorine in the distillate was titrated by a colorimetric determination either with Amadac-F (Burdick & Jackson Laboratory, Muskegon, Michigan) (BELCHER et al, 1959), or with Zirconium-Eriochrome Cyanine R lake (MEGREGIAN, 1954).

RESULT

1. *Microbial Population Resistant to FA and FAA.*

Microbial populations in soils which had been infested or not infested with organic fluorine compounds were compared with each other with different isolation media, and the result is shown in Table 1. There seems to be no major difference between these soils in terms of the number of ingredient resistant microorganisms, although it has been expected that the factory soil would give more bacterial colonies capable of growing on Cme medium containing FAA because a long term selection pressure must have been operating in the factory soil. It should be mentioned here, however, that the factory soil contained more bacteria growing better on the ingredient medium than those of non-infested fields.

Table 1. Fluoroacetate resistant microbes in soils from field and factory

Soil	Isolation medium	Microbial population / g dry soil (x10³)*			
		Bacteria	Fungi	Actinomycetes	Total
Field soil	Basal medium	14,300	310	1,030	15,640
	Basal + FA	215.0	3.2	8.6	226.8
	Basal + FAA	151.4	1.8	5.0	158.2
Factory soil	Basal medium	17,100	223	610	17,933
	Basal + FA	251.2	2.4	7.4	261,0
	Basal + FAA	170.0	1.6	3.8	175.4

* Average of 3 replicates, 5 plates per replicate.

2. *Absence of Specific Selection in the Soils from Citrus Orchards*

Experiments were carried out to know whether or not a selection had occurred in soils of citrus orchards repeatedly sprayed with FAA. The FAA resistant populations in these soils are summarized in Table 2. It is evident that no detectable selection has so far occurred in these soils in spite of repeated sprays of the pesticide. It should be mentioned that the selection was absent irrespective of the physical properties of soils. Therefore the selection of microorganisms resistant to these toxic compounds seems to require an accumulation of tremendous amount of these compounds in soil, which may not be encountered in the ordinary orchards.

Table 2. Ingredient resistant microbial population in some soils of citrus orchard repeatedly sprayed with FAA.

Soil harvested from	Spray of FAA	Microbial population / g dry soil (x10³)											
		5 cm				10 cm				20 cm			
		B	F	A	T	B	F	A	T	B	F	A	T
Shizuoka	−	184.7	3.2	6.7	194.6	91.4	3.1	7.0	101.5	60.5	7.4	6.7	74.6
	+	171.2	1.8	6.2	179.2	97.4	2.6	7.2	107.2	76.1	1.6	5.5	83.2
Osaka	−	86.1	3.6	5.4	95.1	79.6	2.7	5.0	87.3	45.2	3.2	5.1	53.5
	+	89.5	3.1	6.5	99.1	86.4	3.5	4.1	94.0	40.6	2.5	6.3	49.4
Kanagawa	−	105.7	2.1	6.5	114.3	107.2	3.0	8.2	118.4	53.2	2.7	6.0	61.9
	+	123.1	3.4	7.8	134.3	99.5	2.2	5.8	107.5	61.5	2.0	4.9	68.4
Tottori	−	90.1	3.7	7.3	101.1	80.7	3.7	7.2	91.6	35.8	5.4	7.9	49.1
	+	82.3	4.1	8.2	94.6	85.1	4.4	7.0	96.5	26.4	6.2	9.3	41.9

Average of 3–5 soil samples.
B: Bacteria, F: Fungi, A: Actinomycetes, T: Total population
Isolation media: Basal media containing 5.0 g of FAA per liter

3. *Microbial Population in Soil Sprayed with Large Amount of Pesticide Ingredient*

Analyses of microbial population were made of soils in pot which had been

sprayed with relatively large amount of fluorine compounds (1g per pot) 10 days before the sampling. The result is represented in Table 3. It is evident that no appreciable change of microbial population occurred after spraying of these ingredients. BFAA was extremely toxic when it was added to the medium, but not so when it was sprayed on soils, as is illustrated by the number of colonies on

Table 3. Bacterial population capable of growing on media containg pesticide ingredients

Isolation medium	soil sprayed with	Bacterial populations / g dry soil at the depth		
		Surface	5 cm	10 cm
Cme*	water	516	485	518
	FA	529	465	556
	BFAA	468	477	425
	FAA	489	473	543
Cme + FA**	water	248	261	340
	FA	265	350	464
	BFAA	315	344	386
	FAA	319	284	307
Cme + BFAA***	water	18	16	18
	FA	14	20	26
	BFAA	8	18	17
	FAA	8	20	22
Cme + FAA**	water	468	390	479
	FA	336	409	377
	BFAA	403	470	383
	FAA	510	372	384

* Population x 10^5

** Population x 10^3, medium added with 5g of ingredient per liter

*** Population x 10^3, medium added with 2.5g of BFAA per liter

Average of 3 soil samples

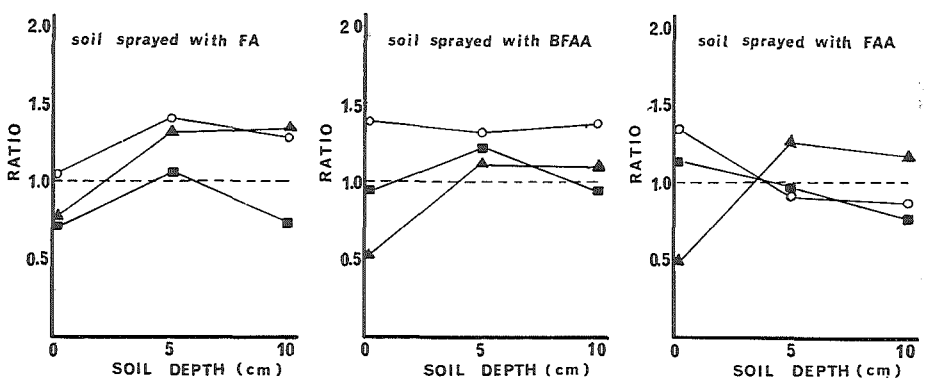

Fig. 1. Ingredient resistant bacterial population in soil sprayed with FA FAA, or BFAA. Soil was harvested 10 days after spraying. The ratio of ingredient resistant population in sprayed soil to that of non-sprayed soil appears on ordinate. ○——○ : grown on Cme + FA, ▲——▲ : grown on Cme + BFAA, ■——■ grown on Cme + FAA.

the non-ingredient medium. Figure 1 illustrates the absence of specific selection in terms of the ratio of bacterial population resistant to these pesticide ingredients in the sprayed soils to that in the non-sprayed soils. It apparently shows that no perceptible selection of the ingredient resistant population occurrs in the sprayed soils at least within 10 days after the treatment. Large variance of bacterial population in the surface soil might probably be attributed to the variability in moisture content of the sample soils.

The factory soil was different from those of non-sprayed fields in microial population, especially in the number of bacteria which are capable of utilizing FA as a sole source of carbon (Table 4). Thus it is reasonable to assume that a specific selection proceeds in the presence of these pesticide ingredients. It is important then to elucidate the dynamic change of microbial population in the sprayed soil in regard to the time sequence and the extent. Soil samples were taken from pots on 2, 5, 10, 15, and 30 days respectively after the spray of the pesticide ingredients. The result is represented in Figure 2. The ratio of the

Table 4. Microbes capable of growing on defined medium containing FA as a sole source of carbon

Soil	Microbial pupulation / g dry soil (x 10^2)				*
	Bacteria	Fungi	Actinomycetes	Total	
Field soil	6.6	0.4	1.4	8.4	
Factory soil	87.2	1.2	1.8	90.2	

* Average of 2 samples, 10 plates per sample

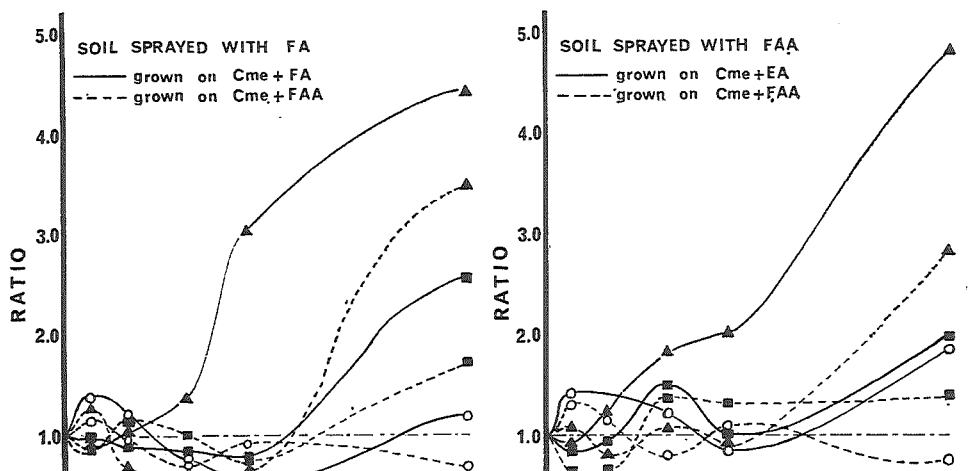

Fig 2. Increase of the ingredient resistant bacterial population in soil sprayed with FA or FAA. The ratio of ingredient resistant population in sprayed soil to that of non-sprayed soil was shown on ordinate. Samples were collected from surface (○), 5 cm (▲), 10 cm (■) in depth respectively.

ingredient resistant bacterial population in sprayed soil to that in non-sprayed soil was plotted as 1.0 (broken line). The result indicates that the ingredient resistant bacterial population in soil increases within 15–30 days after spraying. It also shows that the increase is most remarkable with soils at 5–6 cm in depth.

Although slight increase of the resistant population was observed of soils at 10–11 cm in depth, the shift was apparently delayed comparing to those at 5–6 cm. This might possibly be due to the delayed infiltration of the pesticide ingredients. No perceptible change occurred in soils at the surface under the experimental condition.

4. Defluorination in Soil

In the light of the above finding, it seems to be essential to measure the extent of defluorination in soils sprayed with the pesticide ingredients. Fluorine in the distillate of soil sample was determined colorimetrically and the result is shown in Table 5. As it shows, no specific increase in the amount of fluorine was

Table 5. Fluorine in soil sprayed with organic fluorine compounds.

soil sprayed with	Days after spray	ug F/g dry soil at the depth		
		Surface	5 cm	10 cm
Control (water)	2	3.20	1.86	2.01
	5	2.51	3.02	2.10
	10	2.13	1.67	1.88
	15	2.84	2.07	2.31
	30	2.14	1.85	1.82
FA	2	3.27	3.42	1.17
	5	3.51	3.08	2.51
	10	3.82	2.01	1.25
	15	5.85	4.91	1.50
	30	4.13	6.85	3.25
BFAA	2	5.01	3.13	1.34
	5	4.52	2.63	1.84
	10	3.34	1.67	1.70
	15	3.08	1.84	1.75
	30	3.58	2.55	1.94
FAA	2	2.87	3.34	2.26
	5	2.33	2.94	4.69
	10	2.84	2.67	3.83
	15	3.12	2.27	2.52
	30	3.71	6.10	5.46

Average of 3 soil samples, 3–5 titrations per sample.

observed of soils sprayed with the ingredients, although soil sampes taken 30 days after the spray gave slightly higher titers. As the increment of titer of fluorine was not high enough to account for the increase of the ingredient resistant population, it seems to be reasonable to assume that most of the resistant microbes are merely capable of negating the toxic effect of these organic fluorine compounds in culture media. Some of these resistant bacteria are, however, not only able to grow on the medium containing fluorine compound, but also capable of splitting

the extremely stable C-F bond of FA (OUCHI et al., 1971).

DISCUSSION

Monofluoroacetate is a potent inhibitor of TCA cycle by virtue of its incorporation into citrate to form fluorocitric acid, an inhibitor of aconitase (MORRISON et al., 1954; PETERS et al., 1953). FA and FAA have therefore been used as insecticide or rat poison. Although the C-F link in these compounds is of remarkably stable nature, yet some bacteria belonging to *Pseudomonas* are capale of splitting the bond.

In the light of the current trends in pesticide research and usage, investigations seem to be necessary for negating the toxic residues in agricultural products.

Several literatures are available in regard to the defluorination of organic fluorine compounds. MAZUR (1946) found in animal tissues an enzyme which accelerates the hydrolysis of dialkylfluorophosphate to dialkyl phosphoric acid, fluorine and hydrogen. Thus the enzyme has since been recognized in tissues of various species of animals and microorganisms (MOUNTER et al., 1955, 1955). KAUFMAN (1961) reported that the purified phenylalanine hydroxylation systems from rat and sheep liver catalyzed an oxidative defluorination. SAUNDERS (1957) described an enzymic defluorination of fluoroaniline. HORIUCHI (1961, 1962) found a defluorination enzyme in a soil bacterium, *Pseudomonas indoloxidans*, though the bacterium seemed to be somewhat unstable in the defluorination capacity. TONOMURA et al. (1965) made an extensive search for bacteria which defluorinate FA to glycolic acid and fluorine. They isolated a bacterium which is stable and strong in defluorination activity and characterized the enzyme in terms of induction pattern and the substrate specificity. These workers suggested that the organic fluorine compounds would probably be defluorinated in soil through these bacterial activities.

The result in this paper, however, shows that microbial population in orchard soils did not appreciably change after spray of the pesticide containing FAA, suggesting that the selection of the ingredient resistant population has not so far occurred in these orchard soils. This would probably be due to the fact that the amount of pesticide sprayed so far is not enough to cause such a specific selection as is observed of the factory soil. The factory soil apparently contained more microbes capable of utilizing FA comparing with the orchard soils. The result of pot experiments seems to illustrate how the shift of bacterial population occurs in the pesticide sprayed soils. As the ingredient resistant bacteria increased in the sprayed soils, at least population selection as to the resistance to the ingredient must be assumed. In the light of finding that liberation of fluorine in these sprayed soils was almost negligible in quantity, it is suggested that the

bacteria increased in these sprayed soils might not be involved in the defluorination of the organic fluorine compounds. Some bacteria isolated from these soils are, however, indeed capable of defluorinating FA in an *in vitro* system (OUCHI et al., 1971). Therefore it is conceivable that certain portion of the ingredient resistant bacteria would actually be involved in the splitting of C-F bond in soils, if these organic fluorine compounds accumulated enough to induce the enzyme system. Further analyses are necessary for justify this prediction.

SUMMARY

Microbial population in soils was studied in relation to defluorinaton of organic fluorine compounds, monofluoroacetate (FA) and monofluoroacetamide (FAA). No significant difference in microbial pooulation was observed between the soils collected from FAA-sprayed and non-sprayed citrus orchards. Soils of the FA-producing factory contained more bacteria which are capable of growing in a medium containing FA or FAA as a sole source of carbon. The soils sprayed with relatively large amount of the pesticide ingredients contained more ingredient resistant bacteria, by 30 days after sprayïng, suggesting that a temporary seleection to the ingredient degradation occurs in soils. The defluorination in soils, however, was not as high as had been expected.

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有機ふっ素化合物の脱ふっ素に関与する土壌微生物について

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モノフルオロ酢酸（FA）はTCA回路の著しい阻害剤であって，そのアミド化合物（FAA）とともに農薬として広く利用されている。

本報告は，これら有機ふっ素化合物を主成分とする農薬を撒布した土壌において，微生物が如何なる量的変動を示すかについて若干の検討を加えた結果を報告する。

神奈川，静岡，京都，大阪，鳥取の各県柑橘園より採集した撒布並びに無撒布土壌における微生物数の解析から，これまでのところ撒布による微生物相の変動はないことが明らかになった。

有機ふっ素化合物の製造工場から得た土壌については，FAを炭素源として利用し得る細菌数が，無汚染土壌よりも多い。したがって，農薬添加土壌においては分解菌の選択が考えられなければならないが，ポット試験の結果，かなり多量のFAまたはFAAを添加した土壌では，添加後15～30日で，これら薬剤に耐性のある細菌が多くなることが明らかとなった。しかし，これら添加土壌中の遊離ふっ素の増加が認められないことから，土壌微生物の脱ふっ素活性を高めるには，かなり多量の有機ふっ素化合物の添加蓄積が必要であろうと考える。