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Uptake and Dechlorination of an Organochlorine Fungicide, Tetrachloroisophthalonitrile, Daconil, by Some Soil Fungi*

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Introduction

Organochlorine pesticides are generally stable and resistant to physical, chemical, and other environmental reactions, and some of them persist in environment for a long time and contribute significantly to a harmful side effect as a contaminant in man's food chain. For example, aldrin and heptachlor were reported to be detected in soil and in crops grown on the soil 10 years after treatment[16]. DDT was also recovered 15 years after application[11,15,26].

Another important side effect of these compounds is to change ecosystems including of soil, and this has been the subject of many reseaches[2,8,20].

On the other hand, if one turns his eyes to the natural environment, one might find how saprophytic soil microorganisms contribute in cleaning environment, by breaking down various organic substances. This is also true of agricultural chemicals[4,10,12,17]. Mechanisms of the degradation of DDT[4,5,7,21], dieldrin[4,10,12,17], and pentachloronitrobenzene[18,19], which proceed in soil were studied extensively. Studies on fates of pesticides show clearly that the period of persistence of pesticides is largely affected by properties and conditions of soils[9,12,14]. This may be attributable, in part, to the difference of soil microflora.

The present authors intended to examine an utility of the metabolic activity of some soil microorganisms to encourage the degradation of pesticide residues in soil.

This paper deals with a part of series of these experiments, survay of soil fungi which absorb and degrade an organochlorine fungicide, tetrachloroisophthalonitrile (Daconil), especially with the dechlorination of the fungicide by some fungi.

Concerning the disappearance and metabolism of Daconil in soil, it was reported that this compound disappeared quickly from the soil[3,23]. Two metabolites of Daconil, 2, 4, 5-trichloroisophthalonitrile[3,24] and 2, 3, 5-trichloro-4, 6-dicyanophenol[3] were identified from soil, but the organisms responsible for this conversion were not known.

Materials and Methods

Collection of soil samples

Various soil samples of differnt nature were collected to isolate soil microorganisms.

Volcanic ash soil was collected from a paddy field and vegetable farm in Hirusen highland, Okayama prefecture. Calcareous soils were collected from paddy field, vegetable farm and forest in Niimi-city, Okayama prefecture.

Soils in plastic houses in which tomato and turnip were grown were collected from

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the experimental field of the Agricultural Experiment Station of Ishikawa prefecture and from a sand hill in Ishikawa prefecture, respectively. Plants grown in these plastic houses were sprayed with Daconil for six years in order to control diseases, and therefore the presence of many Daconl tolerant fungi was expected.

Isolation of fungi from soil samples

To obtain Daconil tolerant fungi which may have high activity of Daconil decomposition, Daconil was added into the isolation medium. Namely, 15ml of Martin's medium (1% glucose. 0.5% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, and 2% agar) containing 0.5% commercial Daconil (wettable powder, active ingredient was 75%) and 30 ppm of streptomycin were cooled to about 45°C and mixed with 0.2 ml of soil extract (3 g of soil sample was agitated with 30 ml of sterilized water) in petri dish and incubated at 28°C for 7 days. Number of colonies grown on the medium was counted as Daconil tolerant fungi. Colonies which were considered to be apparently different fungi were isolated and stored on potato sucrose agar slant. The marks and the origins of isolated fungi were shown in Table 1.

Table 1. Marks of fungal isolates and their origin

Mark of isolate	Soil sample from which fungi were isolated
V-P-	Volcanic ash soil from paddy field in Hirusen highland
V-F-	Volcanic ash soil from vegetable farm in Hirusen highland
C-P-	Calcareous soil from paddy field in Niimi-city
C-F-	Calcareous soil from vegetable farm in Niimi-city
C-M-	Calcareous soil from forest in Niimi-city
Pl-E-	Plastic house soil of Ishikawa Agricultural Experiment Station (Tomato)
Pl-S-	Plastic house soil of Ishikawa sand hill (Turnip)

Preparation of pure tetrachloroisophthalonitrile

Pure tetrachoroisophthalonitrile (TCPN) was prepared from commercial Daconil (wettable powder, Takeda Chemical Industries, Ltd.). TCPN was extracted with hot acetone, concentrated under reduced pressure, and recrystallized twice from hot acetone. This pure TCPN gave single spot on TLC plate.

Determination of the uptake of TCPN by soil fungi

Isolated soil fungi were cultured in the liquid medium containing 20 g glucose, 5 g peptone, 2 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 6mg thiamine mononitrate, 2 mg pyridoxine-HCl, 2 mg riboflavin, and 3mg calcium pantothenate in 1000 ml tap water. One hundred ml of the nutrient solution in 500-ml shake flasks were inoculated with a loopful of mycelia and agitated on a shaking machine which is kept at 20°C. At the time of the maximum growth (log phase), mycelia of each fungus were collected in a Büchner funnel. One gram of the fresh mycelia and 1.4mg of TCPN were again put into a shake flask with an incubation mediun (above medium without glucose and peptone) and shaken at 20°C. The disappearance of TCPN from the incubation medium was determined periodically with a spectrophotometer measuring the absorbance at 260 nm, and the amount taken up by fungi was calculated.

Survay of fungal metabolites of TCPN

Fungal isolates which showed good absorption of TCPN were examined to know whether or not these fungi degrade this compound.

Two days after incubation with TCPN, fungal mycelia were separated from the filtrate by filtration. The mycelia were sonicated with 8 volumes of distilled water for 30 min with Kubota Insonicator Model 200M at 200w. The sonicated mycelia were extracted three times with the same volume of *n*-hexane. The extract was concentrated under reduced pressure and the residue was dissolved in a small amount of acetone and chromatographed by TLC with silica-gel GF_{254} as the adsorbent and a mixture of *n*-hexane and chloroform (35/65, v/v) as the solvent. The filtrate was extracted three times with the same volume of *n*-hexane, and the extract was treated and chromatographed as same above. After developing the TLC plate, metabolites of TCPN were searched under the UV light.

Isolation, purification and identification of a metabolite of TCPN

A new spot which is considered to be a metabolite of TCPN was collected by preparative TLC. The spot correspond to the metabolite on TLC was scratched, concentrated, and recrystallized from hot acetone. This substance was subjected to mass spectrometry.

Identification of fungal isolates

The fungal isolates which have the activity to convert TCPN were cultured on Czapek agar medium and idintified according to Gilman[6], and Raper and Thom[22].

Results and Discussion

Population of Daconil tolerant fungi in various samples

Populations of Daconil tolerant fungi in soil samples were assessed by calculating the number of colonies which developed on the Daconil containing media. The results were shown in Table 2.

Table 2. Population of TCPN tolerant fungi in various soil samples

Soil sample	Number of fungi counted [a]
Volcanic ash soil from vegetable farm [b]	55
Volcanic ash soil from paddy field [b]	160
Calcareous soil from vegetable farm [b]	35
Calcareous soil from paddy field [b]	5
Calcareous soil from forest [b]	25
Plastic house soil on which tomato was grown [c]	365
Plastic house soil on which turnip was grown [c]	285

a : Number of colonies developed from 1 ml of soil extract on the TCPN containing media (1500 ppm).
b : Fungicides used for disease control is unknown.
c : Daconil (TCPN) has been applied for six years.

The population of tolerant fungi was extremely large in the soil of plastic houses, where Daconil was applied for six years for the control of diseases. This fact suggests the change of soil microflora by fungicide treatment owing to the decline of Daconil sensitive competitors.

Volcanic ash soil of paddy fields also contain many tolerant fungi, though the application of fungicides is not known.

Uptake of TCPN by soil fungi in submerged condition

Table 3. Uptake of TCPN in submerged condition by the most active ten fungal isolates

Fungal isolate	Amount of Daconil taken up (mg/g dry weight of mycelia)			
	1 day	2 days	3 days	10 days
V-F-1	>1.89	>1.89	>1.89	>1.86
V-P-5	1.87	1.75	1.75	—
C-F-1	1.80	1.74	1.75	—
C-F-4	1.59	1.76	1.81	1.82
C-P-4	1.75	1.75	>1.77	>1.77
C-P-6	>1.86	1.85	>1.86	>1.86
Pl-E-2	1.48	1.67	1.69	>1.77
Pl-E-10	1.74	1.72	1.74	>1.75
Pl-S-7	1.59	1.63	1.60	>1.71
Pl-S-12	1.76	1.81	1.83	>1.85

—: not determined.

Uptake of TCPN by tolerant soil fungi was examined using over than 100 isolates. The results varied between isolates.

Table 3 shows the time course of uptake of TCPN by ten isolates which absorbed actively. Uptake of TCPN by these active isolates reached plateau within one day.

Four of the most active ten were the isolates from calcareous soil, the other 4 from plastic house soil, and the remaining 2 were the isolates from volcanic ash soil.

Another soil fungicide, pentachloronitrobenzene, was reported to be absorbed more rapidly by sensitive fungi than tolerant fungi[18,19]. The tolerant fungi accumlated only a small amount of pentachloronitrobenzene within the mycelium and the greater portion of absorbed compound was converted into pentachloroaniline and pentachlorothioanisole and these metabolites were gradually excreted from the mycelium into the culture medium[18].

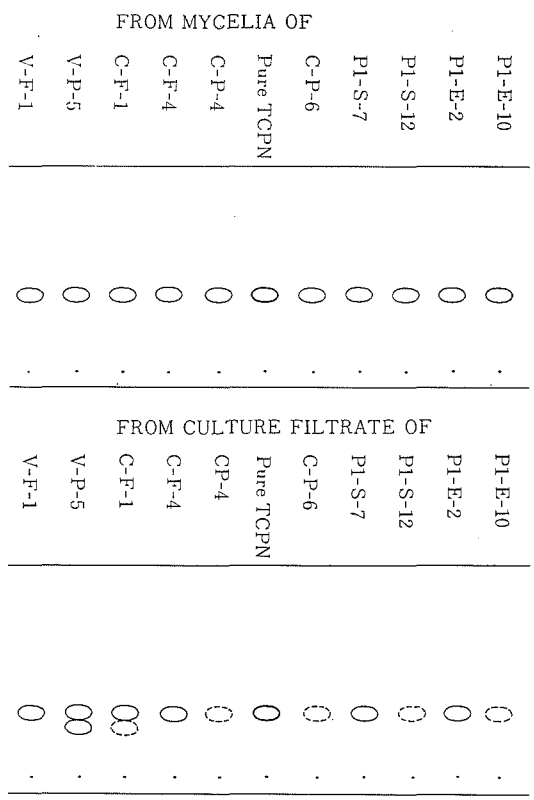

Fig. 1. Thin layer chromatograms of the extracts from mycelia and culture filtrates of fungal isolates which were incubated with TCPN.

Detection, isolation and identification of the metabolite of TCPN

Of all fungal isolates tested for uptake of TCPN, ten isolates which

showed active uptake listed in Table 3 were examined for the ability to metabolize TCPN. The results were summarized in Fig. 1.

As indicated in thin layer chromatogram, a metabolite was found in both culture filtrates of C-F-1 and V-P-5 after 2 days incubation with TCPN.

Four mg of the pure metabolite was isolated by repeated preparative TLC and recrystallization.

Mass spectrum of this metabolite clearly showed that this compound has three Cl atoms in the molecule and the molecular weight is 230, showing parent peak at m/e 230.

From these results the metabolite was identified as trichloroisophthalonitrile. In other words, TCPN was dechlorinated to trichloro-compound by the biological activity of C-F-1 and V-P-5 isolates.

As a dechlorination product of TCPN, 2, 4, 5-trichloroisophthalonitrile was reported by Endo et al.[3], but they did not describe whether the dechlorination reaction is biological or chemical.

In regard to the dechlorination of other pesticides, dechlorination mechanism of DDT was well established. This is probably due to the pollution problem of DDT residue.

Conversion of DDT to DDD was reported in the culture of *Escherichia coli*[5], *Pseudomonas aeruginosa*[5], *Aerobacter aerogenes*[21], and others (species were unidentified)[13]. The conversion was more rapid in anaerobic conditions. Baker's yeast also dechlorinated DDT when a reducing agent, dithionite was added to the culture medium[27]. The same conversion also occured in silage[10], and in flooded soil[11]. All reports suggested that the dechlorination of DDT was predominant under anaerobic and reducing circumstances.

γ-BHC was reported to be dechlorinated to γ-3, 4, 5, 6-tetrachlorocyclohexene in a submerged soil[25].

The detailed mechanism of dechlorination from TCPN to trichloroisophthalonitrile is under way.

Identification of fungal isolates, C-F-1 and V-P-5, which dechlorinate TCPN

Morphological characterietics of C-F-1 on Czapek agar medium was as follows.

Vegetative mycelium ·····septate, colorless.
Conidiophore·····enlarging upward and bearing phialide.
Conidiophore wall ·····smooth.
Conidial head ·····chocolate-brown.
Phialide ·····one series.
Length of phialide ·····5-8 μ.
Conidial diameter·····about 4 μ.
Length and width of conidiophre·····1000-2000 × 10-20 μ.
Diameter of conidial head·····80-150 μ.

These data coincide with those of *Aspergillus luchuensis* described in Gilman's "A Manual of Soil Fungi"[6].

Morphological characteristics of V-P-5 isolate was as follows.

Vegetative mycelium·····septate, colorless.
Penicilli·····strongly divaricated and assymetrical.

Colonies on Czapek agar grow restrictedly, attaining a diameter about 2.5 cm in 14 days at 23°C, consisting tough felt, strongly folded and wrinkled, central area raised, predominantly white but gray-green shade in the sporulating area, odor lacking, reverse dull orange color; conidiophores variable in size, 15-100 μ in length by 2.2 μ in di-

ameter, conidia subglobose to elliptical, 2.5–3 μ in diameter with smooth wall.

Colonies on malt extract agar grow more rapidly up to 6 cm in 14 days at 23°C, thin, plane except central area, largely submarged, gray-green color with slight network structure, reverse in yellow-orange shade; conidiophores 50–200 μ in length by 2.5 μ in diameter, metulae 10–15 μ in length by 2.5 μ in diameter with narrow tip, conidia subglobose to elliptical, 2.5–3 μ in diameter.

From these data, this fungus seemed to belong to a group of *Penicillium godlewskii* described in "Manual of the Penicillia" authored by Raper and Thom[22].

Therefore, C-F-1 isolate was identified as *Aspergillus luchuensis*, and V-P-5 as *Penicillium godlewskii*.

Summary

Fungi tolerant to an organochlorine fungicide, tetrachloroisophthalonitrile (TCPN), was selected and isolated from various soil samples, and examined for the activity to metabolize TCPN. The population of tolerant fungi was extremely large in the plastic house-soil on which TCPN has been used for six years to control diseases. This is probably due to the change of soil microflora owing to the decline of TCPN-sensitive competitors. Many tolerant fungi took up TCPN very rapidly from culture filtrate. Of ten tolerant isolates tested, two isolates, C-F-1 and V-P-5, were found to metabolize TCPN. The metabolite was isolated in pure form by preparative TLC, and identified as trichloroisophthalonitrile by mass spectrometry. C-F-1 was identified as *Aspergillus luchuensis* and V-P-5 as *Penicillium godlewskii*. Namely, these soil fungi dechlorinate from tetrachloroisophthalontrile to trichloroisophthalonitrile.

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土壌菌による有機塩素殺菌剤，ダコニールの吸収と脱クロールについて

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有機塩素殺菌剤，tetrachloroisophthalonitrile（TCPN）に対して耐性を有する土壌菌を選別して分離し，TCPN を代謝する活性をしらべた．TCPN 耐性菌の密度は，病害防除の目的でダコニール（原体 TCPN）を６年間にわたって使用したというビニールハウス内の土壌中に非常に高かった．これは TCPN に対して感受性の拮抗菌が減少して土中のミクロフローラが変化した結果と考えられる．多くの耐性菌は培養液中に添加した TCPN を急速に吸収する．TCPN を非常によく吸収する10株の菌についてテストした結果，２株，C-F-1 と V-P-5 が TCPN を代謝した．その代謝産物を調製用 TLC によって純粋に分離して，マススペクトル分析にかけた結果 trichloroisophthalonitrile と同定した．また C-F-1 および V-P-5 の２株の菌は，その形態学的性質から，それぞれ *Aspergillus luchuensis* および *Penicillium godlewskii* と同定した．即ちこれらの2株の菌は，tetrachloroisophthalonitrile を脱クロールして trichloroisophthalonitrile に変化させる．