Original Article

Effect of a Fungicide, Chlorothalonil, on Cellulose Decomposing Process in Soil

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The effect of chlorothalonil (Daconil® 1000) application on cellulose decomposing process in soil, and the impact and duration of the effect were estimated in laboratory experiments at 25°C and 13°C under upland conditions. In the soil just after the application, the pesticide caused the retardation of cellulose decomposition and the shifts in the balance of fungal flora on the cellulose sheets at doses above 75 µg a.i./g-soil at 25°C and at doses above 15 µg a.i./g-soil (recommended rate of the pesticide for drenching) at 13°C. In the soil after 10 weeks of the application, no retardation in cellulose decomposition and incomplete recovery of the fungal flora were observed at doses of 75 µg a.i./g-soil at 25°C. Relative dominance and frequency of a fungal species, *Rhizoctonia solani*, on the cellulose sheets at 13°C were high in the control soil but low in applied soil. The retardative effects of the chlorothalonil application on cellulose decomposition in the soils at low temperature seem to be attributable to reduction in the dominance of *R. solani* on the cellulose sheets. Since the species is one of the target pathogen for the drenching of chlorothalonil, the retardative effect on cellulose decomposition should be defined as a side-effect in respect of phenomenon, whereas, as a objective effect in respect of mechanism. These results represent the importance of research on the structure of microflora and consideration on the environmental condition such as temperature in assessment on effect of pesticide on soil ecosystem.

INTRODUCTION

A lot of studies have been done on the side effect of pesticide application on populations of soil microorganisms^{1,2)} and microbial activities in soil such as respiration,³⁾ nitrification⁴⁾ and nitrogen fixation.⁵⁾ However, there are few studies on the effect of pesticides on the interaction between the microflora and the microbial activities in soil ecosystems. In addition, most of the studies deal with sieved soil from which plant residue has been removed, although plant residues containing cellulose are one of the most widely distributed organic sources of energy and suitable habitat and active site for soil microorganisms in soil environment.⁶⁾ Indeed, Katayama *et al.*⁷⁾ reported that the microscopic observation on

cellulose sheets enabled to detect minimal change in the microflora by trichlamide application compared with the plate count of bulk soil. Torstensson & Wessen⁸⁾ reported that benomyl application caused changes in composition of fungal flora on straw. Wardle & Parkinson⁹⁾ detected that shifts in fungal species composition on organic particles in soil occurred by glyphosate application.

There are some reports on the suppressive effect of application of a fungicide, chlorothalonil (TPN, 2,4,5,6-tetrachloro-1,3-isophthalonitrile) on cellulose decomposition in soil.^{10,11)} However, the microbial ecological mechanisms of the suppressive effect were not known. On the other hand, we found that the cellulose decomposition in the soil was suppressed in winter and recovered in summer by

long-term application of chlorothalonil at the recommended rate in a field experiment.¹²⁾ The fact indicates that environmental condition, probably temperature, dominated the effects. Little attention has been given to the relationship between temperature and the side effects of pesticide despite the seasonal fluctuation of microbial activities.

In this study, we attempted to: 1) compare the fungal flora on cellulose sheet as a model of plant residue in the pesticide-applied soil with that in the control soil, because the fungi are the major group of organisms responsible for cellulose decomposition¹³⁾ and 2) examine the impact and duration of the effects on cellulose decomposition and fungal flora on cellulose sheet at 25°C (assuming summer condition) and 13°C (assuming winter condition).

MATERIALS AND METHODS

1. Soil

The soil sample used was collected from a field, where no pesticide had ever been applied, in the experimental farm in Shimane University, Matsue, Japan. The soil was carefully dried, but never had less than 12% of water content (about 33% of maximum water holding capacity), and passed through a 2-mm mesh sieve. Some of the soil properties were as follows: soil texture CL, pH (H₂O) 7.1, maximum water holding capacity 36.9%.

2. Pesticide

The fungicide chlorothalonil in flowable formulation was used (Daconil® 1000, 40% a.i.).

3. Application of Pesticide

Soil sample (4 kg) was preincubated at a moisture content of 12% for 20 days at 13 and 25°C. Then, the pesticide suspended in distilled water was mixed with the soil uniformly at the same (15 µg a.i./g dry soil), fivefold (75 µg a.i./g dry soil) and tenfold (150 µg a.i./g dry soil) of the recommended rate for the drench, assuming a uniform distribution in the top 10 cm of soil and a soil bulk density of 1.0 g/cm³. Distilled water was mixed with the control soil. The final moisture content in all soil samples was 50% of maximum water holding capacity. Half of the soil (about 2 kg)

was used immediately for the estimation of cellulose decomposing activity (immediate test). The other half was incubated in an air tight plastic bucket (capacity 13 l) at the respective temperatures for 10 weeks, and then, used for other estimation in the experiment (persistence test). The bucket lid was opened for a moment to replenish oxygen in every week.

4. Measurement of Cellulose Decomposition in Soil

The Benchkote® sheet method¹⁴⁾ was used for the estimation of cellulose decomposition in the soil. Benchkote® (Whatman), a polyethylene backed filter paper, was cut into pieces of 4 cm×4 cm (called a sheet), then desiccated and weighed. The burying of sheets was achieved as follows. Sixty grams of the soil sample was placed in a plastic cup (93 mm diameter, 55 mm depth). After the soil surface was made even and pressed by a weight of 679 g (10 g/cm²). The sheet was put on the soil with filter paper side down. Sixty grams of the soil sample was overlaid in the cup. This procedure was repeated until 5 sheets were buried in a cup (called a set). Five replicates of the set were provided at every treatment and incubated at the same temperature as the preincubation in a humid chamber to maintain the initial moisture content in soil throughout the incubation period. After appropriate intervals of incubation, the sheets in a set were taken out. Two of them were desiccated and weighed for the estimation of the decomposition, the other 3 sheets were used for the examination of fungal flora on the sheets.

5. Isolation and Assortment of Fungi on Sheets

Each sheet was homogenized with 50 ml of sterilized water for 5 min at 10,000 rpm by a waring-blender. Tenfold serial dilution of the homogenate was made. One milliliter of the suitable dilute was inoculated in 12 ml of Martin's rosebengal agar. Triplicate plates were made. After incubation for 4 days at 25°C, the colonies formed were enumerated. Some dominant colonies on the plates were transferred onto potato sucrose agar (PSA)

Fig. 1 Cellulose decomposition in the soils applied with chlorothalonil at the same (■), fivefold (○) and tenfold (□) of the recommended rate (15 μg a.i./g dry soil) for the drench, and in the control soil (●): a), immediate test at 25°C; b), immediate test at 13°C; c), persistency test at 25°C; d), persistency test at 13°C.

Plots within tests and incubation periods followed by the same letter do not differ significantly ($p=0.01$) by Duncan's new multiple range test.

slants. All isolates were given the grade of relative dominance based on the number of colonies on the plates and classified into groups of fungi according to the appearance of colony on PSA, malt extract agar and Czapek agar. Some of the groups were identified according to some manuals for identification of fungi.¹⁵⁻¹⁷⁾

RESULTS

1. Effect on Cellulose Decomposition

Figure 1a) shows the time course of cellulose decomposition in the immediate test at 25°C. In the control soil, the cellulose decomposition began within 1 week of incubation and progressed linearly. In recommended rate soil, the cellulose decomposition was suppressed only in the first week of incubation, after then no obvious suppression was observed. In the fivefold rate soils, the cellulose decomposition was suppressed except for an

estimation (4 weeks). In the tenfold rate soils, the cellulose decomposition was retarded until 3 weeks and suppressed during the following incubation periods. Figure 1b) shows the results obtained in the immediate test at 13°C. In the control soil, the cellulose decomposition began within 2 weeks of incubation and progressed linearly. In the recommended rate soil, the cellulose decomposition was retarded until 3 weeks of incubation and then progressed at the same rate as the control. In the five and tenfold rate soils, the retardation of cellulose decomposition was highly retarded, while the following decomposition was progressed at the same rate as control.

In the persistency test at 25°C (Fig. 1c)), no retardation of cellulose decomposition was observed in the recommended and fivefold rate soils. In the tenfold rate soil, cellulose decomposition was suppressed completely until 5 weeks. Figure 1d) shows the result in the persistency test at 13°C. No retardation of cellulose decomposition was observed in the recommended rate soil. In the fivefold rate soil, the cellulose decomposition was retarded until 3 weeks in the same way as in the immediate test. In the tenfold rate soil, the duration of the suppression was prolonged to 4 weeks.

2. Occurrence and Dominance of Fungi on Sheet

It is well known that the number of fungal colony on an agar plate may not reflect their activity in soil and organic debris in a quantitative way because some or most colonies appearing on the plate may be derived from spores. Fungal sporulation, however, originates from mycelial activity, so in certain situations such as in this study, the relative number of colonies is most likely to reflect the degree of dominance of a fungal species on the cellulose sheet.

Table 1(a) shows the occurrence of fungi on sheets at each incubation period in the immediate test at 25°C. Although 7 fungal species (above the dotted line) occurred on the control sheets, no species was always dominant throughout the incubation period. No. 18 (*Trichoderma* sp.) dominated on all of the three sheets at 4 weeks of incubation. It is

Table 1 Fungi isolated from the cellulose

(a) Immediate test at 25°C		Control					No.	Recommended		
Fungi	No. ^{a)}	1 ^{b)}	2	3	4	5		1	2	3
<i>Rhizoctonia solani</i>	19	○△○ ^{c)}	— ^{d)}				19	◎	—	
Not identified	2	○	—				2	◎◎	—	
Not identified	13		—	△		△○	13		—	
Not identified	15			○			15		—	
Not identified	14		—	○			14		—	
<i>Trichoderma</i> sp.	18		—		◎◎◎	◎	18		—	○
Not identified	9		—			○	9		—	
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Not identified	17		—				17		—	◎◎
<i>Stysanus stemonites</i>	20		—				20		—	
<i>Penicillium</i> sp.	3		—				3		—	
Sterile hyphae	6		—				6		—	
<i>Phoma</i> sp.	11		—				11		—	
<i>Cephalosporium</i> sp.	23		—				23		—	
Not identified	12		—				12		—	
Not identified	25		—				25		—	
<hr/>										
(b) Immediate test at 13°C							No.			
Fungi	No.	1	2	3	4	5		1	2	3
<i>Rhizoctonia solani</i>	19	◎◎◎	◎◎◎	◎◎◎	◎◎◎	◎◎	19	○	○○	○
Not identified	2						2	○	○	
<i>Phoma</i> sp.	11					○	11	◎	△	△ ○
<i>Verticillium</i> sp.	37						37			○
<i>Penicillium</i> sp.	40						40			
<i>Cephalosporium</i> sp.	23						23			
<i>Fusarium</i> sp.	31						31			
Not identified	9						9			
<hr/>										
(c) Persistency test at 25°C							No.			
Fungi	No.	1	2	3	4	5		1	2	3
<i>Rhizoctonia solani</i>	19	○○○	○				19	△△	△	
Not identified	13	○	△○○	◎◎◎	○△○	△	13	△○△	△ ○	◎◎○
Not identified	16	△					16	△		
Not identified	55				△○		55			
<i>Penicillium</i> sp.	56				○	◎◎	56	△		△
<hr/>										
<i>Stysanus stemonites</i>	20						20	△	△	
Sterile hyphae	6						6		△	
<i>Cephalosporium</i> sp.	23						23			
<i>Phoma</i> sp.	11						11			
Not identified	63						63			
Not identified	14						14			
<hr/>										
(d) Persistency test at 13°C							No.			
Fungi	No.	1	2	3	4	5		1	2	3
<i>Rhizoctonia solani</i>	19	◎◎◎	◎◎◎	◎◎◎	◎○○	○	19	◎◎◎	◎◎◎	○○
Not identified	6					◎	6			
Not identified	64					○	64			
<i>Phoma</i> sp.	11					△	11			△○
<i>Verticillium</i> sp.	37						37	△		○

^{a)} Species No. ^{b)} Incubation period (weeks). ^{c)} Relative dominance of the fungus on triplicate of the Within (a) and (c), fungal species below a dotted line dominated only on the sheets in the soil applied

sheets in the soils applied with chlorothalonil.

Application															
rate		Fivefold rate							Tenfold rate						
4	5	No.	1	2	3	4	5	No.	1	2	3	4	5	No.	
○○		19	△	—	○	△		19		—				19	
		2		—				2		—				2	
	○	13		—				13		—				13	
		15		—		△		15		—				15	
		14		—				14		—		⊙	⊙	14	
		18		—				18		—				18	
		9		—				9		—				9	
⊙		17		—			⊙	17		—				17	
		20		—				20		—				20	
	○	3		—				3		—				3	
	○	6		—				6		—				6	
		11	⊙○○	—	⊙△○			11	⊙○○	—	⊙○○	⊙ △	⊙ ⊙	11	
		23		—		○△⊙	⊙⊙	23		—				23	
		12		—		○		12		—		⊙		12	
	25		—				25	○	—				25		
		19	⊙	○	○		△○	19		⊙				19	
		2						2						2	
	△○	11	⊙	⊙ ⊙	⊙⊙○	○○○	○△	11	⊙ ⊙	△	⊙△⊙	⊙○○	△△⊙	11	
	○○	37				△	⊙	37			△	△	⊙○	37	
	○	40						40						40	
		23						23	⊙					23	
		31	⊙					31						31	
	9						9		○				9		
		19	△		△ △	△○	△	19	⊙					19	
		13	△△					13						13	
		16						16						16	
	△△	55			△	△○		55						55	
	○△	56					○	56						56	
		20	⊙	○○⊙				20		○			⊙	○	20
		6	○	△				6	⊙					6	
	23						23			⊙⊙	⊙⊙	○○	23		
	11						11		○○			⊙ △	11		
	63			⊙			63						63		
	14					⊙	14						14		
		19			⊙○			19			○			19	
		6						6						6	
		64						64						64	
	○	11		⊙○○	○ △	⊙⊙⊙	○○○	11		⊙○○	⊙⊙	△△	⊙	11	
		37					△ △	37				⊙⊙○	⊙⊙	37	

sheet: ⊙, ≥70%; ○, ≥40%; △, ≥20%. ^{d)} Not determined.

with chlorothalonil and those above dominated on the control sheets.

not clear whether a fungal succession had occurred on the sheets or the fungal species colonized initially on the sheets varied for each sheet. In the recommended rate soil, some of the 7 species and some others (below the dotted line) appeared on the sheets for which the decomposition was not obviously suppressed as observed in Fig. 1a). Occurrence of the 7 species decreased with an increase in the application rate, while other species (below the dotted line) occurred. Especially, species No. 11 (*Phoma* sp.) was noted to be in dominance on most sheets throughout the incubation period in the five and tenfold rate soils in which the cellulose decomposition was retarded (Fig. 1a)). Table 1(b) shows the results obtained in the immediate test at 13°C. In contrast to 25°C incubation, species No. 19 was noted to be in dominance throughout the incubation period on most of the control sheets. Species No. 19 was identified as *Rhizoctonia solani*. This indicates that the cellulose decomposition in the soil at low temperature depends on a specific fungi *i.e.* *Rhizoctonia solani*, in agreement with the observation by Tribe^{18,19)} that *Rhizoctonia* was dominant and strongly cellulolytic on cellophane and filter paper buried in soil. In the pesticide applied soils in which the cellulose decomposition was retarded, occurrence of species No. 19 decreased with an increase in the application rate, while the dominance of species No. 11 increased.

Table 1(c) shows the results obtained in the persistency test at 25°C. On the control sheets, species No. 13 was one of the predominant species throughout the incubation period on most sheets. Species No. 19 was also dominant at the early periods of incubation and 3 additional species occurred. Occurrence of these 5 species (above the dotted line) decreased slightly in the recommended and fivefold rate soils although the cellulose decomposition was not suppressed (Fig. 1c)). However most of those species did not occur in the tenfold rate soil in which the cellulose decomposition was suppressed as shown in Fig. 1c). In any of the soils, species No. 11 which dominated remarkably in the immediate test least occurred. Table 1(d) shows the results obtained in the persistency test at 13°C. As in the immediate test, species No.

19 was dominant throughout the incubation period on most of the control sheets. In the recommended rate soil (Fig. 1d)) in which the cellulose decomposition was not suppressed, the occurrence of species No. 19 did not decrease as much as in the immediate test, while species No. 11 was also dominant throughout the incubation period. In five and tenfold rate soils, species No. 11 and species No. 37 (*Verticillium* sp.) was noted to be in dominance. The dominance of species No. 37 was distinguished at the latter part of incubation.

DISCUSSION

It was reported that the degree of suppressive effects of trichlamide¹¹⁾ and hydroxy-isoxazol²⁰⁾ on cellulose decomposition under upland conditions differs from that under flooded conditions. We found in a previous field experiment¹²⁾ that an environmental condition, probably temperature dominated the degree of the suppressive effect of chlorothalonil on cellulose decomposition. Also, we demonstrated in this study that the effect of chlorothalonil on cellulose decomposition on the sheet was more persistent in low temperature. A high amount of pesticide residue itself at low temperature could be one of the possible reasons for the persistence of the effect, because Sato & Tanaka indicated that degradation of chlorothalonil in soil was markedly suppressed at 10°C.²¹⁾ In addition, in this study, it is considered that the cellulose decomposition at low temperature depends on a limited fungal species (No. 19). These observations indicate that environmental condition, such as moisture and temperature should be important factors to assess the effect of pesticide.

The results in this study indicate that the effect of chlorothalonil on the cellulose decomposing process in soil is not only the suppression and retardation in the activity but also the changes in the origin of the activity (*i.e.* fungal community). The suppression of cellulose decomposing activity and changes in fungal flora on cellulose sheet in this study correspond to those observed in the field to which chlorothalonil had been applied for 6 years (unpublished data) in some degree. Therefore, it seems that the laboratory ex-

periments in this study can be a suitable model of soil ecosystem to estimate the effect of pesticides on the cellulose decomposition in soil.

In addition, these findings mentioned above indicate importance to research on microflora in the assessment of the effect of pesticides on soil ecosystem. Even though microbial activity which is affected by pesticide application may have been recovered, the origin of the activity may not. In persistency test at 25°C, the cellulose decomposition in the five-fold rate soil progressed at the same rate as the control (Fig. 1c). If only these facts are based on the discussion, the duration of the effect must be assessed to be less than 10 weeks. However, the fungal flora on the sheets in the fivefold rate soil was not the same as in the control (Table 1(c)). On the basis of the results of fungal flora, the persistence period of the effect should be assessed to be no less than 10 weeks. The difference in fungal flora on the sheets indicates that the carbon from cellulose was imparted to different microbes in part. Although the difference in fungal flora must be important ecologically, the significance on total soil activity cannot be discussed at the current stage of soil microbial knowledge.

The effect of chlorothalonil on cellulose decomposition and fungal flora may be assessed to be tolerable in the case of recommended rate but critical in the cases of higher rate. The retardative effects on cellulose decomposition at low temperature seem to be attributable to reduction in the dominance of *R. solani* (species No. 19), a major decomposer at the condition, on the cellulose sheets. This microbial mechanism of the effect is identical in any application rate. Anyway, the population decrease and/or the inactivation of the species in soil by an application of the pesticide is not defined as a side-effect but an objective effect since the species is one of the target pathogen for the drenching of the pesticide. On the contrary, the retardative effect on cellulose decomposition should be defined as a side-effect since an inhibition of cellulose decomposition in soil is not an object of the pesticide. This "inevitable side-effect" mentioned above indicates that it is essential to

give careful considerations involving a research on the mechanism of the effect, at the time of risk assessment on the side-effects of pesticide, especially fungicide.

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要 約

土壌中におけるセルロース分解過程に及ぼす殺菌剤クロロタロニルの影響

巢山弘介, 山本広基, 黒川順司, 駒田 旦
畑地土壌中におけるセルロース分解過程に及ぼす殺菌剤クロロタロニル (ダコニール® 1000) の土壌灌注施用の影響およびその持続性を 25°C および 13°C の温度条件下で容器内試験により評価した。施用直後の土壌にセルロースシートを埋設した場合, 25°C では 5 倍量以上, 13°C では常用量以上の施用区でセルロース分解の遅延

が認められ, シート上の糸状菌相は対照区のそれとは明らかに異なった。また, 施用 10 週間後にシートを埋設した場合には, 25°C では 5 倍量以下の施用区で分解の遅延は認められなかったが, 糸状菌相の回復は不完全であった。一方, 13°C で対照区のシート上に培養期間を通じて優占する糸状菌の優占度および頻度が施用区では低下することが低温下におけるセルロース分解に及ぼす供試農薬の遅延的影響の機構であると推察された。その菌は, 供試農薬の標的病原菌の一つである *Rhizoctonia solani* であることから, 低温下におけるセルロース分解の遅延は現象的には side-effect である一方, 機構的には標的微生物の減少あるいは不活性化であると推察される。これらの結果より, 土壌生態系に及ぼす農薬の影響およびその持続期間を評価するにあたっては, 温度等の環境要因ならびに影響発現機構に関する十分な配慮が必要であることが示された。