Effect of Long-Term Application of a Fungicide, Chlorothalonil, on Cellulose Decomposition and Microflora in Soil under Upland Conditions

Kousuke SUYAMA, Hiroki YAMAMOTO, Kadzunori TATSUYAMA
and Hajimu KOMADA

Laboratory of Environmental Microbiology, Faculty of Agriculture, Shimane University,
Nishi-Kawatsu-cho, Matsue 690, Japan

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INTRODUCTION

Extensive studies have been done on side-effects of pesticide application on soil microbial activities. In most cases, pesticide was applied to soil only once and the effects were evaluated. A few papers have reported changes in microbial community such as enrichment of pesticide degrading and/or tolerant microorganism by repeated application. There is a strong presumption that such changes in microflora could result in changes in microbial activities. It is therefore important to investigate effects of repeated application of pesticide on soil-microbial activities, and also to monitor seasonal fluctuations in microbial activities following environmental changes.

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. Sun et al. have reported that cellulose decomposition was markedly suppressed in an upland field by chlorothalonil repeatedly applied for a long period. Katayama & Kusakabe have demonstrated that chlorothalonil inhibited the cellulose decomposition not only in upland soil but also in flooded soil in laboratory experiments. In their studies, however, effects were monitored for a relatively short period of time and only under relatively high-temperature condition. In our study, chlorothalonil was applied repeatedly to a field and effects on cellulose decomposition in the field were monitored in various seasons for six years.
MATERIALS AND METHODS

1. Experimental Field and Application of Chlorothalonil

An experimental field was set up in the experimental farm, Shimane University, Matsue, Japan on October 4, 1985. Three plots (control, normal dosage and high dosage; each 2.4 m²) had ever received any pesticides before. Some properties of the soil are shown in Table 1. Chlorothalonil was used in wettable powder formulation (Daconil®, 75% a.i.) until 1989 and thereafter in flowable formulation (Daconil® 1000, 40% a.i.). Application rates to each plot are shown in Table 1. Prior to every application, the plots were plowed. An appropriate amount of Daconil® or Daconil® 1000 was suspended in 10 or 3 l of tap water, and sprinkled evenly with a watering can. First application was on November 16, 1985, and thereafter, twice a year, in April or May (spring application) and October or November (autumn application). No crops were cultivated in the experimental field and weeding was performed periodically during the experiment.

2. Measurement of Cellulose Decomposition in Soils

The Benchkote sheet method7) was used to estimate cellulose decomposition in the soil. Benchkote® (Whatman) polyethylene-backed filter paper was cut into 5×10 cm and desiccated (sheet). For each plot, thirty sheets were weighed and inserted lengthwise at intervals of about 3 cm vertically to the plow layer (0–10 cm). After 22–45 days, those sheets were taken out, and the amount of decomposed cellulose was determined by the method of Tatsuyama et al.7) Cellulose decomposition in each plot was expressed by the mean value of 30 sheets. Effect of chlorothalonil application on cellulose decomposition was expressed by relative decomposition rate to the control plot.

3. Microflora in Soils

The population of microorganisms was estimated four times in autumn, 1991, before the 13th application of chlorothalonil when no sheets were applied to the plots. Five soil subsamples were collected for each plot randomly from the plow layer. The sub-samples (totaling about 500 g) were composited, passed through a 2-mm mesh sieve and used immediately.

Microorganisms in the soil samples were enumerated with the dilution plate technique. Sodium albuminate agar was used for aerobic bacteria and actinomycetes, and for gram-negative bacteria crystal violet (5 mg/l) was added to agar. These plates were incubated at 25°C for 7 days or at 13°C for 21 days. Martin’s rosebengal agar was used for fungi. To enumerate cellulase-producing microorganisms, CMC agar-Congo Red staining method9) was used. Modified CMC agar medium was used for actinomycetes, and for fungi streptomycin (100 mg/l) was added. These plates were incubated at 25°C for 4 days or at 13°C for 12 days. All plate counts were done in triplicates.

Table 1 Application rates of chlorothalonil and some soil properties in experimental plots.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rate of application(*)</th>
<th>MWHC(&gt;)</th>
<th>pH(H2O)</th>
<th>pH(KCl)</th>
<th>T-C(&gt;)</th>
<th>T-N(&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>37.4</td>
<td>6.4</td>
<td>5.1</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Normal dosage</td>
<td>2.25(*)</td>
<td>37.4</td>
<td>6.6</td>
<td>5.4</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>High dosage</td>
<td>11.25(&gt;)</td>
<td>37.4</td>
<td>6.4</td>
<td>5.1</td>
<td>1.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

(*) g a.i./m²/application.
(>) Maximum water holding capacity (%).
(>) Total carbon (%).
(>) Total nitrogen (%).
(#) Equivalent to the recommended application rate of Daconil® for drench.
(?) Fivefold of the recommended application rate of Daconil® for drench.
4. Cellulose-Decomposing Activity of Fungi Isolated from Soil

A representative plate was chosen from the triplicates of Martin's rosebengal agar. All fungal colonies on the plate were isolated onto Czapek yeast agar slants. Cellulose-decomposing activity of those isolates was estimated by the following two methods:

4.1 Filter-paper strip method

Four milliliters of medium C and filter paper strip (10 × 50 mm) in a test tube (15 × 105 mm) were autoclaved. Each isolate was inoculated onto the filter paper in the test tube. After incubation for 4 weeks at 25°C, the degree of breakdown of the filter paper was determined into four grades by the naked eye.

4.2 Avicel® suspended agar method

Five milliliters of medium C amended with Avicel® (cellulose microcrystalline, Merck, 2 g/l) and agar (10 g/l) in a test tube (15 × 105 mm) were autoclaved, kept at 50°C, agitated with a vortex mixer to suspend Avicel® and, immediately, kept standing vertically in ice water to solidify the agar. Each isolate was inoculated onto the surface of Avicel®-suspended agar. After incubation for 4 weeks at 25°C and 13°C, clearness of a layer in which Avicel® was decomposed was determined into three grades by the naked eye, and the depth of the layer was measured.

RESULTS AND DISCUSSION

1. Effect on Cellulose Decomposition

Figure 1 shows the changes in cellulose decomposition rates in the control and chlorothalonil-applied plots. Seasonal variations were observed in all plots. In the control plot, the rates were about 2–3% day⁻¹ in summer and about 0.5–1% day⁻¹ in winter. Seasonal variations in cellulose decomposition rate differed between the chlorothalonil-applied plots and the control plot. Figure 2 shows relative values of the applied plots to the control. In normal-dosage plot, decomposition was suppressed (less than 60% of the control) from November to March (winter suppression), and recovered (more than 60% of the control with two exceptions) from April to October (summer recovery). In the high dosage plot, winter suppression was observed, while summer recovery was incomplete. These results indicate that environmental condition, probably soil temperature, influenced the suppressive effect of chlorothalonil on cellulose decomposition in soil. To the best of our knowledge this study is the first to demonstrate the influence of temperature on the degree of side-effect of pesticide. Relative decomposition rates in winter decreased in both plots in the initial 2 or 3 years and then reached minimum in the following 3 or 4 years. This indicates that the suppressive effect remained and accumulated year after year. The accumulation of the effect may be equivalent to that of chlorothalonil itself, since it was found that the dissipation of chlorothalonil was suppressed in several soils after repeated applications compared with the first application and that chemical accumulated in the soils.10,11 Such quite unusual findings and our results suggested that estimation of effect of long-term application is indispensable and that certain microbial activity should be monitored.
continuously for a long period in order to assess effects of pesticide on microbial ecosystem in soil by field experiment.

2. Effect on Microbial Population

Population levels, assumed to be potential inoculum sizes to sheets inserted in soil, of microorganisms in normal- and high-dosage plot soils are shown in Table 2. It has been reported that population levels of total and gram-negative bacteria were increased by long-term application of chlorothalonil. In our experiment, gram-negative bacteria increased remarkably in the high-dosage plot soil at 25°C. Conversely, population levels of actinomycetes and cellulase producing actinomycetes were low in the high-dosage plot soil at both incubation temperatures. Major cellulose decomposers in soil are fungi. It seems that the decrease in the inoculum size of cellulase-producing actinomycetes did not cause inhibition of cellulose decomposition in high-dosage plot. Indeed, population levels of actinomycetes on sheets taken out from all plot were very low (data not shown). It seems that the population levels of Cx-cellulase producing fungi were not low enough to inhibit decomposition in the normal- and high-dosage plots at both incubation temperatures. Accordingly, the suppression of cellulose decomposition in the applied plot was not interpreted by an inoculum size of Cx-cellulase producing fungi. Then, total activity of the cellulase system (activity to decompose filter paper or Avicel®) of each fungi isolated from soil in each plot was examined.

3. Cellulose-Decomposing Activity of Fungi Isolated from Soil in Each Plot

Table 3 shows the filter paper-decomposing activity by fungi isolated from soil in each plot. The order of fungal population having vigorous decomposing activity was control>normal dosage>high dosage. This suggests that the long-term application decreased the fungal population having vigorous activity to decompose filter paper.

Table 4 shows crystalline cellulose-decomposing activity of the fungal population in each plot. The activity is expressed as total depth of layer in which Avicel® was decomposed. The order was control=normal dosage.

Table 2 Effect of long-term application of chlorothalonil on microbial population in soil.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Total</th>
<th>Cx-cellulase producing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal dosage</td>
<td>High dosage</td>
</tr>
<tr>
<td>Aerobic bacteria</td>
<td>133**</td>
<td>185</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>186</td>
<td>494</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>75</td>
<td>24</td>
</tr>
<tr>
<td>Fungi</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

Table 3 Filter paper-decomposing activity of fungi isolated from each plot soil.

<table>
<thead>
<tr>
<th>Decomposition of filter paper*</th>
<th>Control</th>
<th>Normal dosage</th>
<th>High dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vigorous</td>
<td>6**</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Medium</td>
<td>20</td>
<td>35</td>
<td>26</td>
</tr>
<tr>
<td>Weak</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>50</td>
<td>38</td>
</tr>
</tbody>
</table>

* Vigorous: the whole paper strip was decomposed to suspend in the form of fiber, medium: up to half the paper strip was decomposed, weak: only a part of the paper strip was decomposed slightly, -: not decomposed.

** Number of isolates.
Fungi were isolated from the soil collected on Sep. 4, 1991.

Soil samples were collected on Sep. 4, Oct. 16, Oct. 18, Nov. 11 and Nov. 20, 1991.
high dosage at an incubation temperature of 25°C, while it was control>normal dosage>high dosage at 13°C. It is suggested that the suppression of cellulose decomposition throughout the years in the high-dosage plot was attributed to the decline in the fungal inoculum potential to decompose crystalline cellulose at 25°C and 13°C. It is suggested that the suppression of cellulose decomposition in normal-dosage in winter was attributed to the decline of fungal inoculum potential in crystalline cellulose decomposition at low temperature.

The results on the microflora mentioned above were obtained from the study on the soil collected in autumn. Since microflora in winter and summer may differ from that in autumn, it is debatable whether phenomena in summer and winter could be explained by the results obtained on microflora in autumn. Further research on microflora in various seasons is needed to clarify the microbiological mechanism of suppressive effect of chlorothalonil on cellulose decomposition.

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REFERENCES

要　約
殺菌剤クロロタロニルの長期連用が畑地土壌中におけるセルロース分解および微生物相に及ぼす影響

巻山弘介，山本広基，速山和紀，駒田 且
クロロタロニルを土壌灌漑常用量で年2回，6年間連用した圃場において，セルロース分解活性が冬期に抑制され，夏季には回復するという季節変化が認められた。また，その抑制の程度は連用開始3年までは年々增大した。5倍連用圃場でも同様の傾向が認められたが，夏期の回復は不完全であった。このことから，土壌中におけるセルロースの分解に対するクロロタロニルの抑制的影響の発現においては施用量だけでなく温度も重要な因子であると考えられた。25および13℃を培養温度として連用区と対照区の土壌微生物相を比較したところ，糸状菌数はほぼ同程度であったが，結晶性セルロースを完全に分解する糸状菌数およびその活性の和は，25℃では5倍連用区でのみ，13℃では両連用区で低い傾向が認められた。したがって，連用区におけるセルロース分解の抑制は，セルロース基質に対する糸状菌イノキュラムの量的な減少ではなく，質的な変化が原因であることが示唆された。