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論　文

Calorimetric Analysis of Effects of a Soil-Stress Compound on Soil-Microbial Activity

Akiyo K. Sakamiya, Natsuki Origuchi, and Akiyoshi Tanaka

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Stressing effects of the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) on soil-microbial activity were analyzed by a calorimetric method. Thirty-six mg glucose was added to 10 g of upland, orchard, paddy, sand-dune, natural-forest, and cedar-forest soils, respectively, in the presence of various concentrations of 2,4-D, and the evolved heat due to the glucose degradation was recorded at 25 ℃. The heat-evolution curves were analyzed on the basis of Richards growth model to evaluate several parameters that characterize the process of glucose degradation. The apparent degree of resistance of the soil microbes to 2,4-D was estimated from the 50 % stressing concentration K_i and the minimum inhibitory concentration MIC, which were evaluated by analyzing the 2,4-D-concentration-dependence of the degradation parameters. It was shown that the degree of resistance of the two kinds of forest soils were of an order of magnitude greater than that of the sand-dune soil in terms of the value of K_i. These stressing parameters, K_i and MIC, are valid measures for quantitative estimation of the stressing effects of pesticides on soil microbial activity.

Keywords: soil microbes; soil-stress compound; calorimetry; microbial activity

1. Introduction

Numerous microbes inhabit soil and play important roles in carbon cycling in the biosphere. Soil-microbial activity is sensitive to the soil environment and is suppressed by soil pollutants such as chemicals and heavy metals; therefore, the degree of suppression of soil microbial activity can be a measure of soil pollution. However, most of the soil microbes are difficult to cultivate, making it difficult to measure the activity of the microbes directly by conventional methods such as media cultivation.

When soil microbes degrade carbon sources, metabolic heat is generated and the process of this heat generation can be recorded by a calorimeter. Using the calorimetric method, it is possible both to measure the velocity of the carbon-source degradation, which can be regarded as soil microbe activity,[1-6] and to evaluate several parameters that characterize the process of degradation. In this study, we analyze possible stressing effects of an agricultural chemical on soil-microbial activity, using 2,4-dichlorophenoxyacetic acid (2,4-D) as the model compound.

2. Materials and Methods

2.1 Chemicals and soil samples

The glucose (D-glucose, pure grade) and 2,4-D used in this study are products of Nakarai Tesque Inc. (Kyoto, Japan) and Ishihara Sangyo Kaisha, Ltd. (Osaka, Japan), respectively.

The soil samples of orchard, paddy, and upland were collected at the farm station of Mie University (Tsu, Mie, Japan); natural- and cedar-forest soils at the forest station of Mie University (Tsu, Mie, Japan), and sand-dune soil at the Niigata-sand dune (Niigata, Niigata, Japan). Additional upland soil was collected at the farm of the University of Tokyo (Bunkyo-ku, Tokyo, Japan). To discriminate between these two upland samples, the former is referred to as upland-1 soil, and the latter as upland-2 soil. Altogether, seven kinds of soil samples were examined in this study. The samples were collected from the soil surface to a depth of about 15 cm, and the air-dried samples were sieved through a 2.0 mm screen and mixed thoroughly.

2.2 Calorimetry

Soil samples (10 g/sample as dry weight) were placed into 30-mL glass vials and various concentrations of 2,4-D solution were added to each of the samples such that each sample contained water at half the maximum water holding capacity.[7] After the samples were pre-incubated at 25 ℃ for 3 weeks,[8] glucose solutions (1.0 M, 0.2 mL = 36 mg glucose) were added to the soil samples. The vials were then plugged with silicone stoppers and placed in the multiplex isothermal calorimeter.[9] Exothermic calorimetric signals resulting from glucose degradation were recorded at 25 ℃ until the signals returned to the baselines. Referential calorimetric signals were obtained by adding 0.2 mL distilled water to the soil samples instead of the glucose solution. For each soil sample, we confirmed that no exothermic signal was observed when the soil samples were autoclaved before use.

3. Results and Discussion

3.1 Glucose-degradation thermograms

Fig.1 shows examples of the raw calorimetric signals $g(t)$ of glucose degradation after the subtraction of the referential signal obtained for four soil samples in the presence of various concentrations of 2,4-D. In the following discussion, we use $g(t)$ or, simply, thermogram to refer to the glucose-degradation thermogram. With increasing concentration of 2,4-D, the peak height of $g(t)$ became smaller and the time needed to reach the peak became longer. For upland-2 soil (**Fig.1-c**), however, no prominent effect on the peak height or on the time to reach the peak was observed using the experimental concentrations. Although we have no evident explanation for the results at present, it is possible that 2,4-D was decomposed during the pre-incubation. If this is the case, further systematic investigation are needed concerning the effects of the length of pre-incubation on the stressing parameters of 2,4-D which will be described below. In the following discussion we analyze the effects of 2,4-D on the other six soil samples.

3.2 Analysis of the thermograms

The time dependences of the total heat evolution $q(t)$ was obtained from $g(t)$ by the method described earlier.[9,10] The $q(t)$ curves were analyzed on the basis of Richards growth-curve model, a best suitable model

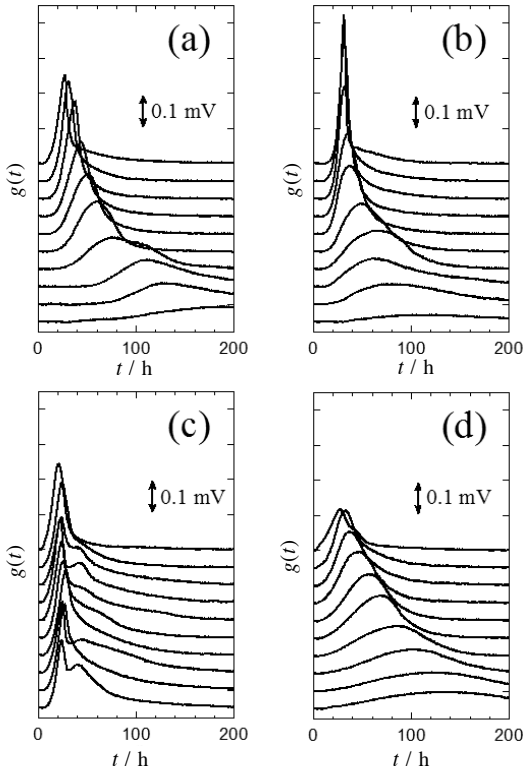

Fig.1 Thermograms of glucose degradation by soil microbes observed in the presence of various concentrations of 2,4-D.
Soils used were (**a**) paddy, (**b**) upland-1, (**c**) upland-2, and (**d**) natural-forest. Observed at 25 ℃. 2,4-D concentrations from top to bottom in the figures were (**a**) 0, 1.0, 1.2, 1.8, 2.0, 2.5, 2.8, 3.0, 3.5 and 4.0 mg/(g soil), (**b**) 0, 0.20, 0.30, 0.60, 0.80, 0.90, 1.2, 1.5, 2.0, and 2.2 mg/(g soil), (**c**) 0, 3.2, 3.8, 4.0, 4.2, 4.8, 6.5, 7.0, 8.0, and 9.0 mg/(g soil), and (**d**) 0, 2.0, 3.0, 4.5, 5.0, 5.7, 6.5, 7.5, 7.7 and 8.0 mg/(g soil), respectively.

microbial calorimetry:[11]

$$q(t) = Q\left(1 + (d-1)\exp\left(-B\left(t - t_{\mathrm{p}}\right)\right)\right)^{\frac{1}{1-d}} \qquad (1)$$

where Q is the total amount of the evolved heat, d is the shape-determining parameter, t_{p} is the time at which the heat-evolution velocity v ($= \mathrm{d}q/\mathrm{d}t = q'(t)$) reached the maximum,* and B is a constant that is related to the maximum heat-evolution rate constant $\mu_{\max}$, that is, $\mu_{\max}$

* Under the experimental conditions of this study, $q'(t)$ is practically proportional to $g(t)$ and, hence, t_{p} also represents the peak time of $g(t)$.

Table 1 Glucose-degradation parameters of upland-1 soil in the presence of various concentrations of 2,4 D evaluated from Richards model.

2,4-D / mg g^{-1}	t_p / h	$t_{1/2}$ / h	v_{max} / mV h^{-1*}	μ_{max} / h^{-1*}
0.0	31.1 ± 0.1**	30.3	1.94×10^3	0.125
0.2	31.1 ± 0.1	31.7	1.77×10^3	0.0720
0.3	31.4 ± 0.1	33.6	1.45×10^3	0.0548
0.6	34.2 ± 0.1	37.0	1.10×10^3	0.0394
0.8	36.3 ± 0.1	41.0	9.44×10^2	0.0296
0.9	47.7 ± 0.1	54.6	6.58×10^2	0.0195
1.2	63.1 ± 0.1	69.5	5.50×10^2	0.0140
1.5	61.7 ± 0.2	85.8	3.62×10^2	0.00948
2.0	80.3 ± 0.1	102	2.75×10^2	0.00834
2.2	112 ± 0.4	133	94.8	0.00684

* Values of $t_{1/2}$, v_{max}, and μ_{max} were caluculated using equations described in the text.
** Figures after ± are standard errors evaluated from the fit.

$= B^{d/(1-d)}$.[11] The values of these parameters were obtained by the least-squares method. The other parameters, $t_{1/2}$ (the time at which half of the total heat evolution is completed) and v_{max} (the maximum heat-evolution velocity), were calculated by $t_{1/2} = t_p - (\ln((2^{d-1}-1)/(d-1)))/B$ and $v_{max} = QBd^{d/(1-d)}$, respectively.[11] Some of the representative values obtained for upland-1 soil are listed in **Table 1**.

3.3 Evaluation of stressing parameters

The values of t_p and $t_{1/2}$ become larger, and those of v_{max} and μ_{max} become smaller with increasing concentration of 2,4-D. It is thus possible to define the specific activity *sa* of soil microbes in the presence of 2,4-D in the following four ways: 1) $sa(i) = t_p(0)/t_p(i)$, 2) $t_{1/2}(0)/t_{1/2}(i)$, 3) $v_{max}(i)/v_{max}(0)$, and 4) $\mu_{max}(i)/\mu_{max}(0)$, where i and 0 refer to the concentration of 2,4-D. Open circles in **Figs.2** and **3** show examples of the concentration dependence of the specific activity *sa* evaluated for the orchard and upland-1 soils using the four parameters mentioned above.

Typically, the stressing effects of 2,4-D can be analyzed in two ways: first, with the bacteriostatic model, the specific activity $sa(i)$ is expressed as follows:[1,12-16]

$$sa(i) = \frac{1}{1+\left(\frac{i}{K_i}\right)^m} \qquad (2)$$

where K_i is the 50 % stressing concentration. m is the cooperativity parameter that characterizes the cooperativity of the stressing effect: When m is large, the value of $sa(i)$ is close to 1 in the lower concentration range of

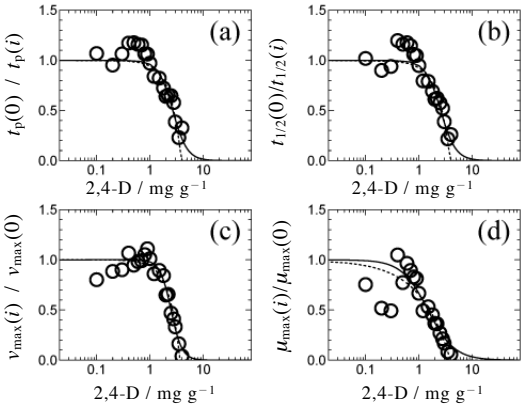

Fig.2 2,4-D-Concentration dependence of the specific activity *sa* of glucose degradation evaluated for the orchard soil.
The vertical scale shows the specific activity *sa* obtained from (**a**) t_p, (**b**) $t_{1/2}$, (**c**) v_{max}, and (**d**) μ_{max}. For each figure, the open circles represent the experimental results, and the solid and dashed lines show theoretical curves obtained on the basis of the bacteriostatic model (equation 2) and the bacteriocide model (equation 3), respectively, using the values of the stressing parameters listed in **Table 2**.

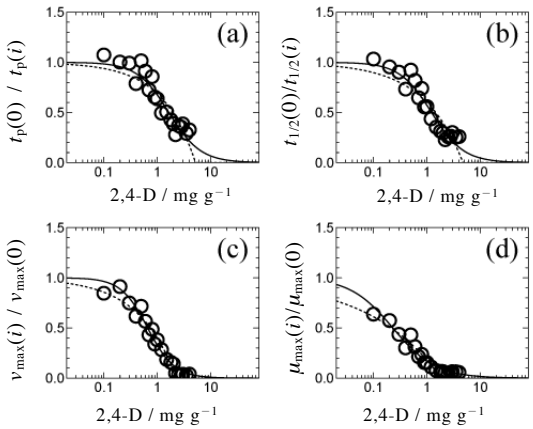

Fig.3 2,4-D-Concentration dependence of the specific activity *sa* of glucose degradation evaluated for the upland-1 soil. For details, see the legend for **Fig.2**.

2,4-D ($i < K_i$), decreases sharply with a small increase of i in the vicinity of $i = K_i$, and then approaches zero in the higher concentration ($i > K_i$).
Second, with the bactericidal model, the specific activity $sa(i)$ is expressed as follows:[1, 12-16]

Table 2 Stressing parameters of 2,4-D.

Soil	Stressing parameter	Degradation parameters used for analysis			
		t_p	$t_{1/2}$	v_{max}	μ_{max}
cedar-forest	K_i / mg g^{-1}	5.1 ± 0.2*	5.5 ± 0.2*	7.6 ± 0.6*	5.8 ± 0.2*
	m	3.2 ± 0.4	3.2 ± 0.4	9.6 ± 7.7	4.2 ± 0.6
	MIC / mg g^{-1}	8.7 ± 0.5	9.0 ± 0.5	8.5 ± 0.8	8.6 ± 0.4
	n	1.4 ± 0.2	1.6 ± 0.2	7.1 ± 5.4	2.0 ± 0.3
natural-forest	K_i / mg g^{-1}	4.6 ± 0.1	4.5 ± 0.1	7.9 ± 0.5	5.6 ± 0.1
	m	2.2 ± 0.1	1.8 ± 0.2	14 ± 14	2.8 ± 0.2
	MIC / mg g^{-1}	9.8 ± 0.3	10 ± 0.4	8.4 ± 0.8	9.5 ± 0.2
	n	0.97 ± 0.05	0.88 ± 0.05	11 ± 10	1.4 ± 0.1
orchard	K_i / mg g^{-1}	2.7 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	1.5 ± 0.2
	m	3.2 ± 0.7	3.1 ± 0.6	4.2 ± 0.6	1.8 ± 0.4
	MIC / mg g^{-1}	3.9 ± 0.3	3.9 ± 0.3	3.7 ± 0.2	3.9 ± 0.6
	n	2.1 ± 0.4	1.9 ± 0.4	2.1 ± 0.3	0.77 ± 0.15
paddy	K_i / mg g^{-1}	1.0 ± 0.05	0.94 ± 0.05	1.6 ± 0.1	0.92 ± 0.05
	m	1.5 ± 0.1	1.5 ± 0.1	2.9 ± 0.5	2.0 ± 0.2
	MIC / mg g^{-1}	3.6 ± 0.3	3.7 ± 0.3	2.9 ± 0.3	2.5 ± 0.2
	n	0.57 ± 0.06	0.59 ± 0.06	1.5 ± 0.4	0.76 ± 0.09
sand-dune	K_i / mg g^{-1}	0.42 ± 0.03	0.44 ± 0.03	0.60 ± 0.06	0.43 ± 0.06
	m	2.7 ± 0.5	2.7 ± 0.5	3.1 ± 1.1	3.0 ± 1.1
	MIC / mg g^{-1}	0.78 ± 0.07	0.93 ± 0.09	1.1 ± 0.1	0.86 ± 0.12
	n	1.3 ± 0.3	1.1 ± 0.2	1.6 ± 0.5	1.3 ± 0.5
upland-1	K_i / mg g^{-1}	1.6 ± 0.1	1.2 ± 0.11	0.66 ± 0.03	0.23 ± 0.02
	m	1.5 ± 0.2	1.4 ± 0.1	1.7 ± 0.1	1.1 ± 0.1
	MIC / mg g^{-1}	5.2 ± 0.7	4.7 ± 0.6	2.3 ± 0.1	1.8 ± 0.2
	n	0.70 ± 0.10	0.59 ± 0.08	0.62 ± 0.05	0.33 ± 0.03

* Figures after ± are standard errors evaluated from the fit.

$$sa(i) = 1 - \left(\frac{i}{\mathrm{MIC}}\right)^m \quad (3)$$

where MIC is the minimum inhibitory concentration at which $sa(i)$ becomes zero and n is the cooperativity parameter of the stressing effect similar to m.

The values of these stressing parameters, K_i, m, MIC, and n, which characterize the effects of 2,4-D on each soil type, were evaluated for each sample by the least-squares method on the basis of the equation 2 (bacteriostatic model) and equation 3 (bactericidal model), and are listed in **Table 2**. In the table, the values of K_i and MIC are expressed as mg weight of 2,4-D in 1 g of soil (mg g^{-1}). The solid and dashed lines in these figures show the theoretical curves drawn according to equations 2 and 3, respectively, using the values listed in **Table 2**.

Although these two models provided similar goodness of fit to the experimental results (**Figs.2** and **3**), the bacteriostatic model seems likely to be a more suitable model because 2,4-D decreased all the specific activities of t_p, $t_{1/2}$, v_{max}, and μ_{max}. That is, as was pointed out by Takahashi *et al.*,[17, 18] a pesticide that has only bacteriostatic action changes the values of t_p, $t_{1/2}$, v_{max}, and μ_{max}, while a pesticide that has only bactericidal action changes the values of t_p and $t_{1/2}$, but not those of v_{max} and μ_{max}.

As seen in **Table 2**, the values of the stressing parameters calculated using the four kinds of degradation parameters showed reasonable consistency with one another. For example, the values of K_i obtained for the natural-forest soil were 4.6 (from the t_p value), 4.5 ($t_{1/2}$), 7.9 (v_{max}), and 5.6 mg/(g soil) (μ_{max}). A similar degree of consistency was also seen among the four MIC values obtained for each soil. In the case of the orchard soil, for example, the values were 3.9 (from the t_p value), 3.9 ($t_{1/2}$), 3.7 (v_{max}), and 3.9 mg/(g soil) (μ_{max}). Significant discrepancies among the K_i values and among the MIC values were seen in the case of the upland-1 soil (see also **Fig.3**), for which we have no explanation at present.

3.4 Degree of resistance of the soils to the stressing effects of 2,4-D

Judging from the values of K_i obtained from $t_{1/2}$, the apparent degree of resistance to the stressing effects of 2,4-D can be ranked as follows: cedar-forest soil (5.5),

natural-forest soil (4.5), orchard soil (2.6), upland-1 soil (1.2), paddy soil (0.94), and sand-dune soil (0.44 mg/(g soil)). A large difference was seen between the two forest soils and the sand-dune soil. The reason for this may be explained as follows: The forest soils are considered to contain a greater amount of organic matter than the sand-dune soil. The higher content of the organic matter may affect the degree of resistance in two ways. One is that the organic matter absorbs the 2,4-D to some extent, resulting in the lowered concentration of 2,4-D in the soil solution and in the apparent lowered stressing effect of 2,4-D. In other words, the absorption of 2,4-D by the organic matter is thought to lower the effective concentration of 2,4-D in the soil solution. No such effect can be expected for the sand-dune soil. The other is that the forest soils likely contain highly diverse and large numbers of microbes due to their higher content of the organic matter; therefore, there may be many chemical-resistant microbes in these soils.

3.5 Conclusion

It was shown in this study that the stressing effects of a pesticide on soil-microbial activity can be quantitatively evaluated by the calorimetric method and that the stressing parameters K_i, MIC, m, and n obtained by this method can be used as valid measures for estimating the stressing effects of pesticides. Since the calorimetric method does not require any chemical treatment or media cultivation, it can, in principle, measure the activity of nonculturable microbes as well as culturable microbes.

The calorimetric method is phenomenalistic in nature and cannot of itself explain the mechanism by which differences in the degree of resistance to pesticides occur. In this regard, we are carrying out a PCR-DGGE/TGGE analysis of the microbial community structure of the soils to gain deeper insight into the mechanism of the experimental results.

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Akiyo K. Sakamiya
Graduate School of Bioresources, Mie University

Natsuki Origuchi
Faculty of Bioresources, Mie University

Akiyoshi Tanaka
Graduate School of Bioresources, Mie University,
E-mail: akiyoshi@bio.mie-u.ac.jp