Discovery and Development of Pyridachlometyl

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Makoto Kurahashi
Yuichi Matsuzaki
Akio Manabe*1
Masato Kawamura*2
Fukumatsu Iwahashi
Mitsuyoshi Yamane
Environmental Health Science Laboratory
Ryoko Matsuyama
Yuko Handa

Pyridachlometyl is a novel fungicide active ingredient that binds fungal tubulin and exhibits antifungal activity based on the modulation of fungal microtubule dynamics. Owing to a binding site that is distinct from that of the existing tubulin polymerization inhibitors, pyridachlometyl shows no cross-resistance to carbendazim and other fungicides (*e.g.*, DMI, QoI, and SDHI). Pyridachlometyl exhibits broad-spectrum antifungal activity, showing high efficacy against the sugar beet leaf spot, soybean purple stain, and powdery mildew, which affects various vegetable crops in field trials. In Japan, registration of 'FUSEKI flowable' as the first product of pyridachlometyl is expected by 2023.

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Introduction

The use of agricultural fungicides is essential to efficiently and stably produce crops. If a specific fungicide-resistant strain spreads, the fungicide loses its effectiveness in fungal control, and other fungicides that operate in the same mode of action also become less effective (cross-resistance), thus leading to potential reductions in crop yield and quality. Therefore, the risk of spreading resistant fungi must be reduced. To achieve this, adhering to proper fungicide-usage guidelines and avoiding the sequential use of fungicides with the same mode of action are recommended. Furthermore, different modes of action or multisite actions should be rotated and mixtures of multiple effective ingredients with different modes of action should be used.

Recently, the global fungicide market has been dominated by three fungicide groups: demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs). Fungicides in these groups are highly effective and exhibit broad-spectrum activities. Furthermore, they have widespread use owing to their high versatility. However, fungal strains resistant to these groups have been detected in several crops and have become problematic. For instance, in Japan, strains of *Cercospora beticola* that cause Cercospora leaf spot in sugar beet and are resistant to benzimidazole fungicides and kasugamycin have been identified. Subsequently, DMIs and QoIs were introduced and became key fungicides. However, strains that are resistant to both groups are increasing, thus creating a demand for novel fungicides with new modes of action.[1)]

Pyridachlometyl is a novel fungicide developed by our company and is classified as a new chemical group (**Fig. 1**). It exhibits antifungal activity by binding to tubulin in filamentous fungi. The binding site of the

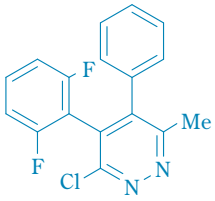

Fig. 1 Chemical structure of pyridachlometyl

*1 Retired before publication of this article
*2 Current affiliations: Oita Factory

compound is different from that of existing tubulin polymerization inhibitors, thus indicating that its activity is not affected by cross-resistance. Furthermore, pyridachlometyl is effective against fungal strains resistant to existing fungicides.

FUSEKI Flowable (35.0% [w/w] pyridachlometyl suspension concentrate) is expected to become the first fungicide in Japan to contain pyridachlometyl by 2023. The invention process, manufacturing method, biological effects, mode of action, formulations, and safety of pyridachlometyl in mammals and the environment are reported herein.

Invention process

The process of pyridachlometyl discovery through chemical structural transformations is summarized in **Fig. 2**.

Compound 1, with its unique chemical structure (**Fig. 2**), exhibits broad-spectrum antifungal activity against various plant pathogens[2]. Additionally, it has a substantially different structure from that of existing fungicides, and our evaluation confirmed the absence of cross-resistance to major fungicides. Therefore, we conducted structural transformation research using compound 1 as a lead to achieve significant structural changes with the goal of developing a novel broad-spectrum fungicide with a new mode of action. To identify the pharmacophore (the partial structure essential for the antifungal activity) of this novel compound, compound 2 was synthesized[3]. In compound 2, the central condensed heterocyclic ring of compound 1 was changed from [1,2,4]triazolo[1,5-*a*]pyrimidine to imidazo[1,2-*a*]pyrimidine, and the 4-methylpiperidin-1-yl group was converted to a 4-methylphenyl group. Compound 2 exhibited almost the same antifungal activity as compound 1. Based on this information, we hypothesized that to secure antifungal activity, two nitrogen atoms from the condensed heterocyclic ring are required (as shown in the lower part of **Fig. 2**); moreover, this part interacts with the amino acids of the target protein in plant pathogens as a hydrogen bond acceptor, along with the two phenyl groups, to constitute the pharmacophore.

Based on this hypothesis, we postulated that a condensed complex ring may not be essential. Considering compound 2 as a proprietary new lead compound and applying our previous knowledge, we designed and synthesized compound 3, which contains a monocyclic heterocyclic ring, the 2(1*H*)-pyrimidinone ring[4]. The antifungal activity of compound 3 was essentially the same as that of compound 2, although the former was slightly lower depending on the target pathogen species. The oxygen atom in the pyrimidinone ring of compound 3 is thought to play a role similar to that of the nitrogen atom of compound 2.

Therefore, compound 3 was modified by replacing its oxygen atom with a nitrogen atom to design and synthesize a new compound, compound 4, which contained a pyridazine ring, a monocyclic heterocyclic ring with two adjacent nitrogen atoms[5]. The antifungal activity of compound 4 was higher than that of compound 3, and its excellent efficacy in disease control was confirmed in field trials.

We investigated the relationship between the structures of various related compounds and their antifungal activity and safety in mammals, using compound 4 as a

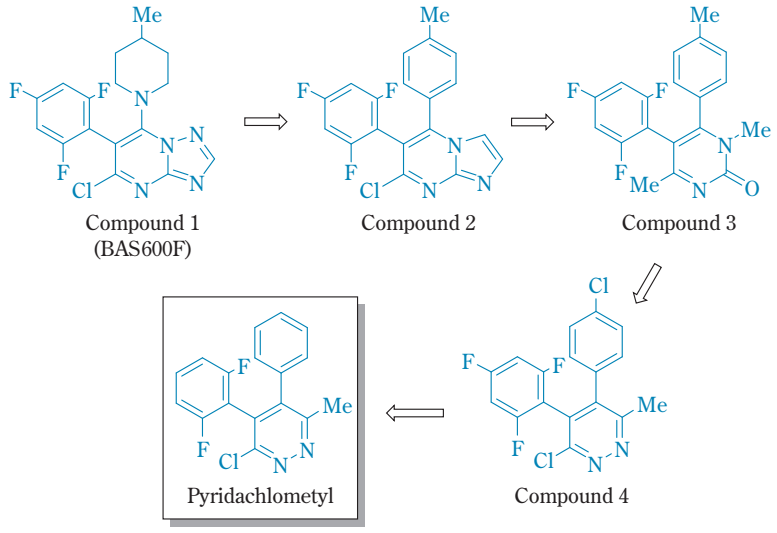

Fig. 2 Invention pathway of pyridachlometyl

new lead compound and focusing on compounds with a pyridazine ring. Based on this analysis, we finally selected pyridaclometyl[5)–8)], which has a simplified chemical structure, as a candidate compound for development.

Manufacturing method

Pyridachlometyl is a pyridazine derivative bearing the 2,6-difluorophenyl group. It can be synthesized by the transformation of a precursor with a 2,6-difluorophenyl group via a pyridazinone intermediate, or by a coupling reaction with Grignard reagent to directly introduce a 2,6-difluorophenyl group into the pyridazinone structure (**Fig. 3**). We explored these methods in detail and established an industrially efficient manufacturing process for pyridachlometyl.

Biological effects

1. Antifungal spectrum

Pyridachlometyl exhibits antifungal activity against the phytopathogenic fungi that infect various crops. For example, in antimicrobial tests using artificial media, it exhibited high antifungal activity (half-maximal effective concentration (EC_{50})) against pathogens, such as *Fluvia fulva, Cercospora kikuchii,* and *Microdochium nivale* (**Table 1**).

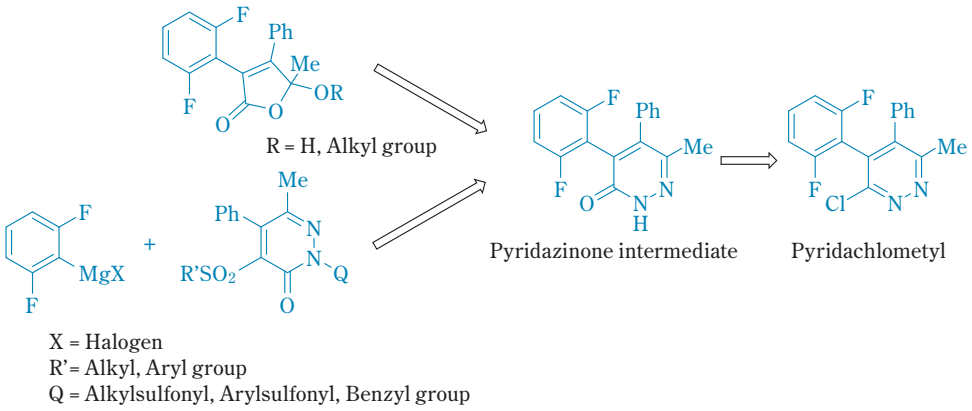

Fig. 3 Synthesis route of pyridachlometyl

Table 1 Pyridachlometyl antifungal spectrum

Kingdom	Division	Class	Species	EC_{50} (ppm)
Fungi	Ascomycota	Dothideomycetes	*Zymoseptoria tritici*	0.056
			Fulvia fulva	0.034
			Cercospora beticola	0.17
			Cercospora kikuchii	0.047
			Alternaria solani	0.22
			Cochliobolus miyabeanus	0.21
			Venturia inaequalis	0.040
		Sordariomycetes	*Pyricularia oryzae*	0.055
			Colletotrichum gloeosporioides	0.023
			Colletotrichum acutatum	1.2
			Microdochium nivale	0.075
			Fusarium graminearum	1.4
		Leotiomycetes	*Botrytis cinerea*	0.059
			Sclerotinia sclerotiorum	1.7
			Clarireedia homoeocarpa	0.038
		Eurotiomycetes	*Penicillium digitatum*	1.4
			Penicillium expansum	2.4
	Basidiomycota	Agaricomycetes	*Rhizoctonia solani* (AG4)	0.66
			Athelia rolfsii	0.40
		Ustilaginomycetes	*Ustilago maydis*	0.12
Stramenopiles		Oomycetes	*Phytophthora infestans*	> 10
			Pythium ultimum	> 10

2. Cross-resistance with existing fungicides

The cross-resistance between pyridachlometyl and major existing fungicides was examined using field isolates of *Cercospora beticola*. Antimicrobial tests were conducted on 67 field isolates from Hokkaido, Japan, using pyridachlometyl, carbendazim (a tubulin polymerization inhibitor that is an active metabolite of benomyl and thiophanate-methyl), difenoconazole (DMI), and trifloxystrobin (QoI) to determine the sensitivity of isolates to each fungicide. The sensitivity distribution of carbendazim and trifloxystrobin exhibited a bimodal distribution: one group with EC_{50} values of 2 ppm or higher indicated resistant strains, and another group with lower EC_{50} values suggested sensitive strains. By contrast, the sensitivity to difenoconazole varied significantly among the strains, with some strains showing EC_{50} values of 2 ppm or higher, thus indicating resistance. However, pyridachlometyl exhibited a unimodal distribution with a clear EC_{50} peak in the range of 0.02–0.2 ppm. Based on these results, pyridachlometyl demonstrated high antifungal activity against a group of strains, including those resistant to carbendazim, difenoconazole, and trifloxystrobin, confirming the absence of cross-resistance to these fungicides (**Fig. 4** and **Table 2**). Furthermore, pyridachlometyl demonstrated high disease control efficiency in a field trial conducted in the presence of DMI- and QoI-resistant strains (**Fig. 5**).

3. Fungicide properties

We investigated the activity characteristics of pyridachlometyl through greenhouse pot tests[*3] on Cercospora leaf spot of sugar beet. Representative examples of the test results are presented below.

(1) Protective effects

In a protective effect test on Cercospora leaf spot on sugar beet, pyridachlometyl exhibited high efficacies of 97% and 98% control at 200 ppm and 133 ppm, respectively. This performance was comparable to that of mancozeb, which is one of the most important fungicides used to control sugar beet Cercospora leaf spot in Hokkaido (**Fig. 6**).

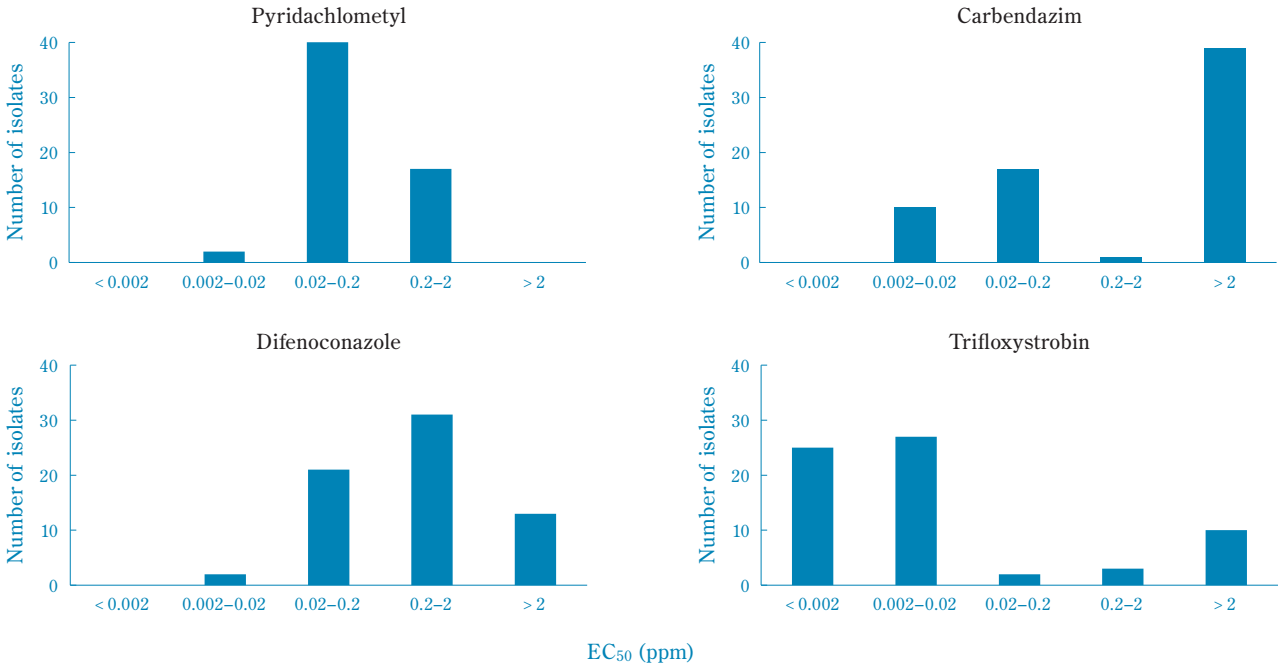

Fig. 4 Histograms of the EC_{50} values of *C. beticola* isolates for pyridachlometyl, carbendazim, difenoconazole, and trifloxystrobin

A total of 67 isolates obtained from sugar beet fields in Hokkaido were tested by microtiter plate assay and the corresponding EC_{50} values (ppm) were calculated for the 4 fungicides.

*3 Pot test of Cercospora leaf spot on sugar beet: Sugar beet seedlings were cultivated in plastic pots (500 mL) until four or five true leaves were unfolded, and then subjected to the experiment. For the chemical treatment, the test agents at specified concentrations were sprayed onto sugar beet leaves. For inoculation, a conidial suspension of *Cercospora beticola* (1×10^4 spores/mL) was prepared and sprayed onto sugar beet leaves.

Table 2 Fungicide sensitivity of *C. beticola* isolates obtained from Hokkaido fields

	EC_{50} (ppm)			
	Pyridachlometyl	Carbendazim	Difenoconazole	Trifloxystrobin
Strain 1	0.0291	0.0482	0.0217	0.0017
Strain 2	0.0398	1.362	0.0079	0.0007
Strain 3	0.0305	> 2.0	1.727	0.0017
Strain 4	0.0978	0.0885	0.0193	> 2.0

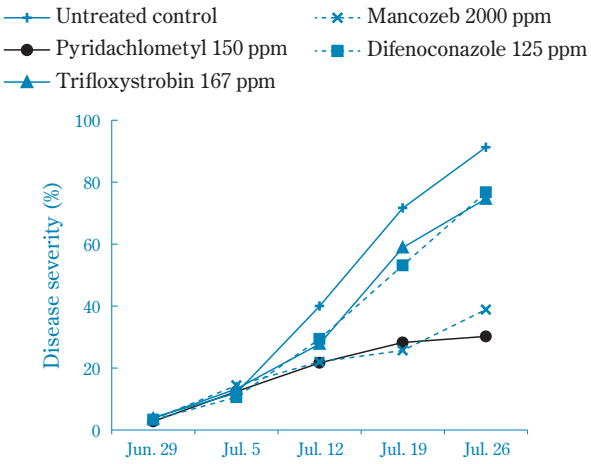

Fig. 5 Performance of pyridachlometyl against the cercospora leaf spot on sugar beet in a field in which the efficacy of difenoconazole and trifloxystrobin are drastically reduced
Treatment dates: 29th June and 7th and 16th July, Spray volume: 1000 L/ha

(2) Inhibitory effect on disease progression

Pot sugar beet seedlings infected with *Cercospora beticola* were treated with pyridachlometyl and disease progression in each plant was observed. Pyridachlometyl exhibited efficacies of 41 and 39% control at 200 and 133 ppm, respectively. The inhibitory effects of pyridachlometyl on disease progression were comparable to those of mancozeb, a 'contact fungicide' (whose primary action is a protective effect) (**Fig. 6**).

(3) Long-lasting efficacy

In a 17-day long-term activity test, pyridachlometyl at 200 and 133 ppm demonstrated efficacies of 86 and 79% control, respectively, against the sugar beet Cercospora leaf spot, which were high and comparable to those of mancozeb (**Fig. 7**).

(4) Rainfastness

Under a total of 100 mm (20 mm/h) of artificial rainfall treatment for 1 h after spraying, pyridachlometyl

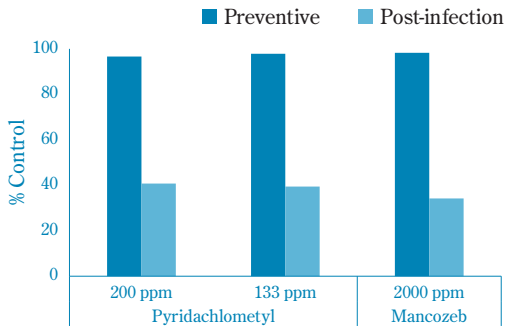

Fig. 6 Preventive and post-infection efficacy on sugar beet seedlings of pyridachlometyl against the cercospora leaf spot
Fungicides were applied 3 days before the *C. beticola* inoculation (preventive) or 2 days post inoculation (post-infection).
Disease severity in the untreated control: 67% (preventive) and 32% (post-infection).

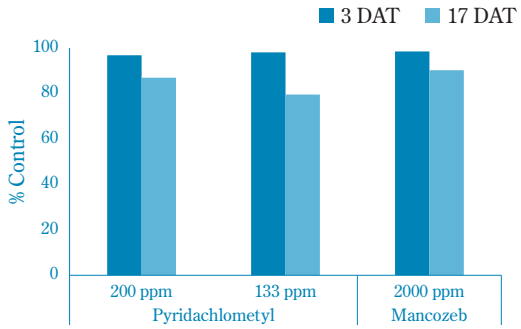

Fig. 7 Long-lasting efficacy of pyridachlometyl against the cercospora leaf spot
Inoculation: test plants were inoculated with conidial suspensions of *C. beticola* 3 days or 17 days after the fungicide treatment (DAT).

exhibited high efficacies of 98 and 88% control at 200 and 133 ppm, respectively, against the sugar beet Cercospora leaf spot, demonstrating higher efficacy than mancozeb (**Fig. 8**).

(5) Translaminar activity

In translaminar tests (wherein the plant was treated with the fungicide on the abaxial side of the leaf and

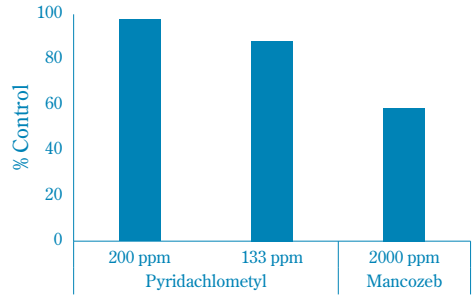

Fig. 8 Rainfastness of pyridachlometyl in the cercospora leaf spot control
Tested plants received artificial rainfall (100 mm/5 hours) 1 hour after the fungicide application. Inoculation: tested plants were inoculated with a conidial suspension of *C. beticola* 3 days after the artificial rainfall.

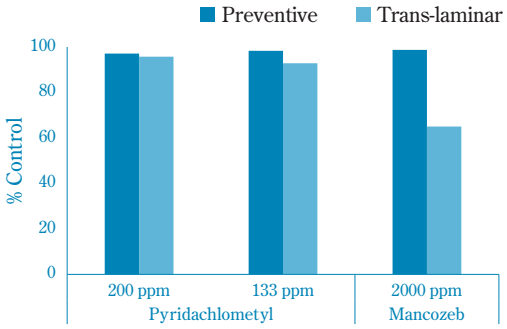

Fig. 9 Translaminar activity of pyridachlometyl against the cercospora leaf spot
Preventive treatment: tested plants were inoculated with a conidial suspension of *C. beticola* on the adaxial side of the leaves 3 days after the fungicide application on the same side.
Trans-laminar treatment: tested plants were inoculated on the adaxial side of the leaves 3 day after the fungicide application on the abaxial side.

then inoculated with the pathogen on the adaxial side), pyridachlorometyl exhibited efficacies of 95 and 92% at 200 and 133 ppm, respectively, against the sugar beet Cercospora leaf spot, thus demonstrating higher efficacy than mancozeb (**Fig. 9**). These results suggest that pyridachlometyl is absorbed into the treated leaf tissues and can translocate to untreated areas of the leaf.

(6) Conidiation-inhibitory action

Pyridachlometyl was applied to the immature lesions of the sugar beet Cercospora leaf spot before conidial formation, after which the number of conidia formed on the lesions was counted. The conidiation inhibitory rates of pyridachlometyl relative to those of the untreated control were 97% at 200 ppm and 92% at 133 ppm; and its inhibitory effect on conidiogenesis was higher than that of mancozeb (**Fig. 10**).

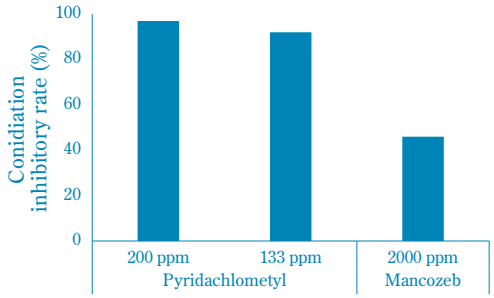

Fig. 10 Conidiation-inhibitory action of pyridachlometyl against *C. beticola* on the lesions
Fungicides were applied 11 days post inoculation. The number of conidia in the untreated control was 2426 conidia/cm^2 per lesions.

4. Field and greenhouse evaluation for practical use

(1) Field trials of sugar beet Cercospora leaf spot

In Japan, sugar beets are cultivated on approximately 55,000 ha of land in Hokkaido (as per FY2022, Ministry of Agriculture, Forestry, and Fisheries). The most significant disease affecting them is Cercospora leaf spot. This disease occur throughout the cultivation period and required approximately five fungicide applications per season. As mentioned previously, *Cercospora beticola* is becoming increasingly resistant to benzimidazole fungicides, Kasugamycin, DMI, and QoI, emphasizing the need for agents with novel modes of action. Pyridachlometyl demonstrated high efficacy in controlling sugar beet Cercospora leaf spot in field trials, suggesting its promising performance in controlling this disease in practical applications (**Fig. 11**).

(2) Field trials of soybean purple seed stain

Similar to sugar beet Cercospora leaf spot, resistance

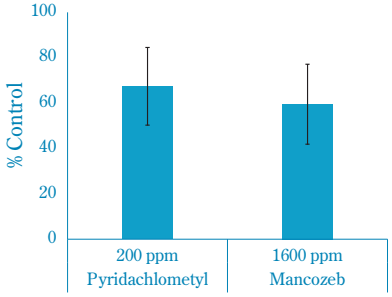

Fig. 11 Efficacy of pyridachlometyl against sugar beet cercospora leaf spot in field trials
A total of 7 field trials were conducted in 2016–2022 in Japan.
Bars represent the mean values; error bars represent the 95% CI

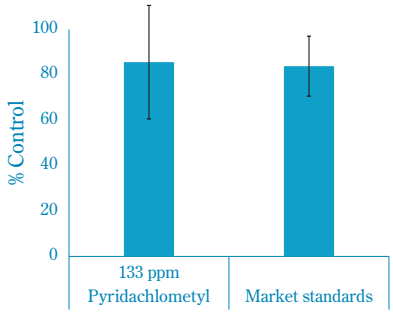

Fig. 12 Efficacy of pyridachlometyl against soybean purple stain in field trials
A total of 6 field trials were conducted in 2015–2016 in Japan.
Market standards: imibenconazole 100 ppm, 2 trials; azoxystrobin 100 ppm, 2 trials; thiophanate-methyl + diethofencarb 525 + 125 ppm, 2 trials
Bars represent the mean values; error bars represent the 95% CI

of *Cercospora kikuchii* to QoI fungicides has been detected and has become a problem in Japan in recent years[9)–11)]. *Cercospora kikuchii* causes purple lesions on the seed surface, thus significantly reducing grain quality and serving as a source of infection for the next generation through seed transmission. Therefore, effective therapeutic agents are needed to control this disease. Pyridachlometyl demonstrated high efficacy in controlling soybean purple stain in the field trials, suggesting its utility in controlling this disease (**Fig. 12**).

(3) Field trials of various powdery mildew

Powdery mildew pathogens infect various crops (including vegetables) and can cause significant damage. Moreover, strains of powdery mildew fungi resistant to various fungicides, including QoI and DMI, have been detected, thus indicating that powdery mildew is a pathogen with a high risk of developing resistance[12)–14)]. Pyridachlometyl is highly effective against powdery mildew in various crops. For example, greenhouse trials against powdery mildew on cucumbers, tomatoes, and strawberries confirmed the high effectiveness of pyridachlometyl (**Fig. 13**).

Mode of action

1. Phenotype of pyridachlometyl-treated *Zymoseptoria tritici*

The conidia (yeast-like cells) of *Zymoseptoria tritici* were treated with pyridachlometyl, and the phenotypic changes caused by pyridachlometyl during conidial germination and mycelial elongation were examined. Twenty-four hours post treatment, the conidia exhibited a characteristic phenotype, with swelling at both ends. This phenotype was similar to that observed when the conidia were treated with carbendazim and benomyl, which are known tubulin polymerization inhibitors, suggesting that the mode of action of pyridachlometyl plausibly affected microtubule dynamics.

2. Generation of laboratory-evolved fungicide-resistant mutants and the identification of mutations associated with fungicide resistance

The wild-type strain of *Zymoseptoria tritici* was used for ultraviolet mutagenesis and the screening of fungicide-resistant mutants. Fungicide-resistant mutants under pyridachlometyl or carbendazim treatment on potato dextrose agar (PDA) were selected. Using this process,

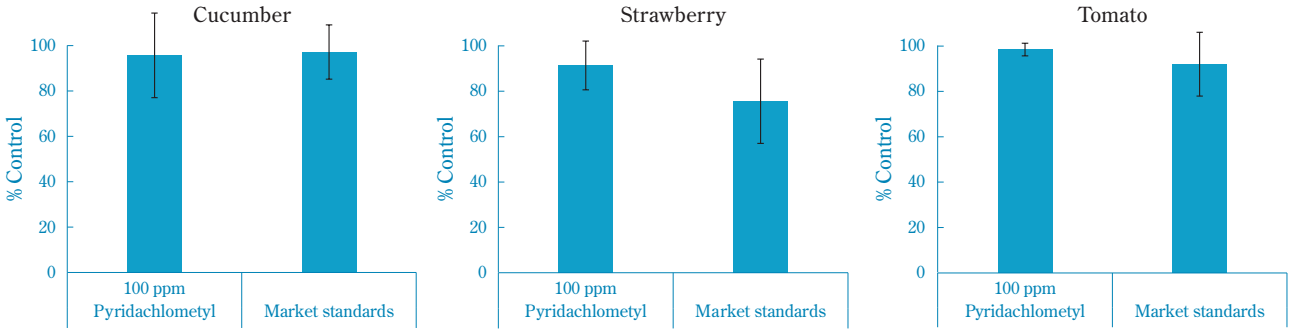

Fig. 13 Efficacy of pyridachlometyl against the powdery mildew of cucumber, strawberry, and tomato in field trials
A total of 7 field trials were conducted for each of the three crops in 2015–2016 in Japan.
Market standards: quinomethionate 125 ppm, 4 trials, and 83 ppm, 2 trials; chlorothalonil 400 ppm, 1 trial (cucumber only); triflumizole 100 ppm, 3 trials; DBEDC 400 ppm, 4 trials (strawberry only); penthiopyrad 100 ppm, 1 trial; DBEDC 400 ppm, 2 trials; potassium hydrogen carbonate 100 ppm, 4 trials (tomato only)
Bars represent the mean values; error bars represent the 95% CI

we selected several mutants resistant to each compound. Subsequent antifungal tests performed using the resistant mutants showed no cross-resistance between pyridachlometyl and carbendazim, indicating that the compounds belonged to different cross-resistance groups.

Tubulin gene sequence analysis of the resistant mutants revealed that all carbendazim-resistant strains harbored mutations in the β-tubulin gene (Q134K, E198A/D/E, F200Y). By contrast, in some pyridachlometyl-resistant strains, mutations in the β-tubulin gene were confirmed (N219K, Y222N/S); mutations in the α-tubulin gene (P325H/S/T) were observed in the remaining strains. Sequence analysis of these mutants and homology modeling and docking simulation analysis of the X-ray crystal structure of the tubulin heterocomplex indicated that the binding site of carbendazim was in the interior region of β-tubulin, whereas that of pyridachlometyl was located near the interface between α-tubulin and β-tubulin when they polymerize (**Fig. 14**). Furthermore, the Y222 mutation in β-tubulin, found in pyridachlometyl-resistant mutants, was involved in π–π stacking with the pyridazine ring and formed hydrogen bonds with the nitrogen atom of the pyridazine ring. Additionally, the P325 mutation in α-tubulin was thought to result in hydrophobic interactions with the pyridazine ring of pyridachlometyl and interactions with the fluorine atom on the 4-phenyl ring. We inferred that these amino acid residue mutations contributed to pyridachlometyl resistance (**Fig. 14**).

3. Binding assay using tubulin derived from *Zymoseptoria tritici*

To validate the mutation data and results of the docking simulation analysis, a competitive binding assay with the tubulin of *Zymoseptoria tritici* was conducted. Tubulin derived from *Zymoseptoria tritici* was treated with stable isotope-labeled carbendazim ([^{13}C, ^{2}H$_3$]-carbendazim) and detected using mass spectrometry. Subsequently, a competitive binding assay was performed using unlabeled carbendazim or pyridachlorometyl groups. Evidently, the amount of stable isotope-labelled carbendazim decreased considerably after carbendazim treatment, whereas the amount of stable isotope-labelled carbendazim remained the same pyridachlometyl treatment. No competition was observed between carbendazim and pyridachlorometyl. The data confirmed that the binding sites of pyridachlometyl and carbendazim in tubulin were different, which was consistent with the predictions from the aforementioned genetic analysis of resistant mutants and docking simulation analysis (manuscript in preparation).

Based on these results, we propose that pyridachlometyl binds to tubulin in phytopathogenic fungi and exerts its antifungal effects by altering tubulin dynamics. Furthermore, because pyridachlometyl binds to a site different from that of existing tubulin polymerization inhibitors, such as carbendazim, it has no cross-resistance with these compounds. This was demonstrated by cross-resistance tests using carbendazim-resistant strains isolated from the field. Considering its structure and binding site, pyridachlometyl may act as a tubulin

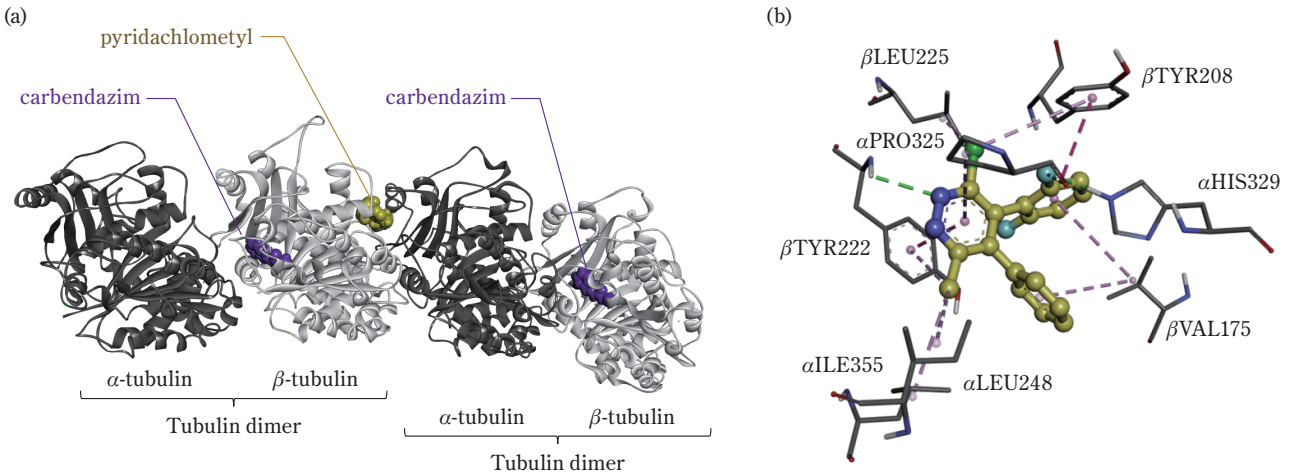

Fig. 14 Homology model of *Z. tritici* tubulin based on the X-rays of *Bos taurus*, *Sus barbatus*, and *Gallus gallus* (PDB, 5NJH, and 5C1A1) with the locations of substitutions leading to resistance against pyridachlometyl and carbendazim highlighted in yellow and violet, respectively
(a) Overall view of the tubulin dimer unit with putative binding sites of pyridachlometyl and carbendazim.
(b) Close-up view of the putative binding site of pyridachlometyl.

depolymerization inhibitor/polymerization promoter. However, further validation is required to confirm these hypotheses. At the time of preparation of this manuscript, pyridachlometyl is registered under the Fungicide Resistance Action Committee (FRAC) code list as a tubulin dynamics modulator (FRAC code 53)[15].

Formulation

A formulation containing pyridachlometyl is currently under development in the Japanese market, with the intent of registering it as a pesticide under the name FUSEKI Flowable (35% [w/w] pyridachlometyl suspension concentrate (SC)) in 2023. This formulation mainly targets sugar beet and soybean; however, the development of mixtures for fruit, vegetable crops, and wheat is also underway.

We refer to the FUSEKI Flowable as the representative formulation. While developing the formulation of this product, we referred to expertise gained in formulation design and optimized the blending ratio and quantity of the excipients. Therefore, we established a formulation with excellent efficacy, low phytotoxicity, user-friendly handling, and safety. In terms of user-handling, this formulation exhibited excellent dispersibility when added to water during dilution (**Fig. 15**). Furthermore, similar to conventional flowable formulations, this formulation was designed to effectively suppress foaming and ensure easy discharge from the container.

Fig. 15 Dispersion behavior of FUSEKI flowable in water

Toxicity, metabolism, and residue

1. Mammalian toxicity

(1) Acute toxicity, irritability, and skin sensitization

The lethal dose (LD_{50}) values of both pyridachlometyl technical grade (TG) and 35% SC exceeded 2,000 mg/kg body weight in rats for oral administration and dermal application. No clinical signs were observed even after inhalation exposure to pyridachlometyl TG, and the lethal concentration (LC_{50}) value exceeded 5,450 mg/m^3. The skin-irritation potential of pyridachlometyl TG was minimal. Furthermore, the eye irritation potential of pyridachlometyl was minimal and reduced by washing. Moreover, 35% SC did not cause irritation to the skin or eyes. Regarding skin sensitization, pyridachlometyl-TG showed positive results in the maximization test, whereas 35% SC showed negative results in the Buehler test (**Table 3**).

(2) Subacute toxicity, chronic toxicity, and carcinogenicity

In subacute, chronic, and carcinogenicity studies using rats, dogs, and mice, repeated administration of pyridachlometyl TG resulted in decreased body weight gain and food consumption, with toxic effects mainly observed in the liver and thyroid. In the liver and thyroid, an increase in liver weight and hepatocyte hypertrophy were observed along with thyroid follicular cell hypertrophy.

In carcinogenicity studies, increased incidences of thyroid follicular cell adenomas/carcinomas in rats and hepatocellular adenomas/carcinomas in mice were observed. Further investigation of the carcinogenic mechanism of action of pyridachlometyl in the liver and thyroid indicated that its effects on the liver were caused by the induction of drug-metabolizing enzymes mediated by nuclear receptors, similar to phenobarbital. The

Table 3 Acute toxicity results for pyridachlometyl

Test type	Pyridachlometyl	Pyridachlometyl 35%SC
Rat acute oral (LD_{50})	> 2000 mg/kg	> 2000 mg/kg
Rat acute dermal (LD_{50})	> 2000 mg/kg	> 2000 mg/kg
Rat inhalation (LC_{50})	> 5450 mg/m^3 of air(4h, nose only exposure)	–
Eye irritation (Rabbit)	Minimally irritant	Non-irritant
Skin irritation (Rabbit)	Minimally irritant	Non-irritant
Skin sensitization (Guinea pig)	Sensitizer	Non-sensitizer

Table 4 Subacute and chronic toxicity results for pyridachlometyl

Species	Administration route and duration	Dose	NOAEL (mg/kg/d)
Dog	Oral (in capsule), 13 weeks	100, 300, 1000 mg/kg/d	Male: 100 Female: 100
Dog	Oral (in capsule), 12 months	10, 50, 300 mg/kg/d	Male: 10 Female: 10
Rat	Oral (in diet), 13 weeks	1000, 5000, 20000 ppm	Male: 291 (5000 ppm) Female: 351 (5000 ppm)
Rat	Oral (in diet), 24 months	200, 2000, 10000, 20000 ppm	Male: 8 (200 ppm) Female: 10 (200 ppm) No carcinogenicity
Mouse	Oral (in diet), 13 weeks	1500, 3500, 7000 ppm	Male: 517 (3500 ppm) Female: 650 (3500 ppm)
Mouse	Oral (in diet), 18 months	700, 2000, 7000 ppm	Male: 83 (700 ppm) Female: 317 (2000 ppm) No carcinogenicity

effects on the thyroid were attributed to a mechanism of action secondary to hepatic enzyme induction, rather than direct effects on the synthesis of thyroid hormones. Based on the known information on drugs with the same mechanism of action, it is considered that these tumor findings in rodents have no human relevance and thus do not indicate carcinogenicity in humans. (**Table 4**).

(3) Reproductive and developmental toxicities

In developmental toxicity (teratogenicity) tests using rats and rabbits, no teratogenicity was observed in fetuses. In the two-generation reproductive toxicity test using rats, the reproductive performance and nursing behavior were not affected (**Table 5**).

(4) Neurotoxicity

No evidence of neurotoxicity was observed in acute, subacute, and chronic toxicity or carcinogenicity tests using pyridachlometyl TG.

(5) Genotoxicity

The result of a reverse mutation test using *Salmonella typhimurium* and *Escherichia coli* for pyridachlometyl TG was negative. Although pyridachlometyl TG showed a positive effect in the chromosome aberration test using Chinese hamster lung-derived CHL/IU cells, a negative result was obtained in the mouse micronucleus test (**Table 6**).

2. Animal/plant metabolism

(1) Metabolism in animals

When ^{14}C-labeled pyridachlometyl was orally administered to rats, it was rapidly absorbed and distributed throughout the body. Subsequently, it was quickly metabolized and primarily excreted in the feces. The oral

Table 5 Developmental and reproductive toxicity results for pyridachlometyl

Study	Species	Administration route and duration	Dose	NOAEL (mg/kg/d)	
Developmental toxicity	Rat	Oral (gavage) Days 6–19 of gestation	250, 500, 1000 mg/kg/d	Maternal Fetal	1000 1000
	Rabbit	Oral (gavage) Days 6–28 of gestation	250, 500, 1000 mg/kg/d	Maternal Fetal	250 1000
Two-generation reproductive toxicity	Rat	Oral (in diet)	600, 4000, 20000 ppm	Parental Offspring Reproductive	Male: 218 (4000 ppm) Female: 329 (4000 ppm) Male: 267 (4000 ppm) Female: 362 (4000 ppm) Male: 1145 (20000 ppm) Female: 1697 (20000 ppm)

Table 6 Mutagenicity results for pyridachlometyl

Study	Study design	Results
Reverse mutation (Ames test)	*S. typhimurium*: TA98, TA100, TA1535 and TA1537 *E. coli*: WP2*uvrA* −/+S9 mix: 156–5000 μg/plate	Negative
In vitro chromosomal aberration	Chinese hamster CHL/IU −S9 mix (6 h): 0.938–7.50 μg/mL +S9 mix (6 h): 30.0–50.0 μg/mL	Positive
Bone marrow micronucleus	CD-1 mice 500, 1000, 2000 mg/kg	Negative

absorption rate was estimated to be 90% or higher, with no residue or tissue accumulation. The main metabolic reactions of pyridachlometyl involved the hydroxylation of the methyl group at the sixth position of the pyridazine ring, followed by the formation of carboxylic acid derivatives or glucuronide conjugates of the hydroxyl group, substitution of chloro groups with glutathione, and further metabolism to yield cysteine conjugates, thiols, or mercapturic acid conjugates.

(2) Metabolism in plants

The metabolism of ^{14}C-labeled pyridachlometyl was investigated in three different types of crops (wheat, tomato, and sugar beet). In all of these crops, the metabolic pathways were similar, involving the hydroxylation of the methyl group at the sixth position of the pyridazine ring, followed by conjugation.

3. Environmental behavior and residues

(1) Degradation in water

In the hydrolysis study, ^{14}C-labeled pyridachlometyl was stable in buffer solutions at pH 4, 7, and 9, with an estimated half-life of over one year (at 25 °C). Under light exposure, the photodegradation half-life of pyridachlometyl in buffer solution (pH 7) ranged from 47.6 to 52.7 days (equivalent to the natural sunlight at Tokyo in spring).

(2) Metabolism in soil

In aerobic soil metabolism studies, the degradation half-life of ^{14}C-labeled pyridachlometyl ranged from 273 to 1,450 days (at 20 °C). The metabolic pathway involved the hydroxylation of the methyl group at the sixth position of the pyridazine ring, followed by the formation of carboxylic acids, which were finally bound to the soil matrix or mineralized to carbon dioxide. On the soil surface, the degradation half-life (20 °C) was 133 days in the irradiation conditions and 333 days in the dark control.

(3) Residue in soil

Terrestrial field dissipation studies using a single application of a 1,000-fold diluted solution of pyridachlometyl 35% SC at a rate of 300 L/10 a in the fields in Ibaraki and Kochi demonstrated that the dissipation half-life was estimated to be 33.6–48.8 days, with the maximum residual concentration at 0.65–1.14 mg/kg.

(4) Mobility in soil

The adsorption coefficient ($K_{Foc(ads)}$) and desorption coefficient ($K_{Foc(des)}$) of pyridachlometyl corrected for the organic carbon content were calculated using the Freundlich adsorption isotherm and were 1,521–3,446 and 1,811–5,364, respectively.

(5) Residue in crops

Residue trials for wheat and barley were conducted using two applications of a 2,000-fold diluted solution of pyridachlometyl 35% SC at a rate of 100–139 L/10 a, and the residues in both crops were less than the limit of quantification (<0.01 mg/kg). The same diluted formulation was applied to sugar beets three times with a 7-days interval at a rate of 180–200 L/10 a, and the residues were less than the limit of quantification (<0.01 mg/kg). Residue trials for soybeans were conducted using three applications of a 3,000-fold diluted solution of pyridachlometyl 35% SC with a 7-days interval at a rate of 177–192 L/10 a, and the maximum residue was 0.09 mg/kg. The residue levels for cherry tomatoes, bell peppers, eggplants, cucumbers, watermelons, cantaloupes, and strawberries after four applications of the same diluted formulation with a 7-days interval at rates of 153–300 L/10 a ranged from less than the limit of quantification (<0.01 mg/kg) to 1.64 mg/kg.

Table 7 Ecotoxicological results for pyridachlometyl on non-target organisms

Test substance	Test species		Test type	Results
Pyridachlometyl	Aquatic organisms	*Cyprinus carpio*	Acute (96 h)	LC_{50} > 0.70 mg/L
		Daphnia magna	Acute (48 h)	EC_{50} = 0.50 mg/L
		Green alga*1	Acute (72 h)	ErC_{50} > 0.68 mg/L
	Honeybee	*Apis mellifera*	Acute oral (48 h)	LD_{50} > 23.8 µg/bee
		Apis mellifera	Acute contact (48 h)	LD_{50} > 100 µg/bee
	Bird	*Colinus virginianus*	Acute oral	LD_{50} > 2000 mg/kg
Pyridachlometyl 35%SC	Aquatic organisms	*Cyprinus carpio*	Acute (96 h)	LC_{50} = 68 mg/L
		Daphnia magna	Acute (48 h)	EC_{50} = 0.57 mg/L
		Green alga*1	Acute (72 h)	ErC_{50} = 47 mg/L

*1: *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*)

4. Effects on non-target organisms

The test results for the aquatic organisms, honeybees, and birds are summarized in **Table 7**.

(1) Effects on aquatic organisms

The acute toxicity values (LC_{50}/EC_{50}/ErC_{50}) of pyridachlometyl TG in carp, Daphnia magna, and freshwater green algae were >0.70, 0.50, and >0.68 mg/L, respectively. The acute toxicity values (LC_{50}/EC_{50}/ErC_{50}) of pyridachlometyl 35% SC in carp, Daphnia magna, and freshwater green algae were 68, 0.57, and 47 mg/L, respectively. These values were significantly higher than the environmental concentrations predicted from their intended use, indicating that the effects of pyridachlometyl on aquatic organisms were minimal.

(2) Effects on honeybees

The acute toxicity of pyridachlometyl TG in western honeybees was low in both oral and contact exposure, with LD_{50} values of >23.8 and >100 µg/bee, respectively. Therefore, the effects of pyridachlometyl on honeybees from their intended use were considered insignificant.

(3) Effects on birds

The acute toxicity of pyridachlometyl TG to bobwhite quails was low, with an oral LD_{50} value of >2,000 mg/kg. Therefore, the effects of pyridachlometyl on birds due to its intended use were considered insignificant.

Based on the above results, both pyridachlometyl TG and pyridachlometyl 35% SC exhibited extremely weak acute toxicity in mammals and were expected to have no adverse effects, such as carcinogenicity, mutagenicity, or reproductive toxicity, on the next generation, even with prolonged exposure. Furthermore, considering the behavior of pyridachlometyl in the environment and its impact on non-target organisms, it will not cause negative effects on the environment when used in accordance with the registered methods.

Conclusion

Pyridachlometyl has a broad spectrum of fungal control, low risks of phytotoxicity and toxicity toward mammals, and minimal impact on non-target organisms, such as bees, and is expected to be used in various crop cultivation scenarios. As a new fungicide with a novel mode of action, it is also effective against existing resistant strains. Thus, it is a valuable tool for managing the resistance risks associated with various modes of action, particularly in cases where fungicide options are limited owing to the spread of resistant strains. To delay the development of resistance and maximize the utility of pyridachlometyl, we recommend avoiding its sequential use and promoting its use in fungicide rotation by combining it with other fungicides with different modes of action.

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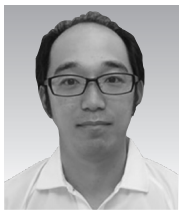

Makoto Kurahashi

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Senior Research Associate

Fukumatsu Iwahashi

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Senior Research Associate, Ph. D.

Yuichi Matsuzaki

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Senior Research Associate, Ph. D.

Mitsuyoshi Yamane

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Researcher

Akio Manabe

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Senior Research Associate, Ph. D.
(Retired by the publication of this article)

Ryoko Matsuyama

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Senior Research Associate

Masato Kawamura

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Senior Research Associate, Ph. D.

Yuko Handa

Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Senior Research Associate