Discovery and Development of a Novel Fungicide, Metyltetraprole

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Yuichi Matsuzaki
Yuya Yoshimoto*
Sadayuki Arimori
Fukumatsu Iwahashi
Makoto Kurahashi
Tatsuya Toriumi
Risa Ito
Environmental Health Science Laboratory
Miwa Kondo
Miho Tabuchi

Metyltetraprole is a novel fungicide developed by Sumitomo Chemical and belongs to the QoI group of fungicides, which target the Qo site of complex III ubiquinol oxidase enzyme in fungal cells. QoI fungicides have been widely used in various crops since the late 1990s for their broad spectrum of activity. However, the spread of QoI-resistant fungal strains has decreased their effectiveness. By contrast, metyltetraprole, despite being a QoI, exhibits equivalent efficacy against both susceptible and QoI-resistant strains because of its unique chemical structure. Therefore, metyltetraprole can manage various diseases in important crops such as wheat, barley, soybean, cotton, and sugar beet. It also has favorable characteristics such as long-lasting activity and rainfastness. It is highly safe for humans, animals, and the environment. The product, called Muketsu DX in Japan, was released in 2023 and is being worked on in Europe and South America.

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Introduction

Modern intensive agriculture has contributed to a stable food supply, which in turn has led to peace through improved health and political and economic stability. The use of pesticides is one of the main means of ensuring high yields in such agriculture[1]. However, pesticides have been criticized for their potential to cause health and environmental damage. Therefore, researchers have sought to invent pesticides that are safe for humans, livestock, and non-target organisms and do not persist in the environment. The synthetic pesticides produced from these efforts are generally effective at significantly lower doses than arsenic, mercury, and other pesticides used in the past and safe based on scientific toxicity evaluation tests[2].

Thus, these efforts have led to the development of pesticides safe for humans, livestock, and non-target organisms, with biological and chemical properties that allow for their decomposition and detoxification in the environment. However, this has provided room for target organisms to acquire resistance to pesticides through genetic mutations.

The recent spread of insects, plant pathogens, and weeds that have acquired resistance has become a major problem in pesticide use[2]. Although pesticides have become safer, they are not risk-free. There are no substances with zero risk in terms of toxicology, whether synthetic or natural; thus, the use of all pesticides involves some risk, even if small[3]. The manufacturing and application of pesticides also use fossil fuels and water, contributing to greenhouse gas emissions and the consumption of precious water resources. Therefore, regardless of whether they are chemically synthesized or naturally derived, pesticides must be shown to have high efficacy and provide societal benefits that outweigh their disadvantages. Maintaining the safety of pesticides at a sufficiently high level while addressing resistance issues and maintaining their effectiveness is a new challenge for pesticide researchers[2].

* Current affiliation: Misawa Works

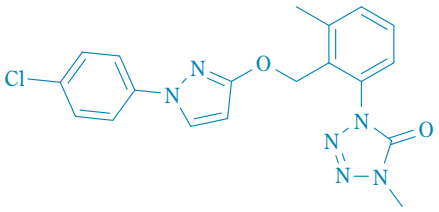


Fig. 1 Chemical structure of metyltetraprole

Given this background, we have developed the novel fungicide metyltetraprole, described in this paper (**Fig. 1**)[4]. Metyltetraprole is classified as a QoI fungicide (ubiquinol oxidase Qo site inhibitor, also known as a strobilurin fungicide), a major fungicide type, but it is also effective against plant pathogens that have acquired resistance to existing QoI fungicides[4]. Metyltetraprole is being developed in many countries under the trademark name "Pavecto," and in Japan, a mixture with mancozeb (non-QoI fungicide) named "Muketsu DX" was launched in 2023 ahead of other countries.

In this paper, we report on the discovery of metyltetraprole, its manufacturing process, mode of action, biological effects, formulations, and safety to mammals and the environment.

Discovery

Fig. 2 summarizes the discovery of metyltetraprole by tracing the transformation of its chemical structure. QoI fungicides, launched in the late 1990s, are versatile fungicides with a broad fungicidal spectrum, having global sales in 2021 reaching $3 billion (approximately 440 billion yen) per year[5),6)]. However, QoI-resistant fungi with reduced sensitivity have become widespread, reducing the QoI efficacy. The emergence and spread of QoI-resistant fungi occur in many crop pathogens, typically caused by a mutation where the 143rd amino acid of the cytochrome *b* protein of the target enzyme, the electron transport complex III (ubiquinol oxidase), is changed from glycine (G) to alanine (A) (G143A-type resistance)[5]. This amino acid substitution causes steric hindrance for existing QoI fungicides, preventing their binding to cytochrome *b* and reducing the activity of the QoI fungicide to less than one-hundredth (**Fig. 3**, **Table 1**). Therefore, we began exploratory research aimed at discovering new QoI fungicides unaffected by G143A resistance[4),7)].

First, we sought seed compounds as a starting point for drug discovery by selecting approximately 200 compounds with various QoI-like pharmacophores (partial structures essential for exhibiting pharmacological activity) that may avoid the steric hindrance from our compound collection while also referring to computer simulation results.

We screened compounds using an antifungal activity test against the G143A-harboring strain of *Zymoseptoria tritici*, and the results showed that compound 1, which has a tetrazolinone ring, had equal antifungal activity against sensitive wild-type and G143A-harboring resistant strains (**Fig. 2**, **Table 1**). Further investigation showed that a group of analogs with a tetrazolinone ring-like compound 1 had a resistance factor, representing the ratio of activity against G143A-harboring and susceptible strains, of generally less than 2 (data not shown). In other words, we found that QoI-like compounds with a tetrazolinone ring as a pharmacophore had almost equal antifungal activity against sensitive wild-type and G143A-harboring resistant strains.

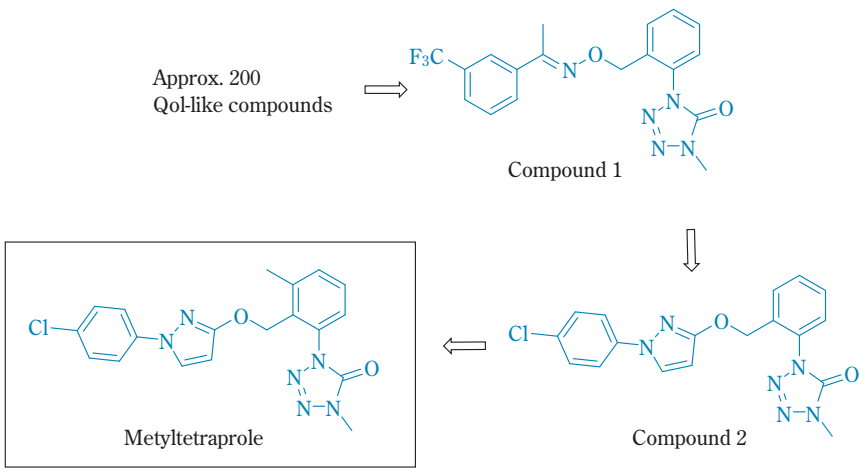


Fig. 2 Structural modifications that led to the discovery of metyltetraprole

Table 1 Activity against *Zymoseptoria tritici* wildtype and G143A-harboring strains

Compound	Sensitive wild-type strain EC_{50} mg/L	G143A-harboring strain EC_{50} mg/L	Resistance factor[a]
Compound 1	0.2	0.4	2.0
Compound 2	0.02	0.02	1.0
Metyltetraprole	0.002	0.002	1.0
Azoxystrobin (reference QoI)	0.02	> 10	> 500
Pyraclostrobin (reference QoI)	0.001	0.2	200

a: Resistance factor, EC_{50} [G143A-harboring strain]/EC_{50} [sensitive wild-type strain]

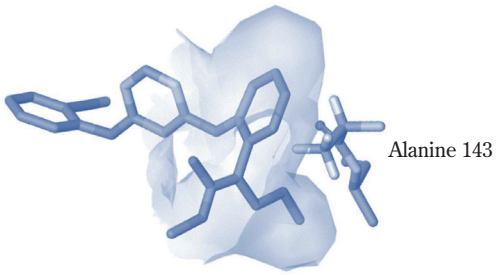


Fig. 3 Steric hindrance between a conventional QoI fungicide (azoxystrobin) and G143A-harboring cytochrome *b*

Therefore, we used compound 1 as a seed compound, and subsequent structural optimization was based on empirical knowledge accumulated at the Health & Crop Sciences Research Laboratory of Sumitomo Chemical. Then, we modified the side chain structure of compound 1 to improve its antifungal activity. Previous QoI fungicide research showed that the side chain was crucial for enhancing activity[5], and we extensively converted it into various chemical structures. As a result, we developed compound 2, which has a phenylpyrazole structure and approximately 10-fold improvement in activity (**Fig. 2**, **Table 1**)[4]. Additionally, modifying the substituents on the central benzene ring showed that introducing a substituent to the 3-position was effective in improving activity[4]. Compounds with various substituents introduced to the 3-position of the central benzene ring were synthesized and evaluated, and as a result, we selected metyltetraprole as a candidate for development because of its high efficacy and favorable safety data for beneficial organisms and the environment (**Fig. 2**, **Table 1**)[4),7)].

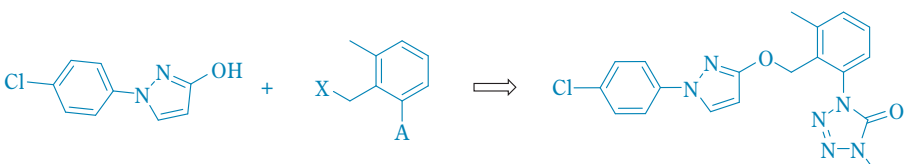
Manufacturing process

Metyltetraprole is a benzene derivative with a pyrazole and tetrazolinone skeleton. The key to developing the manufacturing process was constructing a benzene ring bearing three consecutive functional groups and coupling the pyrazole and tetrazolinone units. Through extensive research, various regioselective reactions were developed to achieve efficient skeleton construction and coupling of the units, leading to the establishment of an industrial manufacturing process (**Fig. 4**).

Mode of action

As described in the "Discovery" section, metyltetraprole was discovered as a QoI fungicide capable of controlling G143A-harboring fungi, and its mode of action, like other QoI fungicides, is the inhibition of the complex III of mitochondrial electron transport chain.

In general, when a compound is structurally modified for a target protein with a specific amino acid substitution, there are concerns that changes in the binding mode associated with a structural change in the compound binding pocket will reduce the binding affinity to the target protein before the substitution and that there may be cases of negative cross-resistance, such



A: NO_2, tetrazolinone, *etc.*
X: Halogen, *etc.*

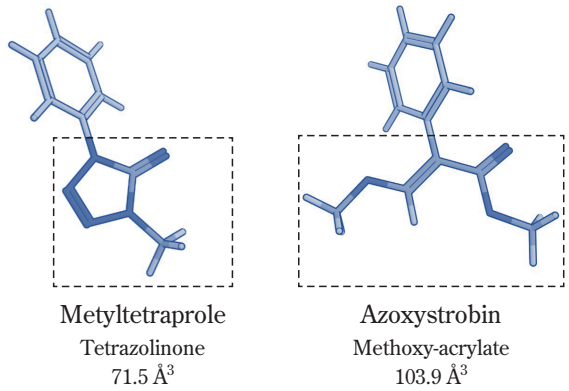
Metyltetraprole

Fig. 4 Synthetic route of metyltetraprole

Table 2 Inhibitory activities of metyltetraprole, azoxystrobin, and pyraclostrobin against mitochondrial electron transport chain of *Z. tritici*

	Metyltetraprole		Azoxystrobin		Pyraclostrobin	
Cytochrome *b* Substitution	IC_{50}[a]	Resistance Factor[b]	IC_{50}[a]	Resistance Factor[b]	IC_{50}[a]	Resistance Factor[b]
Wild-type	0.00025	–	0.0080	–	0.00026	–
G143A	0.00042	1.7	1.9	240	0.20	770

a: Mean of three independent IC_{50} values (mg/L).
b: Resistance factor is the ratio of the IC_{50} of the G143A-harboring QoI-resistant strain to that of the wild-type strain.



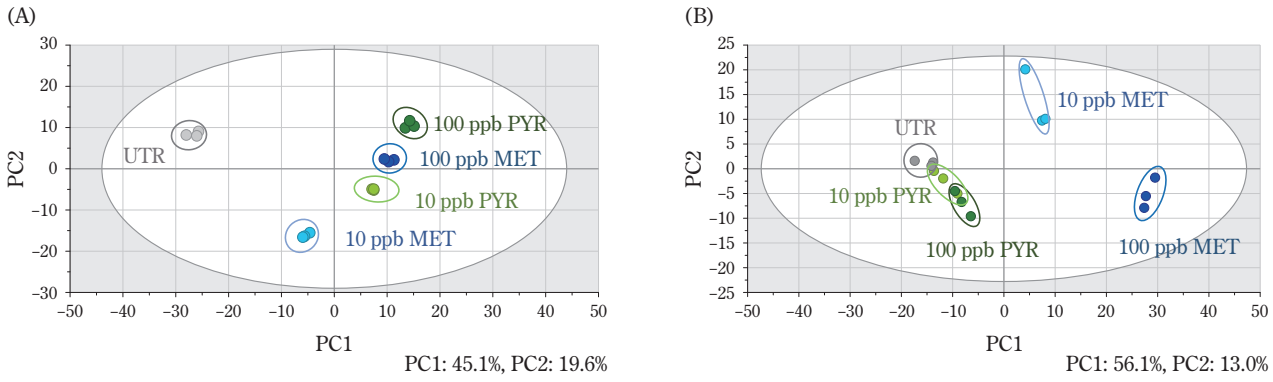
The 3D conformations of simplified partial structures are presented. The central linking rings are subtracted for volume calculation.

Fig. 5 The volume of each pharmacophore

as between benzimidazole fungicides and diethofencarb[8),9)]. However, electron transport chain inhibition tests using mitochondrial crude fractions derived from QoI-susceptible wild-type and G143A-harboring resistant *Z. tritici* confirmed that metyltetraprole had almost the same inhibitory activity at the site of action against both target enzymes (**Table 2**). The main reason for this phenomenon could be attributed to the pharmacophore of this compound, which is the tetrazolinone ring. In other words, the calculated size of the methoxyacrylate structure of the pharmacophore of a representative QoI fungicide is 103.9 Å^3, whereas the calculated size of the smaller tetrazolinone structure is 71.5 Å^3. Conventional QoI fungicides exhibit a significant decrease in activity when the drug binding site is sterically restricted by the substitution of glycine to alanine at the 143rd amino acid of cytochrome *b*. However, metyltetraprole is thought to maintain its binding mode regardless of the G143A substitution because of its compact pharmacophore structure (**Fig. 5**)[4)].

QoI fungicides are believed to bind to the Qo site of the mitochondrial electron transport complex III, inhibiting electron transport between cytochrome bc_1, thereby suppressing ATP production and exerting antifungal activity[10)]. Evaluating the pathways from the manipulation of the target molecule to final phenotypes, such as growth suppression and cell death, requires a comprehensive approach to intracellular metabolic pathways, such as metabolomics.

Metabolome analysis of *Z. tritici* using capillary electrophoresis-mass spectrometry and multivariate analysis (principal component analysis) resulted in



Z. tritici samples were treated with two different concentrations of metyltetraprole or pyraclostrobin, respectively (n = 3). The hydrophilic extract from *Z. tritici* was analyzed by capillary electrophoresis-mass spectrometry, and a total of 573 peaks (286 cations and 287 anions) were detected. (A) PCA score plot for QoI-sensitive strain, (B) PCA score plot for G143A-harboring strain. MET: metyltetraprole, PYR: pyraclostrobin, UTC: untreated control.

Fig. 6 Principal component analysis (PCA) of the targeted metabolomics of *Z. tritici*

a model where compound efficacy was reflected in the first principal component (PC1). Application of the compounds to QoI-susceptible wild-type strains showed that metyltetraprole and pyraclostrobin treatments were plotted in a concentration-dependent manner against PC1. However, this trend was only maintained for metyltetraprole when applied to G143A-harboring resistant strains (**Fig. 6**). These results suggest that metyltetraprole inhibits the mitochondrial electron transport chain complex III, resulting in antifungal activity through the suppression of intracellular energy metabolism in both QoI-susceptible wild-type and G143A-harboring resistant strains. Consequently, metyltetraprole is registered as a "Quinone outside inhibitors subgroup A (FRAC group code 11A)" in the Fungicide Resistance Action Committee (FRAC) code list as an effective QoI fungicide against G143A-harboring fungi[11].

Biological effects

1. Antifungal spectrum

Metyltetraprole exhibited a broad antifungal spectrum, mainly against Ascomycota (**Table 3**).

The same method used to detect antifungal activity in conventional QoI fungicides was effective for metyltetraprole. In other words, the antifungal activity of metyltetraprole could be detected by inhibiting alternative oxidase, which acts as a bypass pathway for ubiquinol oxidase in nutrient-rich conditions, with iron-chelating compounds such as salicylhydroxamic acid or by using a nutrient-poor medium or liquid medium[12),13)].

2. Characteristics of action

We conducted *in planta* tests using potted wheat plants to investigate the efficacy and characteristics of metyltetraprole against septoria leaf blotch. G143A-harboring resistant strains were used in all tests as the inoculum source. Prothioconazole, a non-QoI fungicide widely used for wheat in Europe after the spread of QoI resistance, served as the control fungicide.

(1) Preventative efficacy

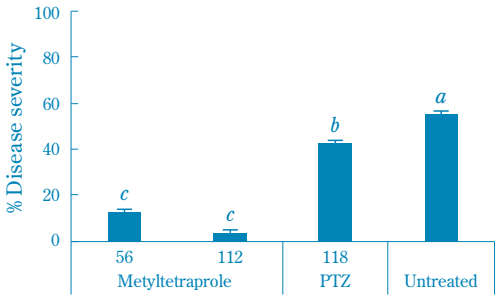
We sprayed wheat plants with metyltetraprole, grew them outdoors (Takarazuka City, mid-April) for 14 days, and then conducted artificial inoculation. We investigated the disease severity of the metyltetraprole-sprayed wheat after waiting for symptoms to appear in the untreated control plant. The results showed that the disease was significantly more suppressed in the metyltetraprole-sprayed wheat than in the control prothioconazole-treated wheat (**Fig. 7**). Metyltetraprole was found to be highly effective in preventative treatment with sufficient long-lasting efficacy even under ultraviolet exposure.

(2) Post-infection efficacy

Metyltetraprole was sprayed 7 days after the artificial inoculation of wheat (*i.e.*, when the wheat was infected with *Z. tritici* and left to progress). We investigated the disease severity in the metyltetraprole-sprayed wheat after waiting for symptoms to appear in the untreated control plant. The results indicated that the disease was significantly more suppressed in the metyltetraprole-sprayed wheat than in the

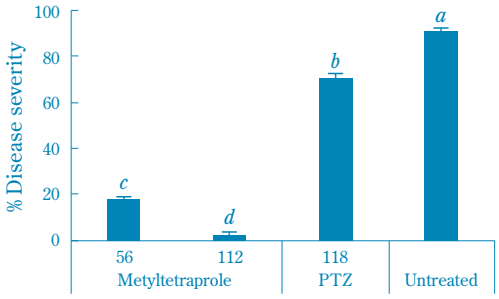
Table 3 Antifungal spectrum of metyltetraprole

Division	Class	Species	Host crop	Disease	EC_{50} (mg/L)
Ascomycota	*Dothideomycetes*	*Ramularia collo-cygni*	Barley	Ramularia leaf spot	0.0020
		Ramulariopsis pseudoglycines	Cotton	Gray mildew	0.0057
		Cercospora beticola	Sugar beet	Leaf spot	0.0016
		Cercospora kikuchii	Soybean	Leaf blight	0.0020
		Pyrenophora teres	Barley	Net blotch	0.0048
		Pyrenophora tritici-repentis	Wheat	Tan spot	0.054
		Parastagonospora nodorum	Wheat	Glume blotch	0.0025
		Corynespora cassiicola	Soybean/Cotton	Target spot	0.013
	Leotiomycetes	*Rhynchosporium commune*	Barley	Scald	0.034
		Botrytis cinerea	Grape/Vegetables	Gray mold	0.026
	Sordariomycetes	*Colletotrichum graminicola*	Corn (Maize)	Anthracnose	0.0068
		Microdochium nivale	Wheat	Snow mold/Head blight	0.0047
Basidiomycota	*Agaricomycetes*	*Rhizoctonia solani* AG4	Various crops	Root rot	2.2
	Ustilaginomycetes	*Ustilago maydis*	Corn (Maize)	Smut	0.040



Fungicides were applied 14 d before inoculation. Water volume was 200 L/ha. Test plants were placed under direct sunlight after application (openair condition). Dose rates of treatments are represented as g/ha. Error bars represent standard errors. Different lower-case letters shown above the bars represent statistical differences (Tukey-kramer, P < 0.05). Artificial inoculation was made with G143A-harboring strain. PTZ, prothioconazole (reference)

Fig. 7 Preventive efficacy against *Z. tritici* on wheat



Fungicides were applied seven d after inoculation. Water volume was 200 L/ha. Artificial inoculation was made with G143A-harboring strain. Dose rates of treatments are represented as g/ha. Error bars represent standard errors. Different lower-case letters shown above the bars represent statistical differences (Tukey-kramer, P < 0.05). PTZ, prothioconazole (reference)

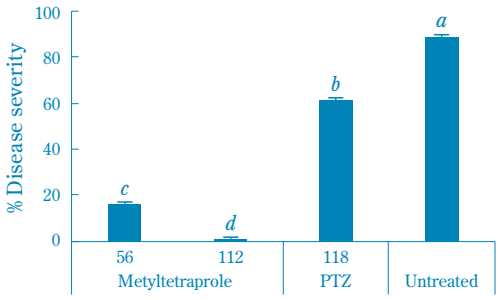
Fig. 8 Post-infection efficacy against *Z. tritici* on wheat

control prothioconazole-treated wheat (**Fig. 8**). Metyltetraprole inhibited pathogen progress and suppressed the disease when applied at early infection stages.

(3) Moisture tolerance and rainfastness

Metyltetraprole was sprayed on wheat, and then the wheat was moved into a mist chamber.

The chamber was humidified for 6 h daily for 14 days until artificial inoculation, and artificial rainfall of 10 mm/h was applied for 3 h for 4 days after spraying. We investigated the disease severity in the metyltetraprole-sprayed wheat after waiting for symptoms to appear in the untreated control plant. The



Fungicides were applied 14 d before inoculation. Water volume was 200 L/ha. Test plants were kept in damp chamber for 6 h/d. Artificial rainfall (10 mm × 3 h) was conducted 4 d after fungicide application. Artificial inoculation was made with G143A-harboring strain. Dose rates of treatments are represented as g/ha. Error bars represent standard errors. Different lower-case letters shown above the bars represent statistical differences (Tukey-kramer, P < 0.05). PTZ, prothioconazole (reference)

Fig. 9 Rainfastness in controlling *Z. tritici* on wheat

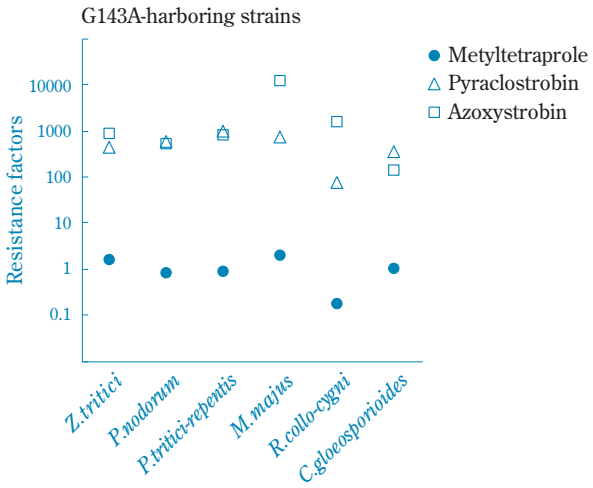
results indicated that the disease was significantly more suppressed in the metyltetraprole-sprayed wheat than in the control prothioconazole-treated wheat (**Fig. 9**). Metyltetraprole exhibited sufficient long-lasting activity even under highly moist conditions and heavy rainfall.

3. Activity against existing QoI-resistant fungi

As described in the "Discovery" section, metyltetraprole was discovered as a new QoI fungicide unaffected by G143A resistance in practical use. Therefore, we conducted a detailed investigation of metyltetraprole's activity against various QoI-resistant strains of plant pathogenic fungi through *in vitro* and *in vivo* tests. Though omitted in this paper, we have also tested some less frequently detected minor types of QoI-resistant strains and published the results (see references)[14].

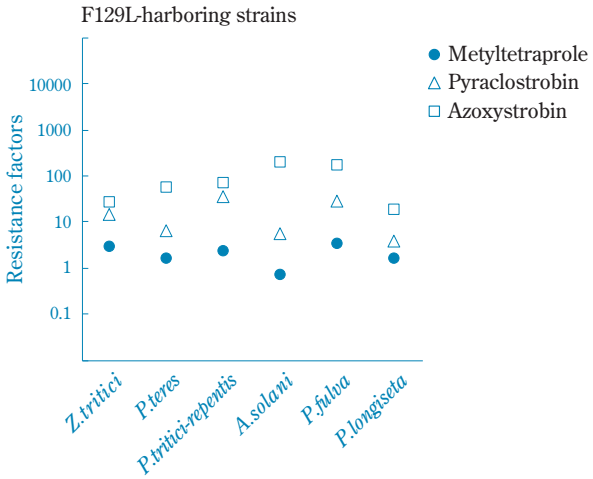
(1) Activity against G143A-harboring strains

We evaluated antifungal activity *in vitro* for six species of plant pathogenic fungi, comparing the antifungal activity between QoI-susceptible strains with wild-type cytochrome *b* and G143A-harboring resistant strains. We calculated the resistance factor as the ratio of these two values, finding that the resistance of G143A-harboring strains to metyltetraprole was around 1 or less, regardless of the fungal species (**Fig. 10**). By contrast, resistance factors of 100–1000 were observed for reference QoI fungicides.



Resistance factor, [EC_{50} of the resistant mutant]/[EC_{50} of the wild type]; *Z. tritici*, *Zymoseptoria tritici*; *P. nodorum*, *Phaeosphaeria nodorum*; *P. tritici-repentis*, *Pyrenophora tritici-repentis*; *M. majus*, *Microdochium majus*; *R. collo-cygni*, *Ramularia collo-cygni*; *C. gloeosporioides*, *Colletotrichum gloeosporioides*

Fig. 10 Resistance factors shown by G143A-harboring strains in six species



Resistance factor, [EC_{50} of the resistant mutant]/[EC_{50} of the wild type]; *Z. tritici*, *Zymoseptoria tritici*; *P. teres*. *Pyrenophora teres*; *P. tritici-repentis*, *Pyrenophora tritici-repentis*; *A. solani*, *Alternaria solani*; *P. fulva*, *Passalora fulva*; *P. longiseta*, *Pestalotiopsis longiseta*

Fig. 11 Resistance factors shown by F129L-harboring strains in six species

(2) Activity against F129L-harboring strains

As mentioned in the "Discovery" section, the G143A type is the most severely resistant type for QoI fungicides in terms of prevalence and resistance intensity. Meanwhile, the prevalence of cytochrome *b* F129L-harboring strains has been reported in some plant pathogenic fungi, which, although not as resistant as the G143A type, still result in reduced practical effectiveness[5]. Therefore, we used the same method as with the G143A type to compare the antifungal activity of QoI-susceptible strains and F129L-harboring strains, calculating the resistance factor. Interestingly, the results showed that F129L-harboring strains also had low resistance to metyltetraprole regardless of the fungal species, with no cases where the resistance factor exceeded 5, a level of concern for practical applications (**Fig. 11**). Thus, metyltetraprole was not substantially impacted by F129L-harboring strains.

By contrast, we observed resistance factors of 5–150 for reference QoI fungicides.

(3) Activity against resistant strains overexpressing multidrug efflux transporters

Research has confirmed the existence of resistant strains that show slight resistance to fungicides because of the overexpression of multidrug efflux transporters (multidrug-resistant [MDR] strains)[15]. Therefore, we investigated the activity and efficacy of metyltetraprole against MDR strains of *Z. tritici* *in vitro* and *in vivo* (*in planta*). The results showed that antifungal activity against the MDR strain was slightly lower than that against the wild-type strain *in vitro*, but there was no substantial difference in efficacy *in vivo* (**Table 4**). A similar phenomenon has been observed with Qi site inhibitors (QiI fungicides) that target ubiquinol oxidase, as well as with QoI fungicides[16]. Multidrug efflux transporters function by consuming energy generated within the cell through

Table 4 *In vitro* and *in planta* activity of metyltetraprole against multidrug resistant[a] isolates of *Z. tritici*

	In vitro	% Control of disease (*In planta*)	
Isolate	EC_{50} (mg/L)	50 g/ha	17 g/ha
non-MDR1	0.007	98	91
non-MDR2	0.004	96	85
non-MDR3	0.006	85	55
non-MDR4	0.002	90	64
Mean	***0.005***	***92***	***74***
MDR1	0.023	95	81
MDR2	0.007	79	77
MDR3	0.005	89	77
MDR4	0.010	88	57
Mean	***0.011***	***88***	***73***

a: Multidrug resistant phenotype (MDR) was confirmed by either of tolnaphtate-resistance or *ZtMFS1* overexpression. Fungicides were applied three days before inoculation. Water volume was 200 L/ha.

respiration and other processes. Pathogen cells were thought to have less energy on the plant tissue (*in vivo* tests) compared to that on a nutrient-rich medium (*in vitro* tests). Considering experimental results, the mechanism of multidrug efflux transporters, and the fact that metyltetraprole is a QoI fungicide that inhibits energy generation, it was thought that metyltetraprole would be less affected by MDR strains of pathogenic fungi in practical situations.

4. Field evaluations

The field efficacy of metyltetraprole against major diseases of various crops was investigated worldwide. Several representative examples are shown below. In this paper, we show the evaluation results of solo metyltetraprole, except for the mixture with mancozeb "Muketsu DX," which is already available in Japan. However, the commercialization of mixtures with other active ingredients is planned in all regions because of factors such as spectrum complementation and resistance management.

(1) Septoria leaf blotch control in Europe

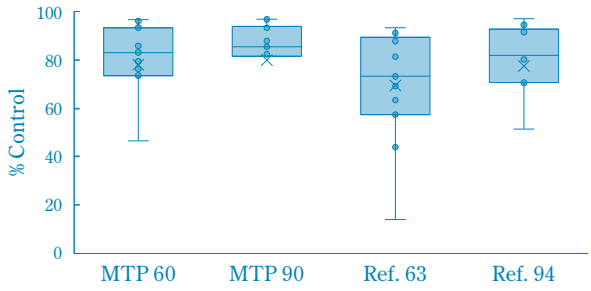
Wheat is the grain with the largest cultivation area in Europe. However, the rate of G143A-harboring resistant strains is nearly 100% for major diseases such as septoria leaf blotch and ramularia leaf spot (*Ramularia collo-cygni*), making disease control difficult[5),12)]. **Fig. 12** shows an overview of field tests on septoria leaf blotch conducted at 11 sites in France, Germany, and the United Kingdom in 2018, when the spread of resistant fungi had already become a problem. The efficacy of metyltetraprole against this disease was superior to that of fluxapyroxad (a non-QoI fungicide), one of the most effective fungicides against septoria leaf blotch in 2018. There were also no cases of phytotoxicity from the fungicide. Evaluations by local public agencies have confirmed that metyltetraprole is superior to other fungicides against ramularia leaf spot[17)].

(2) Soybean disease control in Brazil

Brazil is a leading soybean producer, competing with the United States for the top position in the world, and soybeans are the crop with the largest cultivation area in the country. However, there is a high risk of yield reduction because of rust or late-season diseases, making fungicide spraying essential. A 2015–2016 survey reported that at least 80% of *Cercospora* spp., which cause late-season diseases, harbor G143A strains[18)]. **Fig. 13** shows field test results for soybean late-season diseases conducted at 11 test sites in Brazil in 2020 (five for leaf blight diseases caused by *Cercospora* spp., and six for brown spot disease caused by *Septoria glycines*). Metyltetraprole showed superior efficacy against both pathogens compared to a mixture of azoxystrobin (a QoI fungicide) and cyproconazole (a non-QoI fungicide), widely used in the country. There were no cases of phytotoxicity from the compound.

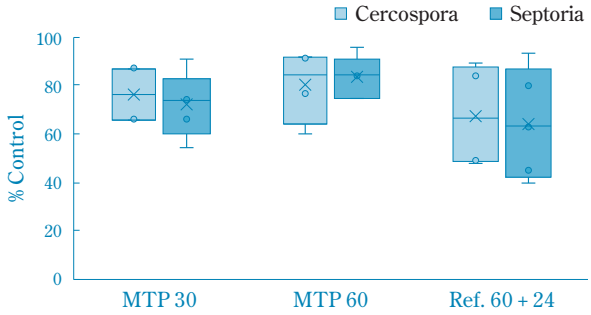
(3) Cotton disease control in Brazil

Brazil is one of the world's major cotton-producing countries and expected to soon surpass the United



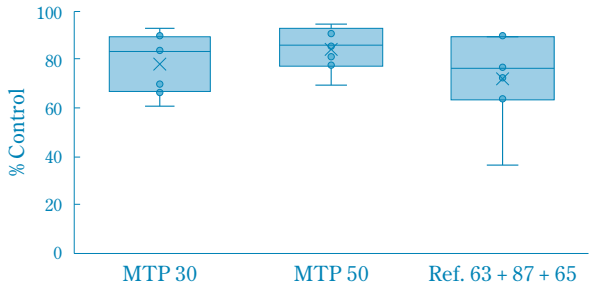
Wheat Septoria leaf blotch field trials in the 2018 season (11 trials in Europe: France, UK, and Germany). Dose, g/ha; MTP, metyltetraprole; Reference, fluxapyroxad; Box, interquartile range; Center cross, mean value; Center horizontal line, median value; Edges of the vertical line, the highest and lowest value

Fig. 12 Efficacy against wheat Septoria leaf blotch (field trials)



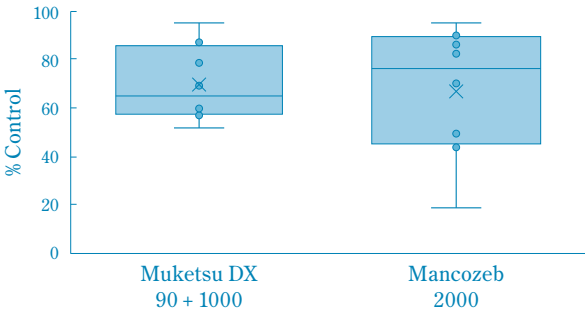
Soybean late season diseases field trials in the 2020/21 season (four *Cercospora kikuchii* and five *Septoria glycines* trials in Brazil). Dose, g/ha; MTP, metyltetraprole; Reference, azoxystrobin + cyproconazole; Box, interquartile range; Center cross, mean value; Center horizontal line, median value; Edges of the vertical line, the highest and lowest value

Fig. 13 Efficacy against soybean late season diseases (field trials)



Cotton areolate mildew field trials in the 2020/21 season (seven trials in Brazil)
Dose, g/ha; MTP, metyltetraprole; Reference, bixafen + prothioconazole + trifloxystrobin;
Box, interquartile range; Center cross, mean value; Center horizontal line, median value; Edges of the vertical line, the highest and lowest value

Fig. 14 Efficacy against cotton areolate mildew (field trials)



Sugar beet leaf spot field trials in 2017–2020 season (eight trials in Hokkaido, Japan)
Dose, mg/L of active ingredient; Muketsu DX, metyltetraprole + mancozeb
Box, interquartile range; Center cross, mean value; Center horizontal line, median value; Edges of the vertical line, the highest and lowest value

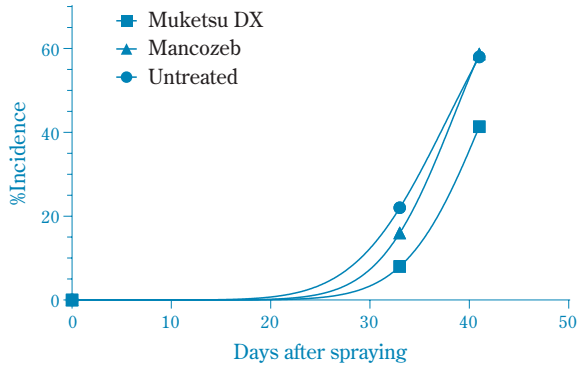
Fig. 15 Efficacy against sugar beet leaf spot (field trials)

States as the largest exporter[19]. However, Brazil's humid climate increases the risk of disease, particularly areolate mildew caused by *Ramulariopsis pseudoglycines* and *R. gossypii*. The frequent use of QoI fungicides has resulted in nearly 100% presence of G143A strains in these pathogens in 2019–2020[20]. **Fig. 14** shows field test results for areolate mildew conducted at seven test sites in Brazil in 2020. Metyltetraprole's efficacy was superior to that of a three-component mixture of bixafen (a non-QoI fungicide), prothioconazole (a non-QoI fungicide), and trifloxystrobin (a QoI fungicide), the best-performing product at the time. There were no cases of phytotoxicity from the compound.

(4) Sugar beet disease control in Japan

Sugar beet is a major sugar crop grown in temperate to cool climates that are unsuitable for sugarcane cultivation. In Japan, sugar beet is produced in Hokkaido, and its cultivation area is larger than that of sugarcane in the country. One report indicated that about half of the *Cercospora beticola* strains, a causal agent of the major disease sugar beet leaf spot, isolated from various parts of Hokkaido in 2014–2015 were G143A strains[21]. **Fig. 15** shows field test results of Muketsu DX, conducted at eight sites in Hokkaido from 2017 to 2020. Muketsu DX's efficacy was more stable than that of the representative reference fungicide, mancozeb. There were no cases of phytotoxicity from the compound.

Additionally, a comparison of the efficacy of a single spray before fungal infection (preventative treatment) showed that Muketsu DX's long-lasting efficacy was superior to that of mancozeb (**Fig. 16**). Sugar beet leaf



Sugar beet leaf spot field trials in Ebetsu, Hokkaido. Single spraying was made on June 21. Muketsu DX, metyltetraprole + mancozeb 90 + 1000 mg/L; Mancozeb, 2000 mg/L

Fig. 16 Comparison of long-lasting efficacy against sugar beet leaf spot/Slow disease progression (SDP) effect

spot is usually controlled by 3–7 applications of fungicide. This slow disease progression (SDP) effect should be considered when incorporating Muketsu DX into spraying programs.

These results show that metyltetraprole is expected to contribute to the control of various crop diseases. Meanwhile, although no resistance to metyltetraprole has been detected, the possibility of the emergence of new fungicide-resistant fungal strains specific to this compound cannot be ruled out. Future tasks include raising awareness of measures to prevent the spread of resistant fungi by mixing or alternating with other fungicides and conducting continuous sensitivity monitoring to ensure the long-term use of metyltetraprole.

Formulation

Muketsu DX (metyltetraprole 4.5% [w/w], mancozeb 50% [w/w] wettable powder) for the Japanese market was registered as a pesticide in May 2021 and has been available for growers since January 2023 (**Fig. 17**). As described in the "Biological effects" section, this product targets sugar beet leaf spot and is highly effective against fungal strains resistant to other fungicides. Another feature is its SDP effect. Its preventative spray exhibits excellent long-lasting activity, contributing to the reliability of spraying programs. In the development study of Muketsu DX, we optimized the types and amounts of inert components, considering high efficacy, low risk of phytotoxicity, and ease of handling based on our past experiences.

Fig. 17 Commercial package of Muketsu DX

Consequently, we established the recipe for Muketsu DX, the formulation with high efficacy and safety for sugar beet, low foaming, and high dispersion stability in spray dilution (**Table 5**).

Toxicity, metabolism, and residues

Here, we present the evaluation results of metyltetraprole technical grade and metyltetraprole 35% [w/w] suspension concentrate (35%SC).

1. Mammalian toxicity

(1) Acute toxicity, irritation, and skin sensitization

The acute toxicity of metyltetraprole technical grade was extremely low, with the median lethal dose (LD_{50}) values exceeding 2000 mg/kg body weight in rats for oral administration and dermal application and the median lethal concentration (LC_{50}) value exceeding 2520 mg/m^3 in rats for inhalation exposure. No deaths or toxic signs were observed following any administration route. The acute toxicity of metyltetraprole 35%SC was also extremely low, with no deaths or toxic signs observed following oral administration or dermal application at 2000 mg/kg body weight or inhalation exposure at 2010 mg/m^3. The eye irritation potential of metyltetraprole technical grade was minimal and reduced by washing. The eye irritation potential of metyltetraprole 35%SC was very slight and practically classified as non-irritant.

Table 5 Physical and chemical properties of Muketsu DX

Items		Typical value
Appearance		Pale yellow powder
Suspensibility[a] (After 15 min)	Metyltetraprole	98%
	Mancozeb	99%
Persistent foam[a] (After 1 min)		2 mm
Stability		The physical and chemical properties after storage at room temperature for 3 years remained unchanged.

a: 250 mL cylinder, 53.6 ppm hard water, 500-fold dilution, 20 °C

Table 6 Acute toxicity summary of metyltetraprole

Test type	Metyltetraprole	Metyltetraprole 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})	> 2520 mg/m^3 of air (4 h, nose only exposure)	> 2010 mg/m^3 of air (4 h, nose only exposure)
Eye irritation (Rabbit)	Minimally irritant	Practically non-irritant
Skin irritation (Rabbit)	Non-irritant	Non-irritant
Skin sensitization (Guinea pig)	Non-sensitizer	Non-sensitizer

Neither metyltetraprole technical grade nor metyltetraprole 35%SC caused skin irritation or skin sensitization (**Table 6**).

(2) Subchronic and chronic toxicity and carcinogenicity

In subchronic, chronic, and carcinogenicity studies using rats, dogs, or mice, no toxic effects were observed, even with repeated administration of metyltetraprole technical grade. No carcinogenicity was observed in rats or mice (**Table 7**).

(3) Reproductive and developmental toxicity

In developmental toxicity (teratogenicity) studies using rats and rabbits, no teratogenicity was observed in fetuses. In two-generation reproductive toxicity study using rats, reproductive performance and nursing behavior were not affected (**Table 8**).

(4) Neurotoxicity

No evidence of neurotoxicity was observed in acute neurotoxicity study using rats (**Table 9**).

Table 7 Subacute and chronic toxicity summary of metyltetraprole

Species	Administration route and duration	Dose	NOAEL (mg/kg/d)
Rat	Dermal, 28 days	100, 300, 1000 mg/kg/d	Male: 1000 Female: 1000
Rat	Oral (in diet), 13 weeks	2000, 6000, 20000 ppm	Male: 1508 (20000 ppm) Female: 1715 (20000 ppm)
Rat	Oral (in diet), 24 months	2000, 6000, 20000 ppm	Male: 852 (20000 ppm) Female: 1190 (20000 ppm) No carcinogenicity
Dog	Oral (in capsule), 13 weeks	100, 300, 1000 mg/kg/d	Male: 1000 Female: 1000
Dog	Oral (in capsule), 12 months	100, 300, 1000 mg/kg/d	Male: 1000 Female: 1000
Mouse	Oral (in diet), 13 weeks	1500, 3500, 7000 ppm	Male: 1060 (7000 ppm) Female: 1360 (7000 ppm)
Mouse	Oral (in diet), 18 months	700, 2000, 7000 ppm	Male: 820 (7000 ppm) Female: 1012 (7000 ppm) No carcinogenicity

Table 8 Developmental and reproductive toxicity summary of metyltetraprole

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	100, 250, 750 mg/kg/d	Maternal Fetal	250 750
Two-generation reproductive toxicity	Rat	Oral (in diet)	2000, 6000, 20000 ppm	Parental Offspring Reproductive	Male: 1385 (20000 ppm) Female: 1544 (20000 ppm) Male: 1385 (20000 ppm) Female: 1544 (20000 ppm) Male: 1385 (20000 ppm) Female: 1544 (20000 ppm)

Table 9 Neurotoxicity summary of metyltetraprole

Study	Species	Administration route and duration	Dose	NOAEL (mg/kg/d)
Neurotoxicity	Rat	Acute oral (gavage)	500, 1000, 2000 mg/kg/d	Male: 2000 Female: 2000

Table 10 Mutagenicity summary of metyltetraprole

Study	Study design	Results
Reverse mutation (Ames test)	*Salmonella typhimurium*: TA98, TA100, TA1535 and TA1537 *Escherichia coli*: WP2*uvrA* –/+S9 mix: 156–5000 μg/plate	Negative
In vitro gene mutation	Chinese hamster V79 –S9 mix: 0.3–4.0 μg/mL +S9 mix: 1.0–62.0 μg/mL	Negative
In vitro chromosomal aberration	Chinese hamster CHL/IU –S9 mix (6 h): 20.0–60.0 μg/mL +S9 mix (6 h): 17.5–70.0 μg/mL –S9 mix (24 h): 2.0–4.0 μg/mL	Negative
Bone marrow micronucleus	CD-1 mice 500, 1000, 2000 mg/kg	Negative

(5) Mutagenicity

In a reverse mutation study using *Salmonella typhimurium* and *Escherichia coli*, chromosomal aberration study using Chinese hamster lung-derived CHL/IU cell lines, gene mutation study using V79 cell lines, and micronucleus study in mice, the results were all negative (**Table 10**).

2. Animal and plant metabolism

(1) Metabolism in animals

Following oral administration of ^{14}C-labeled metyltetraprole to rats, metyltetraprole was rapidly absorbed and distributed throughout the body. Metyltetraprole was then rapidly metabolized and excreted in urine and feces. The oral absorption rate was estimated to be 86% or higher, and no significant residue or tissue accumulation was observed. The main metabolic reactions of metyltetraprole were oxidation and glucuronidation of the methyl group on the benzene ring and N-demethylation of tetrazolinone ring.

(2) Metabolism in plants

In plant metabolism studies, ^{14}C-labeled metyltetraprole underwent similar metabolic pathways among three different crop groups (wheat, soybean, and apple). Namely, metyltetraprole was metabolized *via* photocleavage of the benzyl ether bond on the plant surface followed by intramolecular rearrangement, and hydroxylation of the methyl group on the benzene ring and cleavage of the benzyl ether bond with successive conjugation with the natural components. The metabolites produced were finally incorporated into the constituents of plants.

3. Environmental behavior and residues

(1) Degradation in water

In the hydrolysis study, ^{14}C-labeled metyltetraprole was stable in buffer solutions at pH 4, 7, and 9 with a half-life of over a year (25 °C). The photodegradation half-life of metyltetraprole in a buffer solution (pH 7) was 5.7–10.2 h (equivalent to the natural sunlight at Tokyo in spring). The main photodegradation pathway was considered to be cleavage of the benzyl ether bond followed by intramolecular rearrangement or replacement of the chlorine atom of the chlorophenyl ring with a hydroxyl group and elimination of the chlorophenyl group, and finally mineralization to carbon dioxide.

(2) Metabolism in soil

In the aerobic metabolism study, the degradation half-life (20 °C) of ^{14}C-labeled metyltetraprole was 711–1000 days or greater. Degradation on the soil surface was accelerated by exposure to light, and the half-life (20 °C) was 196–241 days (equivalent to the natural sunlight in Tokyo during spring). The degradation pathway in/on soil was mainly the cleavage of the benzyl ether bond by light exposure followed by intramolecular rearrangement, and finally binding to the soil matrix or mineralized to carbon dioxide.

(3) Residue in soil

With a single application of a 1000-fold diluted solution of metyltetraprole 35%SC to fields in Saitama, Kochi, Kumamoto, and Miyazaki at 300 L/10 a, the maximum residue of metyltetraprole was 1.01–1.58 mg/kg, with a half-life of 22.5–57.7 days in the field dissipation studies.

(4) Mobility in soil

The adsorption coefficient, $K_{Foc(ads)}$, and desorption coefficient, $K_{Foc(des)}$, of metyltetraprole corrected for the organic carbon content were calculated using the Freundlich adsorption isotherm and were 1551–5468 and 7382–12334, respectively.

(5) Residue in crops

With two or three applications of a 2000–3000-fold diluted solution of metyltetraprole 35%SC to sugar beet, apple, and tea plants, the maximum mean residues of metyltetraprole were 0.09, 3.3, and 21.1 mg/kg, respectively, in the crop residue trials.

(6) Residue in succeeding crops

Because the half-life of metyltetraprole in the field dissipation studies was 22.5–57.7 days, the effects on succeeding crops were considered to be minimal.

4. Effects on non-target organisms

Table 11 summarizes the test results for aquatic animals and plants, honeybees, silkworms, natural enemy insects, and birds.

(1) Effects on aquatic animals and plants

The acute toxicity values (LC_{50}/EC_{50}/ErC_{50}) of metyltetraprole technical grade for carp, *Daphnia magna*, and freshwater green algae were >0.15, 0.34, and >0.32 mg/L, respectively. The toxicity values of metyltetraprole 35%SC were 0.85, 1.7, and 410 mg/L for the same organisms, respectively. These values are sufficiently higher than the predicted concentration in environmental water expected from practical use, indicating that the effects on aquatic animals and plants are considered to be low.

(2) Effects on honeybees, silkworms, and natural enemy insects

The LD_{50} values of metyltetraprole technical grade for oral and contact administration to honeybees were >109.3 and >100.0 µg/bee, respectively. The mortality rate of silkworms following acute oral administration of a 2000-fold diluted solution of metyltetraprole 35%SC was 4%. The residual toxicity study using mulberry leaves sprayed with the same agent showed no effect on silkworms 32 days after spraying, indicating a residual toxicity period of less than 32 days.

For natural enemies, the mortality rates of adult *Neoseiulus californicus*, *Propylea japonica*, and *Aphelinus asychis* after 7 days of contact administration of a 2000-fold diluted solution of metyltetraprole 35%SC were 2.0%, 0%, and 6.0%, respectively, which were equivalent to those of the negative control groups. These results indicate that metyltetraprole has a low effect on honeybees, silkworms, and natural enemy insects in practical use.

(3) Effects on birds

The acute toxicity of metyltetraprole technical grade to bobwhite quail was low, with an oral LD_{50} value of >2250 mg/kg, indicating low effects on birds in practical use.

Table 11 Ecotoxicological summary of metyltetraprole on non-target organisms

Test substance	Test species		Test type	Results
Metyltetraprole	Aquatic organisms	Carp	Acute (96 h)	LC_{50} > 0.15 mg/L
		Daphnia magna	Acute (48 h)	EC_{50} = 0.34 mg/L
		Green alga[a]	Acute (72 h)	ErC_{50} > 0.32 mg/L
	Honeybee	*Apis mellifera*	Acute oral (48 h)	LD_{50} > 109.3 µg/bee
		Apis mellifera	Acute contact (48 h)	LD_{50} > 100.0 µg/bee
	Bird	Bobwhite quail	Acute oral	LD_{50} > 2250 mg/kg
Metyltetraprole 35%SC	Aquatic organisms	Carp	Acute (96 h)	LC_{50} = 0.85 mg/L
		Daphnia magna	Acute (48 h)	EC_{50} = 1.7 mg/L
		Green alga[a]	Acute (72 h)	ErC_{50} = 410 mg/L
	Silkworm	*Bombyx mori*	Acute oral (22 d)	Mortality 4% (2000x dilution solution)
		Bombyx mori	Residual toxicity test	< 32 days (2000x dilution solution)
	Natural enemy	*Neoseiulus californicus* (adult)	Acute contact (7 d)	Mortality = 2.0% (2000x dilution solution)
		Propylea japonica (adult)	Acute contact (7 d)	Mortality = 0.0% (2000x dilution solution)
		Aphelinus asychis (adult)	Acute contact (7 d)	Mortality = 6.0% (2000x dilution solution)

a: *Raphidocelis subcapitata*

These results indicate that the acute toxicity of metyltetraprole technical grade and metyltetraprole 35%SC to mammals is extremely low and long-term exposure to metyltetraprole is unlikely to result in any risks to human health such as carcinogenicity, teratogenicity, or reproductive effects. Additionally, given the behavior of the compound in the environment and its effects on non-target organisms, metyltetraprole is considered to have no impact on the environment when used according to the method applied for registration.

Conclusion

Metyltetraprole shows a wide control spectrum and is effective against plant pathogens resistant to conventional QoI fungicides. The risk of phytotoxicity to crops is low, and the compound can improve the yield of grains such as wheat and soybeans and industrial crops such as sugar beet and cotton. The effects on non-target organisms such as honeybees are also sufficiently small, allowing for use throughout the crop growth period.

Based on the results of (eco)toxicity studies, metyltetraprole is considered to be of low toxicity to humans and non-target organisms. We hope that metyltetraprole, a new achievement in modern pesticide science, will be used appropriately and contribute to present and future human society.

Acknowledgments

During our development of metyltetraprole, we received practical evaluation tests and valuable advice from institutions such as the Japan Plant Protection Association, as well as independent administrative institutions and plant protection associations in each prefecture. We express our gratitude and hope for their continued guidance and encouragement.

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PROFILE

Yuichi Matsuzaki

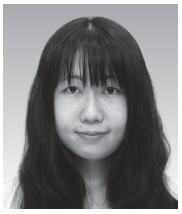
Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Senior Research Associate,
Ph.D. (Agriculture)

Tatsuya Toriumi

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

Yuya Yoshimoto

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Researcher (at the time)
(Current affiliation: Misawa Works, Manager)



Risa Ito

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

Sadayuki Arimori

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

Miwa Kondo

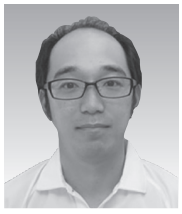
Sumitomo Chemical Co., Ltd.
Environmental Health Science Laboratory
Senior Research Associate,
Ph.D. (Veterinary Medicine)

Fukumatsu Iwahashi

Sumitomo Chemical Co., Ltd.
Health & Crop Sciences Research Laboratory
Fellow, Ph.D. (Agriculture)

Miho Tabuchi

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



Makoto Kurahashi

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