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DISEASE CONTROL

Metyltetraprole activity against plant pathogens with relatively rare cytochrome *b* haplotypes for azoxystrobin resistance

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Abstract

Metyltetraprole is a novel quinone outside inhibitor (QoI) fungicide designed to avoid cross-resistance in cytochrome *b* G143A-harboring QoI-resistant phytopathogenic fungi. The resistance factors of G143A-harboring fungal isolates for metyltetraprole are around 1, but > 200 for the reference QoI fungicide azoxystrobin. In this study of metyltetraprole activities against azoxystrobin-resistant isolates carrying G137R, G137S, L299F, N256S + L299F, or L275F + L299F in cytochrome *b,* metyltetraprole had potent activity against all isolates with these cytochrome *b* haplotypes. The resistance factors ranged from 0.7 to 2.9 for metyltetraprole and from 3.0 to 175.1 for azoxystrobin. We revealed unique metyltetraprole inhibitory activities against QoI-resistant plant pathogens.

Keywords Metyltetraprole · Cytochrome *b* · Fungicide resistance · QoI fungicide

Introduction

Quinone outside inhibitor (QoI) fungicides are widely used on various crops worldwide (Fig. S1). However, QoI-resistant mutants with mutations relevant to G143A or F129L substitutions in cytochrome *b* (Cytb), the target site of QoI fungicides, threaten the sustainable use of these fungicides. G143A is the most frequently detected substitution and induces a resistance factor (RF) > 200 for most QoIs, whereas F129L is the second most frequent substitution and induces (RF: 5–20; Matsuzaki et al. 2020a; Sierotzki 2015). To solve the resistance issue of QoI fungicides, metyltetraprole, characterized by a tetrazolinone pharmacophore and 3-substituent in the central benzene ring, was designed to avoid steric hindrance between the molecule and Cytb with G143A substitution (Matsuzaki et al. 2020b). Interestingly, metyltetraprole retained potent activity against both G143A and F129L types and its practical efficacy was not affected substantially by the two Cytb substitutions (Matsuzaki et al. 2020a; Suemoto et al. 2019). The mean EC_{50} values for metyltetraprole did not differ significantly between the wild-type isolates (0.00027 mg l^{-1}, $N = 19$) and G143A-harboring isolates (0.00022 mg l^{-1}, $N = 358$) in *Zymoseptoria tritici* (Mann–Whitney *U* test). In contrast, the small difference in EC_{50} for metyltetraprole between F129L-harboring isolates of *Pyrenophora teres* and the wild-type isolates was significant (RF = 1.5; mean metyltetraprole EC_{50} value was 0.016 mg l^{-1} for 20 wild-type isolates and 0.030 mg l^{-1} for 15 F129L-harboring isolates).

Although very minor, a few other substitutions in Cytb have been reported, including G137R in *Pyrenophora tritici-repentis* (Sierotzki et al. 2007), G137S in *Venturia effusa* (Standish et al. 2016, 2019), and L299F, N256S + L299F, and L275F + L299F in *Puccinia horiana* (Matsuzaki et al. 2021a, b). These substitutions can confer moderate levels of resistance to a few of the most widely used QoI fungicides, such as azoxystrobin (5–170 RF for azoxystrobin, Matsuzaki et al. 2021a, b; Sierotzki et al. 2007; Standish et al. 2016, 2019). We investigated the inhibitory activities of metyltetraprole against these mutants in each fungal species. In addition, some unique QoI fungicides are available in Japan but are not frequently used in other countries. Among them, pyribencarb is characterized by its benzyl-carbamate pharmacophore and known to retain some activity against G143A-harboring isolates in some fungal species (RF ranges 15–60, Kataoka et al. 2010). Mandestrobin, with a methoxy-acetamide pharmacophore, is characterized by its potent activities against fungi belonging to *Sclerotiniaceae* (Hirotomi et al. 2016). Metominostrobin and orysastrobin with oximino-acetamide pharmacophore are

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highly systemic and mainly used in paddy rice (Masuko et al. 2001; Stammler et al. 2007). Interestingly, their pharmacophore structures are different from globally used QoIs such as azoxystrobin (methoxy-acrylate), kresoxim-methyl (oximino-acetate), picoxystrobin (methoxy-acrylate), trifloxystrobin (oximino-acetate), and pyraclostrobin (methoxy-carbamate) (Fig. S1, FRAC 2021). Therefore, it is interesting to examine these unique QoI fungicides and their inhibitory activities against fungal isolates with Cytb haplotype G137R/S, L299F, L256S + L299F, or L275F + L299F.

We tested the in vitro sensitivity of *P. tritici-repentis* carrying G137R (Sierotzki et al. 2007), *V. effusa* carrying G137S (Standish et al. 2016, 2019), and *P. horiana* carrying L299F, N256S + L299F, and L275F + L299F substitutions in Cytb to metyltetraprole, mandestrobin, metominostrobin, orysastrobin, and pyribencarb (Matsuzaki et al. 2021a, b). The efficacy of the fungicides against *P. horiana* isolates was also assessed on chrysanthemum plants because the degree of resistance of this fungus requires *in planta* assays (Matsuzaki et al. 2021a, b).

Materials and methods

Chemicals

Chemical structures of QoI fungicides and fluazinam are shown in Fig. S1. For in vitro assays, metyltetraprole and mandestrobin were synthesized as previously described (Hirotomi et al. 2016; Matsuzaki et al. 2020b). Azoxystrobin, metominostrobin, orysastrobin, pyribencarb, and fluazinam were purchased as analytical standards (Sigma-Aldrich Japan, Tokyo, Japan). All chemicals were dissolved in 1% (w/v) dimethyl sulfoxide (DMSO) and stored at − 20 °C.

For the in planta assay, metyltetraprole 400 g l^{-1} suspension concentrate (SC) formulation was prepared by Sumitomo Chemical as previously described (Yoshimoto et al. 2013). Sukurea® (mandestrobin 40% SC formulation) and Fantasista® (pyribencarb 40% water dispersible granule formulation) were purchased from Sumitomo Chemical (Tokyo, Japan) and Kumiai Chemical Industry (Tokyo, Japan), respectively.

Fungal isolates

The origin and genotypes of the fungal isolates are shown in Table 1. The *P. tritici-repentis* isolate carrying the G137R substitution in Cytb was provided by Dr. Friedrich Felsenstein of EpiLogic, GmbH, Germany. Wild-type isolates of *P. tritici-repentis* were collected from wheat fields in Germany and Poland in 2018. Haplotypes of the *Cytb* gene of all isolates were checked using a previously described method (Sierotzki et al. 2007). The wild-type and G137S-carrying isolates of *V. effusa* were provided by Dr. Tim Brenneman of the University of Georgia, USA. *Puccinia horiana* monopustule isolates were collected in Japan in our previous studies (Matsuzaki et al. 2021a, b).

In vitro microtiter plate tests for *Pyrenophora tritici-repentis* and *Venturia effusa*

A microtiter plate test (Spiegel and Stammler 2006) was used for *P. tritici-repentis* and *V. effusa* as a suitable method to assess the metyltetraprole sensitivity of fungi (Matsuzaki et al. 2021c).

For *P. tritici-repentis*, conidia of each isolate were obtained from V8 agar plates using published methods (James et al. 1991) and adjusted to 1×10^2 conidia ml^{-1} in YBA (yeast extract 10 g l^{-1}, peptone 10 g l^{-1}, and sodium acetate 20 g l^{-1} in distilled water, Stammler and Speakman 2006). Conidia of *V. effusa* were obtained from malt extract yeast agar (MYA, 10 g malt, 4 g yeast extract, 4 g glucose, 20 g agar) cultured in the dark at 18 °C for 2 weeks and adjusted to 1×10^4 conidia ml^{-1} in potato dextrose broth (PDB, 24 g l^{-1}). A 100-fold dilution series of fungicides (0.03, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 mg l^{-1} in DMSO, corresponding to final concentrations of 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 mg l^{-1} in the medium, respectively) were prepared for each test. Samples (1 μl) of each fungicide were mixed with the fungal culture medium (99 μl) and added to the wells in 96-well microtiter plates and incubated at 23 °C for 3 days (*P. tritici-repentis*) or 7 days (*V. effusa*). The tests were performed with four replicates per treatment. Fungal growth was assessed by measuring the optical density (OD) of each medium at a wavelength of 600 nm using a microplate reader SH-9000 Lab (Corona Electric, Ibaraki, Japan) with a 3 × 3 matrix of scanning points. The OD values were corrected using a blank well containing one of the respective dilutions without the inoculum. The EC_{50} was calculated from the mean of the OD values of each fungicide concentration using the nonlinear regression (curve fit) of GraphPad Prism8. The resistance factor (RF) was calculated as RF = (Mean EC_{50} of less-sensitive isolates with the non-wild-type Cytb haplotype)/(Mean EC_{50} of sensitive isolates with the wild-type Cytb haplotype).

In vitro basidiospore germination tests for *Puccinia horiana*

Because *P. horiana* is an obligate parasite and its mycelium does not grow in isolation, basidiospore germination was tested as previously described (Matsuzaki et al. 2021a, b). DMSO solutions of fungicides were adjusted to final concentrations of 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1,

Table 1 Fungal isolates used in this study

Fungal species (disease)	Isolate	Origin country	Isolation year	Cytochrome *b* haplotype
Pyrenophora tritici-repentis	DE18 Ptr40.12[a]	Germany	2018	Wild type
(wheat tan spot)	DE18 Ptr40[a]	Germany	2018	Wild type
	PL18 Ptr61.5[a]	Germany	2018	Wild type
	DTR06-35/1[b]	Germany	2006	G137R
Venturia effusa	WD-6[c]	USA	2018	Wild type
(pecan scab)	WD-5 [c]	USA	2018	Wild type
	MS-6 [c]	USA	2018	G137S
	MS-4 [c]	USA	2018	G137S
Puccinia horiana	CWR5[d]	Japan	2019	Wild type
(chrysanthemum white rust)	CWR37[e]	Japan	2020	Wild type
	CWR23[e]	Japan	2020	L299F
	CWR32[e]	Japan	2020	L299F
	CWR1[f]	Japan	2015	L275F + L299F
	CWR12[d]	Japan	2020	L275F + L299F
	KTR1[e]	Japan	2020	N256S + L299F
	KTR2[e]	Japan	2020	N256S + L299F

[a]Isolates from our unpublished studies

[b]Isolates provided from Dr. Friedrich Felsenstein of EpiLogic, GmbH, Germany

[c]Isolates provided by Dr. Tim Brenneman of the University of Georgia, USA

[d]Isolates from Matsuzaki et al. (2021a)

[e]Isolates from Matsuzaki et al. (2021b)

[f]Isolates provided by Dr. Akiho Harada of the Kagoshima Prefectural Institute for Agricultural Development, Japan

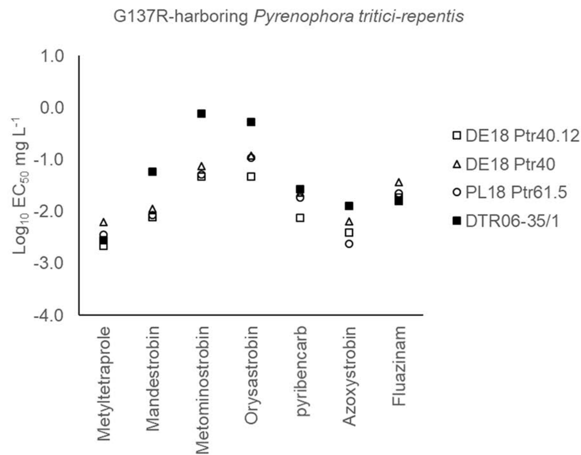
0.3, 1, 3, and 10 mg l^{-1} in melted water agar (WA) and solidified in petri plates; one plate was used for each concentration and isolate. The DMSO concentration in WA was 0.1% (v/v). Cut leaves with fresh teliospore pustules were attached to the lid of Petri dishes and placed 5–6 mm above the WA surface. After 2 h of incubation at 18 °C with 100% humidity to form and disperse basidiospores from teliospore pustules, leaves were removed from the lid of Petri dishes. After 18 h at 18 °C with 100% humidity, 20 basidiospores from each of five independent pustules for each fungicide concentration were assessed for germination with a light microscope. The inhibition of germination (%) was normalized to the percentage germination in fungicide-free 0.1% DMSO controls, and EC_{50} and RF values were determined similarly to microtiter plate tests.

In planta efficacy tests of fungicides against *P. horiana*

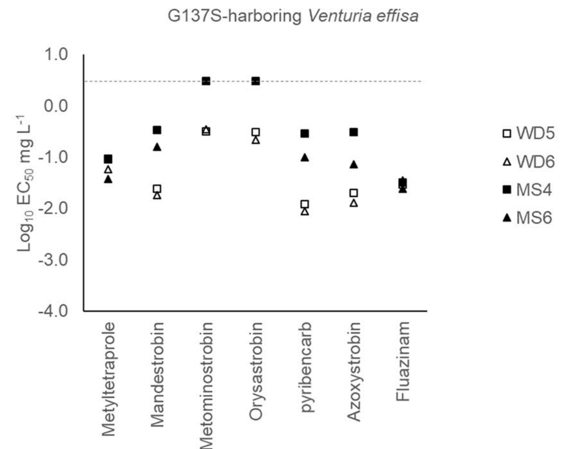
The efficacy of metyltetraprole, mandestrobin, and pyribencarb was tested against *P. horiana* if the trend observed in basidiospore germination tests was also observed in vivo and in planta. The test procedures were similar to those reported by Matsuzaki et al. (2021a, b). Chrysanthemum root cuttings of Shuho-no-Chikara variety were purchased from Misaki-Engei (Gifu, Japan) and grown in a 23 °C greenhouse. Fungicides were sprayed on chrysanthemum plants at the 12- to 14-leaf stage in 8-cm-diameter plastic pots with a sufficient volume of water to cause runoff. Each fungicide was applied at the labeled use rate and 1/3 and 1/9 recommended for Fantasista® (pyribencarb) for the control of chrysanthemum white rust in Japan. Metyltetraprole and mandestrobin were also tested at the same rate as pyribencarb, although neither compound has been registered for chrysanthemum white rust protection. Each fungicide was applied at 13, 44, or 133 mg l^{-1} active ingredient, then 18 h later, the plants were placed adjacent to chrysanthemums with fresh *P. horiana* pustules in a damp chamber with 100% humidity at 23 °C for 18 h. Test plants were then grown in a 23 °C greenhouse for 12–14 days when symptoms on untreated plants were optimal for assessment. The lesion-covered areas (0–100%) of the upper four leaves that had already unfolded during spraying were visually estimated by the same person. The mean lesion-covered areas of each treated and untreated plants were calculated as the disease severity. The control efficacy (%) of each treatment was calculated using the following formula:

$$\text{Control efficacy (\%)} = 100 \times \left[1 - (\text{Disease severity on fungicide} - \text{treated plants} / \text{Disease severity on untreated plants})\right].$$

(a)



(b)



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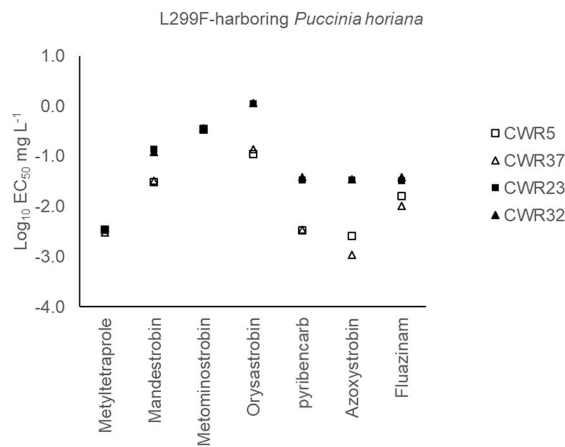

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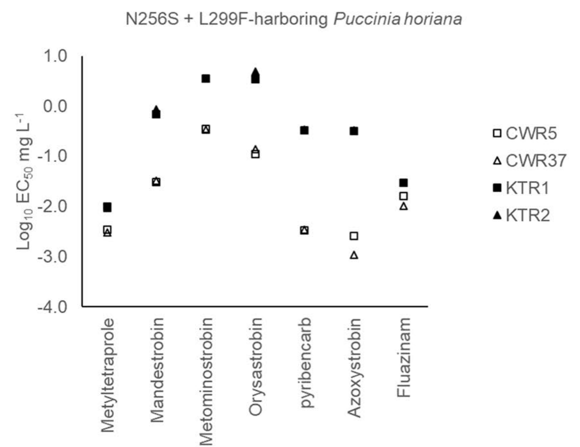
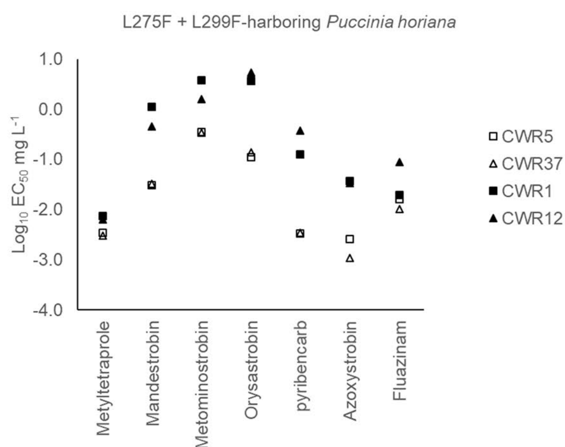


(e)



◂**Fig. 1** The 50% effective concentration (EC_{50}) for QoI fungicides (metyltetraprole, mandestrobin, metominostrobin, orysastrobin, pyribencarb, and azoxystrobin), and fluazinam, an uncoupler of mitochondrial oxidative phosphorylation in in vitro tests. Black squares and triangles represent isolates with Cytb substitutions; white squares, triangles, and circles represent wild-type isolates. **a** *Pyrenophora tritici-repentis* isolates with and without G137R substitution in Cytb. **b** *Venturia effusa* isolates with and without G137S substitution in Cytb. **c** *Puccinia horiana* isolates with and without L299F substitution in Cytb. **d** *P. horiana* isolates with and without N256S + L299F substitutions in Cytb. **e** *P. horiana* isolates with and without L275F + L299F substitutions in Cytb. The black horizontal dotted line indicates the cut-off value in **b**

Four plant replicates (two trials, each with two plant replicates) were used. Dunnett's tests were performed using BellCurve for Excel (Social Survey Research Information Co. Ltd., Tokyo, Japan) with the CWR5 isolate counterparts as the sensitive reference isolate without mutations in *Cytb* gene.

Results

In vitro tests

For all tested species/*Cytb* haplotypes, the EC_{50} of azoxystrobin was higher for azoxystrobin-resistant isolates with non-wild-type *Cytb* than that for wild-type equivalents (Fig. 1a–e, Table S1–S3). For azoxystrobin, the RF was 3.0 for G137R-harboring *P. tritici-repentis* (Fig. 1a, Table S1), 11.6 for G137S-harboring *V. effusa* (Fig. 1b, Table S2), 18.3 for L299F-harboring *P. horiana* (Fig. 1c, Table S3), 175.1 for N256S + L299F-harboring *P. horiana* (Fig. 1d, Table S3), and 19.0 for L275F + L299F-harboring *P. horiana* (Fig. 1e, Table S3). On the other hand, the EC_{50} values for fluazinam, which does not target the Qo site but works as an uncoupler of mitochondrial oxidative phosphorylation (Guo et al. 1991), against these isolates with non-wild-type Cytb, did not substantially differ from that for their wild-type equivalents (RF < 3.0 in all tested species or *Cytb* haplotypes except L275F + L299F-harboring *P. horina* with RF = 4.0) (Fig. 1a–e, Tables S1–S3). EC_{50} values for metyltetraprole were not substantially higher against isolates with substitutions in Cytb compared with that against their wild-type equivalents (RF < 3.0, Fig. 1a–e, Tables S1–S3), although metyltetraprole has been proven to be a QoI. EC_{50} values for mandestrobin, metominostrobin, orysastrobin, and pyribencarb against isolates with the Cytb substitution were higher than that against the wild-type equivalents (RF > 3.0, Fig. 1a–e, Tables S1–S3) except for metominostrobin against L299F-harboring *P. horiana* (RF = 1.0, Fig. 1c, Table S3) and pyribencarb against G137R-harboring *P. tritici-repentis* (RF = 1.7, Fig. 1a, Table S1). The highest RF among the isolates for the respective fungicides was for L275F + L299F-harboring *P. horiana* and mandestrobin (RF = 24.8, Fig. 1e, Table S3), G137R-harboring *P. tritici-repentis* and metominostrobin (RF = 13.2, Fig. 1a, Table S1), by L275F + L299F-harboring *P. horiana* and orysastrobin (RF = 36.0, Fig. 1e, Table S3), and by N256S + L299F-harboring *P. horiana* and pyribencarb (RF = 97.3, Fig. 1d, Table S3).

In planta test for *Puccinia horiana* isolates

The control efficacy of metyltetraprole was > 90%, approximately 90%, and approximately 80% at 133, 44, and 15 mg l^{-1}, respectively (Fig. 2a–c). The efficacies of metyltetraprole did not significantly differ among isolates. Mandestrobin and pyribencarb performed similarly to metyltetraprole in wild-type isolates (Fig. 2d–i). However, their efficacies were significantly lower in most isolates with Cytb substitutions, especially at lower treatment rates (Fig. 2d, e, g, h). For mandestrobin, the reduction in efficacy at 44 mg l^{-1} was greater in L275F + L299F-harboring isolates (Fig. 2e). For pyribencarb at the same rate, the reduction was significant for both L275F + L299F-harboring isolates and N256S + L299F-harboring isolates, whereas the efficacy was not as reduced in single L299F-harboring isolates (Fig. 2h).

Discussion

The chemical structure of metyltetraprole was designed to avoid steric hindrance with Cytb carrying G143A by using a tetrazolinone substructure as a smaller pharmacophore (Matsuzaki et al. 2020b). As a result, the activity of metyltetraprole against the wild-type isolates and the G143A-carrying isolates is very similar (Matsuzaki et al. 2020a, b). Interestingly, this strategy to avoid steric hindrance resulted in a significantly smaller impact of the F129L Cytb substitution on metyltetraprole activity (Suemoto et al. 2019; Matsuzaki et al. 2020a). In this study, metyltetraprole retained potent activity against isolates carrying the G137R/S, L299F, N256S + L299F, and L299F + L299F *Cytb* haplotypes. The structural mechanisms to reduce RF for isolates carrying each substitution remain unclear and should be better elucidated. Nevertheless, a small pharmacophore structure might help mitigate the negative impacts of various amino acid substitutions at the Qo site. Metyltetraprole did not increase the frequency of G143A-carrying isolates in *Zymoseptoria tritici* in the field (Matsuzaki et al. 2020a), because it had sufficient efficacy against these QoI-resistant isolates. However, if isolates with QoI resistance that is mediated by other amino acid substitutions in Cytb are insensitive

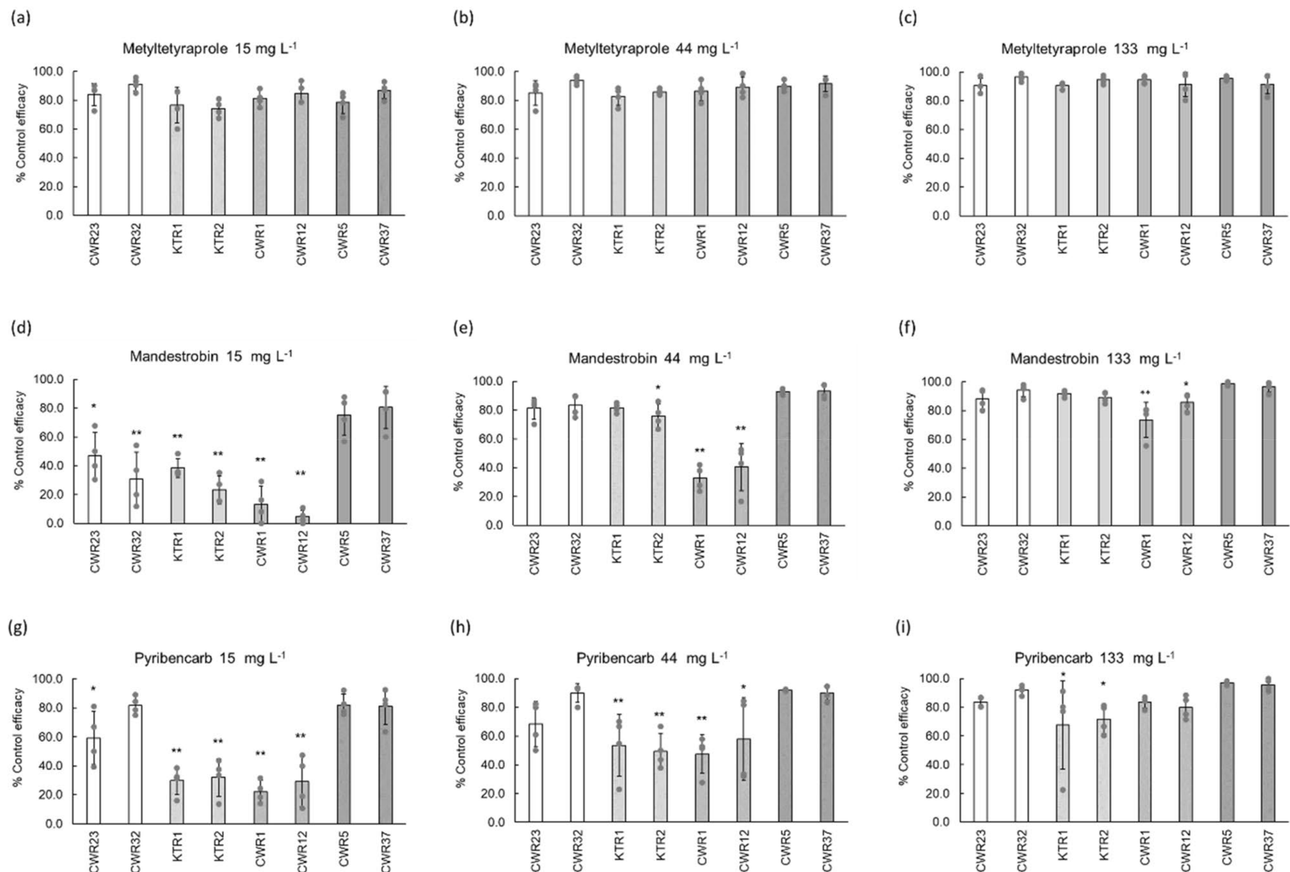


Fig. 2 *In planta* control efficacy (%) of metyltetraprole (**a**–**c**), mandestrobin (**d**–**f**), and pyribencarb (**g**–**i**) against *Puccinia horiana* isolates with L299F, N256S + L299F, L275F + L299F, and wild-type Cytb haplotypes. The error bars represent the standard deviations. Two isolates were tested for each haplotype. The test was performed in four plant replicates. Gray circles represent individual data points. Dunnett's tests were implemented for efficacies of each fungicide against the isolates carrying mutations and the reference CWR5 isolate. Single asterisk means $P < 0.05$. Double asterisks mean $P < 0.01$

to metyltetraprole, they could be rapidly selected when metyltetraprole is used. Our study demonstrated that this situation would not occur for G137R/S, L299F, N256S + L299F, and L275F + L299F. Nevertheless, the sensitivity of fungal strains to metyltetraprole should be continuously monitored in fields to prevent the undesired spread of metyltetraprole-resistant populations. For example, before we began our studies in 2021, Matsuura (2019) first reported *P. horiana* isolates with L275F in Cytb, but their entire *Cytb* sequence has not been elucidated, and the mechanism responsible for low azoxystrobin sensitivities remains unclear. The metyltetraprole activities against those isolates should be investigated in future studies.

Among the four remaining QoI fungicides tested in this study, Kataoka et al. (2010) reported that the efficacy of pyribencarb is less affected by the G143A Cytb substitution in *Botrytis cinerea* and *Corynespore cassicola* (RF 65 and 15, respectively) than that of azoxystrobin (RF 300 and 1200, respectively). However, the reduction in the activity of pyribencarb in G137R-carrying *P. tritici-repentis*, G137S-carrying *V. effusa*, and L299F-carrying *P. horiana* was not substantially smaller (RF > 3.0 except for G137R) than that of mandestrobin, metominostrobin, and orysastrobin (RF > 3.0 in most cases). This finding supports the validity of the QoI use recommendations by the Fungicide Resistance Action Committee (FRAC) to categorize pyribencarb in the same group with other QoI fungicides as Code 11 and assigning metyltetraprole to a distinct subgroup, Code 11A (FRAC 2021).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10327-022-01081-6.

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Declarations

Conflict of interest All authors are employees of Sumitomo Chemical Co., Ltd., the manufacturer of metyltetraprole and mandestrobin.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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