Antifungal activity of metyltetraprole against the existing QoI-resistant isolates of various plant pathogenic fungi

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Abstract

BACKGROUND: Metyltetraprole is a novel quinol oxidation site of Complex III inhibitor (QoI) fungicide that inhibits mitochondrial electron transport at the Qo site of the cytochrome bc1 complex. Previous reports have demonstrated that it is also active against the QoI-resistant (QoI-R) isolates of Zymoseptoria tritici and Pyrenophora teres with the mutations G143A and F129L in their cytochrome b gene, respectively. Further studies on cross-resistance between metyltetraprole and existing Qols were performed using an increased number of isolates of Z. tritici, P. teres, Ramularia collo-cygni, Pyrenophora tritici-repentis, and several other plant pathogenic fungi.

RESULTS: Differences in the EC50 values between the wild-type and QoI-R isolates with the mutations G143A or F129L were always smaller for metyltetraprole compared to those for the existing Qols, and they were never greater than five in terms of resistance factor. The 2-year field experiments showed that the metyltetraprole treatment did not increase the percentage of QoI-R isolates likely to harbor the G143A mutation in a Z. tritici population.

CONCLUSION: The unique behavior of metyltetraprole against the existing QoI-R isolates was confirmed for all tested pathogen species. Our results provide important information to establish a fungicide resistance management strategy using metyltetraprole in combination or alternation with other fungicides.

Keywords: metyltetraprole; QoI; resistance; G143A; F129L; MDR

1 INTRODUCTION

The quinol oxidation site of Complex III inhibitor (QoI) is one of the most important classes of agricultural fungicides with the advantages of high efficacy against a wide range of pathogens, good crop safety, and some favorable physiological effects on crops. However, the efficacy against many pathogens was drastically reduced because of the development of resistance, especially by the mutation corresponding to the amino acid substitution of G143A in the cytochrome b gene (CytB). Before the discovery of metyltetraprole, all previously existing commercial Qols lost their effectiveness against pathogens having a high population of resistant isolates harboring the G143A mutation. For example, Zymoseptoria tritici, one of the representative plant pathogens that affects wheat in Western Europe, has lost sensitivity to existing fungi because of the presence of the G143A mutation. Overcoming this problem of QoI resistance through small structural modification of existing Qols was believed to be difficult because of direct steric interference between the central linking-ring of Qols, which is essential for retaining enough stability under sunlight, and the methyl group of the alanine residue at position 143 of CytB substituted in resistant mutants. Furthermore, the resistant strains harboring the F129L mutation in CytB are known to be a second problematic type of QoI-resistant (QoI-R) mutants, although their strength of resistance is weaker than that of the strains harboring the G143A mutation. The F129L mutation is estimated to obstruct the interaction between the CytB protein and the pharmacophore of Qols. This second type of QoI-R mutants have been reported to show partial resistance to QoI in pathogenic fungi such as Pyrenophora teres, Metyltetraprole, 1-(2-[[1-(4-chlorophenyl)-1H-pyrazol-3-yl]oxy methyl]-3-methylphenyl)-1,4-dihydro-4-methyl-5H-tetrazol-5-one, is a novel fungicide with the unique tetrazolinone pharmacophore found by Sumitomo Chemical Co. Ltd. Its molecule has a side chain very similar to that of pyraclostrobin, a representative of the existing Qols, and it also binds to the Qo site of the cytochrome bc1 complex. A competitive binding assay using radiolabeled metyltetraprole and pyraclostrobin showed that both fungicides

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bound to the same target pocket in the cytochrome bc1 complex.\(^5\) Nevertheless, it was previously demonstrated to be highly active even against the \(Z. \) tritici and \(P. \) teres strains harboring the mutations G143A and F129L, respectively.\(^7\) Therefore, metyltetraprole is considered to be the first molecule of a new innovative generation of QoIs. Although metyltetraprole itself is a highly promising agricultural fungicide, which is expected to be commercialized in the near future (https://sumitomo-chem-agro.com/blog/sumitomo-chemical-submits-a-registration-application-in-the-ee-for-metyltetraprole-a-new-fungicide/), its discovery has paved the way to overcome the mutations G143A and F129L in the history of synthetic QoIs. However, the numbers of tested isolates and pathogen species were limited in our previous reports.\(^7,8\)

In the case of QoIs, it has been reported that the orthologous amino acid mutations result in similar resistance profiles across different organisms with respect to the strength of resistance; the G143A mutation causes very high level of resistance (the resistance factor RF, \([\text{EC}_{50}\text{ of the resistant mutant}] / [\text{EC}_{50}\text{ of the wild type}]\), is always greater than 100) to all commercial QoIs in any known cases of more than 20 pathogen species.\(^1,2\) However, recently some cases of different resistance profiles within the orthologous mutations of different pathogen species were reported in cases of resistance against succinate dehydrogenase inhibitor (SDHI), another important agricultural fungicide class.\(^10–13\) Considering the unique behavior of metyltetraprole in the G143A mutants of \(Z. \) tritici as well as in the F129L mutants of \(P. \) teres, its consistency in orthologous mutations in different species should be investigated.

Furthermore, in recent years, a fungicide resistance mechanism different from the target-site mutations has been reported in some species, including \(Z. \) tritici. It is a multi-drug resistance (MDR) phenotype resulting from the overexpression of a multidrug transporter, such as the major facilitator superfamily (MFS) transporter in the plasma membrane.\(^14–17\) In the case of \(Z. \) tritici, the overexpression of MgMFS1 associated with the insertions to the genomic sequence upstream of its open reading frame (ORF) was detected in field isolates that showed an MDR phenotype.\(^14\) Such isolates have been reported to show a decreased sensitivity to various classes of fungicides, regardless of their structures.\(^14–17\) Such a sensitivity shift due to the lower uptake of chemicals into the cells in an MDR phenotype should be checked for metyltetraprole because its target site is assumed to be located in the mitochondria inside the cell.

We report on the cross-resistance studies between metyltetraprole and previously existing QoIs in various pathogenic fungi. At first, each representative pair of the wild-type (WT) and QoI-resistant isolates harboring the G143A or F129L mutation was used to compare the RFs of metyltetraprole and existing QoIs. Thereafter, the influence of the QoI sensitivity on the metyltetraprole sensitivity was investigated in field populations of \(Z. \) tritici, Pyrenophora tritici-repentis, Ramularia collo-cygni, and \(P. \) teres in Western Europe. Furthermore, the effect of an MDR phenotype on the antifungal activity of metyltetraprole was investigated in \(Z. \) tritici.

2 MATERIALS AND METHODS

2.1 Chemical materials
Salicylhydroxamic acid (SHAM) and 2,3-dihydroxybenzaldehyde (DHBA) were purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Pyraclostrobin, azoxystrobin, fluxapyroxad, prothioconazole-desthio, tolnaftate, and tebufenpyrad were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Metyltetraprole (>=99% purity) was synthesized as previously described.\(^6\) For conducting in vitro experiments, all chemical compounds were dissolved in dimethyl sulfoxide as stock solutions. For carrying out the metyltetraprole treatment in fields, an emulsifiable concentrate (EC) formulation of metyltetraprole was prepared by Sumitomo Chemical.

2.2 Fungal materials
The \(Z. \) tritici, \(R. \) collo-cygni, \(P. \) teres, and \(P. \) tritici-repentis strains were isolated from wheat or barley leaves in the fields of European countries (Table S1). Detached leaves were kept in humid conditions to induce spore formation. A single spore was collected under the microscope and grown on potato dextrose agar (PDA) medium (39 g PDA in 1 L water). The origins of the strains of Parastagonospora nodorum, Microdochium majus, Colletotrichum gloeosporioides, Pycnoria oryzae, Venturia inaequalis, Alternaria solani, Passalora fulva, and Pestalotiopsis longiseta are shown in Table S1.

2.3 Antifungal tests
The antifungal activity of all tested compounds against \(Z. \) tritici, \(R. \) collo-cygni, \(P. \) teres, \(P. \) tritici-repentis, \(P. \) nodorum, \(M. \) majus, \(C. \) gloeosporioides, \(P. \) oryzae, \(V. \) inaequalis, \(A. \) solani, \(P. \) fulva, and \(P. \) longiseta was evaluated by two different methods under the incubation conditions detailed in Table S2. Synergic agents such as alternative oxidase (AOX) inhibitors (e.g., SHAM) were added only for the species in which growth was not completely inhibited by QoI in a low nutrient medium (e.g., YBG) because those agents are themselves harmless to fungal growth. The RF was calculated using the following formula:

\[
RF = ([\text{EC}_{50}\text{ of resistant isolate}] / [\text{EC}_{50}\text{ of WT}]).
\]

2.3.1 Microtiter plate (96-well) method
The growth of \(Z. \) tritici, \(R. \) collo-cygni, \(P. \) nodorum, \(C. \) gloeosporioides, \(V. \) inaequalis, and \(P. \) fulva was evaluated in 96-well microtiter plates. The inoculum of each fungal strain was harvested at more than 100 times higher density in distilled water and suspended in the appropriate medium at the density shown for each species (Table S2). A 100-fold dilution series of the active ingredient at the designated final concentration was prepared for each test (Table S2). Aliquots (1 \(\mu\)L) of each fungicide were mixed with the inoculum (99 \(\mu\)L) prepared in medium. After incubating all fungal strains for the incubation periods listed in Table S2, their growth was determined by measuring the optical density of each culture solution at a wavelength of 600 nm using the microplate reader SH-9000 Lab (Corona Electric, Ibaraki, Japan) with a 3 \(\times\) 3 matrix of scanning points. The optical density values were corrected by the value of the blank well without inoculum. The 50% effective concentrations (EC\(_{50}\)) were calculated by probit analysis.

2.3.2 Agar plate method
Pyrenophora teres, \(P. \) tritici-repentis, \(M. \) majus, \(A. \) solani, \(P. \) oryzae, and \(P. \) longiseta were cultured on agar medium amended with a series of designated concentrations of fungicides (see Table S2). The mean mycelial radial growth (mm) from two different inocula on each agar plate was measured at designated periods after inoculation, and the EC\(_{50}\) values were calculated by probit analysis.
described, and the sensitivity tests were performed for five iso-

phism (PCR-RFLP) was carried out as previously described, using

individual trials. An untreated plot (both were divided into three to four replicates

each pathogenspecies. 3,18–28 DNA sequencing was also performed

for the identification of the genotypes of

USA) according to the manufacturer’s instructions. PCR amplifica-

the PrepMan Ultra Reagent (Applied Biosystems, Waltham,

Kyoto, Japan) based on the previously described methods for

The presence of mutation in each resistant isolate was checked as

previously described.5,18–28 The DNA of each fungi was extracted using

the restriction enzymes (BsaI, Fnu4HI, and MnlI) and primers listed

in Table S3.20 Sequences were analyzed by the GENETYX ver. 12

software (Genetyx Corporation, Tokyo, Japan).

2.4 Sampling of isolates from the field trial

of metyltetraprole

Leaves were collected from wheat plants treated with an EC formu-
lation of metyltetraprole at 120 g hectare\(^{-1}\) and untreated wheat

plants from 20 fields in 2014 and 22 fields in 2015, when the field

efficacy evaluation of metyltetraprole was performed. The treat-

ments were carried out by contractor companies according to the

guidelines of the European and Mediterranean Plant Protection

Organization (http://pp1.eppo.int/). The sampling of leaves was

also done by contractor companies 25–40 days after the metylte-

traprole treatment. Leaves for sampling were selected randomly

from each plot treated with metyltetraprole at 120 g hectare\(^{-1}\) and

an untreated plot (both were divided into three to four replicates

in individual trial sites). Z. tritici isolates were done as previously

described,\(^{7}\) and the sensitivity tests were performed for five iso-

lates from each field, except for a few sites from where only four

strains could be isolated.

2.5 Statistical evaluation

Statistical evaluations were performed with the statistic software

BellCurve for Excel (Social Survey Research Information Co., Ltd,

Tokyo, Japan). The significance of differences in the median EC50

values was analyzed by the Mann–Whitney U-test. The tests for the

equality of two proportions were performed for the percentage of

putative G143A mutants in each of the untreated and metyltetrap-

role (120 g L\(^{-1}\))-treated populations.

2.6 Identification of mutations in each pathogen species

The presence of mutation in each resistant isolate was checked as

previously described.5,18–28 The DNA of each fungi was extracted using

the restriction enzymes (BsaI, Fnu4HI, and MnlI) and primers listed

in Table S3.20 Sequences were analyzed by the GENETYX ver. 12

software (Genetyx Corporation, Tokyo, Japan).

3 RESULTS

3.1 Resistance factors for the representative sets of several

pathogen species

The test results for the G143A mutants are shown in Table 1 and

for the F129L mutants in Table 2. The EC50 values of metylte-

traprole were always lower than 0.1 mg L\(^{-1}\) for the WT isolates

of all tested species. In G143A mutants, the EC50 values of pyra-

clostrobin and azoxystrobin were significantly higher than those

in the WT isolates, regardless of the species tested, as already

reported.1–3,16,21–25 The mean RFs were >2000 and >400 for pyra-

clostrobin and azoxystrobin, respectively. On the other hand, the

EC50 values of metyltetraprole in the G143A mutants were less

than twice of those in the WT isolates for all tested species, except

for V. inaequalis. The RFs of metyltetraprole ranged from 0.17 to

5.17, and the mean was 1.6. In F129L mutants, the EC50 values

of pyraclostrobin and azoxystrobin were also higher than those in

the WT isolates, regardless of the species tested, as those differ-

ences were smaller than those of the G143A mutants, as already

reported.1,2,18,19,25–27 The mean RFs were 15.8 and 90.6 for pyra-

clostrobin and azoxystrobin, respectively. On the other hand, the

EC50 values of metyltetraprole in the F129 L mutants were not so

different from those in the WT isolates among all tested species.

The RFs ranged from 0.7 to 3.3, and the mean was 2.1.

3.2 Z. tritici population in Western Europe

A total of 377 isolates were collected from five countries in June

and July of 2015. In the box-whisker plot for the EC50 values of

metyltetraprole and pyraclostrobin, the population was divided

into two different groups based on their sensitivity to pyra-

clostrobin (Fig. 1). The isolates showing the pyraclostrobin EC50

values greater than 0.05 mg L\(^{-1}\) were classified as the QoI-R isolates

and were expected to harbor the G143A mutation.2,8 The median

EC50 value for metyltetraprole in the QoI-sensitive (QoI-S) popu-

lation (isolates whose pyraclostrobin EC50 values were less than



|                      | Wild type | G143A | Resistance factor² | Wild type | G143A | Resistance factor² | Wild type | G143A | Resistance factor² |
|----------------------|-----------|-------|--------------------|-----------|-------|--------------------|-----------|-------|--------------------|
| Z. tritici           | 0.00048    | 0.00077 | 1.60              | 0.00050    | 0.42   | 840.0              | 0.012      | 5⁰       | >400              |
| P. tritici-repentis   | 0.0067    | 0.0054 | 0.81              | 0.00085    | 0.43   | 505.9              | 0.0042     | 2.5      | 595.2             |
| M. majus             | 0.0068    | 0.013  | 1.91              | 0.00020    | 2.2    | 11000.0            | 0.015      | 10⁰      | >650             |
| R. collo-cygni       | 0.0019    | 0.00033 | 0.17            | 0.00046    | 0.67   | 1456.5            | 0.011      | 0.82      | 74.5              |
| C. gloeosporioides    | 0.010     | 0.010  | 1.00              | 0.0040     | 0.52   | 130.0             | 0.029      | 10⁰      | >300             |
| P. tritici-repentis   | 0.029     | 0.025  | 0.86              | 0.0039     | 3.0    | 769.2             | 0.058      | 50⁰       | >800             |
| P. oryzae            | 0.047     | 0.056  | 1.19              | 0.0072     | 0.61   | 84.7              | 0.040      | 1⁰        | >25              |
| V. inaequalis        | 0.0060    | 0.031  | 5.17              | 0.0030     | 1⁰     | >300              | 0.0030     | 10⁰       | >1000            |

Mean of resistance factor 1.6 >2000 >400

Table 1. Cross-resistance on representative sets of several pathogen species (wild types and G143A mutants)

² Resistance factor (EC50 value for the mutant isolate/EC50 value for the wild-type isolate).
³ Cut-off value.
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observed almost no difference in the metyltetraprole EC50 values were expected to harbor the G143A mutation. The median EC50 values in the QoI-S isolates were 0.0029, 0.0028, 0.0022 mg L−1, whereas that of the WT isolates was 0.019 mg L−1, indicating almost no effect of QoI-resistance on the activity of metyltetraprole against R. collo-cygni. For P. tritici-repentis, 64 isolates were collected in 2015, of these, 45 were highly resistant to pyraclostrobin (QoI-R isolates showing EC50 > 1 mg L−1) and were expected to harbor the G143A mutation. In the isolates whose growth was inhibited by 1 mg L−1 of pyraclostrobin, we checked the presence of the F129L mutation by PCR-RFLP as well as by sequencing of fragments. As a result, seven isolates were found to contain the F129L mutation and 12 isolates did not (QoI-S isolates). The median EC50 value for metyltetraprole in the QoI-R isolates was 0.0065 mg L−1, whereas that in QoI-S isolates was 0.016 mg L−1 (Fig. 2(b)). The result of the Mann–Whitney U-test indicated that the difference of EC50 values for metyltetraprole between the QoI-S and QoI-R isolates was statistically significant (P = 0.0027). Thus, we concluded that the metyltetraprole sensitivity of the QoI-R isolates, which are likely to harbor G143A mutation, is slightly higher than that of the QoI-S isolates of P. tritici-repentis.

### Table 2. Cross-resistance in representative sets of several pathogen species (wild types and F129L mutants)

| Pathogen                        | EC50 (mg L−1)a | Resistance factorb | EC50 (mg L−1)a | Resistance factorb | EC50 (mg L−1)a | Resistance factorb |
|---------------------------------|----------------|-------------------|----------------|-------------------|----------------|-------------------|
| **Metyltetraprole**             |                |                   |                |                   |                |                   |
| Wild type                       | 0.0035         |                   | 0.0013         | 0.00043           | 0.00016         |                   |
| F129L                           | 0.0055         | 1.6               | 0.0086         | 6.6               | 0.016           | 90.6              |
| Pyrenophora teres               | 0.00060        | 1.8               | 0.011          | 9.9               | 0.0057          | 45.9              |
| Zymoseptoria tritici            | 0.0029         | 0.7               | 0.0039         | 35.9              | 0.00057         | 28.1              |
| Alternaria solani               | 0.0029         | 1.6               | 0.0073         | 5.9               | 0.00057         | 28.1              |
| Pestalotiopsis longiseta        | 0.011          |                   | 0.011          | 3.9               | 0.0065          | 1.2               |
| **Pyraclostrobin**              |                |                   |                |                   |                |                   |
| Wild type                       | 0.00060        | 1.8               | 0.00090        | 14.4              | 0.00097         | 166.7             |
| F129L                           | 0.0017         |                   | 0.013          |                   | 0.0031          |                   |
| Pyrenophora tritici-repentis    | 0.00029        | 0.7               | 0.0065         | 45.9               | 0.0045          | 75.7              |
| Zymoseptoria tritici-repentis   | 0.00029        | 0.7               | 0.0057         | 28.1               | 0.0065          | 1.2               |
| **Azoxystrobin**                |                |                   |                |                   |                |                   |
| Wild type                       | 0.00060        | 1.8               | 0.00090        | 14.4              | 0.00097         | 166.7             |
| F129L                           | 0.0017         |                   | 0.013          |                   | 0.0031          |                   |
| Pyrenophora tritici-repentis    | 0.00029        | 0.7               | 0.0065         | 45.9               | 0.0045          | 75.7              |
| Zymoseptoria tritici-repentis   | 0.00029        | 0.7               | 0.0057         | 28.1               | 0.0065          | 1.2               |

a Values represent the mean 50% effective concentration (EC50) values (mg L−1) of each isolate in two replicates. The 95% confidence intervals of all EC50 values ranged between 50% (MIN) and 200% (MAX) of the representative values.
b Resistance factor (EC50 value for the mutant isolate/EC50 value for wild type isolate).

3.3 Putative G143A isolates of R. collo-cygni and P. tritici-repentis

In Fig. 2(a),(b), the box-whisker plots show the EC50 values of metyltetraprole for both pathogens. For R. collo-cygni, 88 isolates were collected in 2015; of these 82 were highly resistant to both azoxystrobin and pyraclostrobin (QoI-R isolates, EC50 > 0.1 mg L−1) and were expected to harbor the G143A mutation. The median EC50 value of metyltetraprole in the QoI-R population was 0.0029 mg L−1, and the EC50 values in the QoI-S isolates were 0.0029, 0.0028, 0.0029, 0.0035, 0.0039, and 0.0051 mg L−1, indicating almost no effect of QoI-resistance on the activity of metyltetraprole against R. collo-cygni. For P. tritici-repentis, 64 isolates were collected in 2015; of these, 45 were highly resistant to pyraclostrobin (QoI-R isolates showing EC50 > 1 mg L−1) and were expected to harbor the G143A mutation. In the isolates whose growth was inhibited by 1 mg L−1...
mutants were collected as previously mentioned. The median EC50 value in the F129L mutants was 0.034 mg L\(^{-1}\), whereas that in the WT isolates was 0.016 mg L\(^{-1}\) (Fig. 3(b)). The result of the Mann–Whitney U-test did not support the statistical significance of this difference (\(P = 0.50\)) of the EC50 values between the WT and F129L strains of \(P. tritici-repentis\).

3.5 Effect of multi-drug resistance on sensitivity to metyltetraprole

For the isolates of \(Z. tritici\) collected in 2015, we identified the isolates showing an MDR phenotype based on their sensitivity to tolnaftate.\(^{14,15}\) As a result, 15 among 377 isolates showed an MDR phenotype based on their sensitivity to metyltetraprole expressed in a log10 scale for each of the WT isolates and F129L mutants of (a) \(P. teres\) and (b) \(P. tritici-repentis\). \(^*P < 0.05\) in the Mann–Whitney U-test.

The results showed a limited effect of enhanced efflux activity on sensitivity to metyltetraprole with RF values <5.

3.6 Effect of the selection pressure of metyltetraprole on pathogen population

Table 3 shows the frequency of QoI-R isolates in the isolates of \(Z. tritici\) obtained from the leaves of the metyltetraprole-treated (two sprays on 20- to 35-day intervals at 120 g hectare\(^{-1}\)) and untreated wheat plants of an individual trial field. No increase was observed in the frequency of QoI-R isolates that showed resistance to pyraclostrobin (EC50 > 0.05 mg L\(^{-1}\)) in both experiments conducted in 2014 and 2015 (\(P = 0.08\) and 0.59, respectively).

4 DISCUSSION

Our study confirmed lower RFs of metyltetraprole in QoI-R isolates of all tested pathogen species. However, the presence of a small, but statistically significant, difference between the WT and QoI-R isolates was not always consistent across the studied pathogen species, though its magnitude in the RFs of metyltetraprole across species was small. For example, the G143A mutation seemed to affect metyltetraprole sensitivity neither in \(Z. tritici\) nor in \(R. collo-cygni\) (Figs 1 and 2(a)). On the other hand, the same G143A mutation seemed to confer a slight, but statistically significant, increase in metyltetraprole sensitivity in \(P. tritici-repentis\) (Fig. 2(b)). Furthermore, the F129L mutation seemed to confer a slight, but statistically significant, reduction of metyltetraprole sensitivity in \(P. teres\) (Fig. 3(a)), although its RF value was much smaller than that of the existing QoIs. The RF calculated by the median EC50 value of the WT and F129L isolates was only 1.6, while that of the existing QoIs has been reported to range from 5 to 20.\(^{1,2,19}\) On the other hand, the reduction in metyltetraprole sensitivity was not statistically significant in the F129L mutants of \(P. tritici-repentis\) in this study, though the number of samples was limited and, therefore, more experiments might be required for solid conclusions with general applicability (Fig. 3(b)). Such inconsistency across species could be attributed to a minor difference in the original structure of the Qo site in each species due to amino acid variations in the Qo site at several positions other than positions 129 and 143 (Fig. S1).

Specifically, the target Qo site structure of the CytB protein is slightly different across the studied pathogen species, even in the WT isolates, thus the structural influence of the mutation G143A/F129L could also be slightly different. Some other factors might also affect the variation in RFs across species, for example the effect of mutations on the expression level of the \(AOX\) gene that encodes a well-known enzyme (alternative oxidase), whose overexpression can reduce the in vitro fungal sensitivity to QoIs as well as QIs.\(^{29-33}\) to maintain the balance of homeostasis might also be variable across species. In fact, we observed that the addition of salicylic hydroxamic acid (SHAM) could increase the sensitivity of \(R. collo-cygni\) to metyltetraprole (Table S4). However, the reduction of sensitivity conferred by \(AOX\) overexpression has been reported to play a minor role in practical efficacy reduction in vivo.\(^{2}\) In addition, differences in the environmental fitness of the G143A and F129L strains across species\(^{34-38}\) suggest a variable physiological effect conferred by these orthologous mutations in each species, and it could be relevant to a slight secondary effect.
Table 3. Impact on pathogen population by the selection pressure from metyltetraprole (Z. tritici)

| Trial site       | Untreated | Metyltetraprole 120 g L⁻¹ | 2014 numbers of QoI-sensitive/resistant isolates | 2015 numbers of QoI-sensitive/resistant isolates |
|------------------|-----------|----------------------------|-----------------------------------------------|-----------------------------------------------|
| France (site 1)  | 0/5       | 0/5                        | 7 (7.1%)                                      | 8 (7.3%)                                      |
| France (site 2)  | 0/5       | 1/4                        | 15 (15.2%)                                    | 10 (9.3%)                                     |
| France (site 3)  | 1/4       | 0/5                        | 92 (92.9%)                                    | 102 (92.7%)                                   |
| France (site 4)  | 0/5       | 0/5                        | 84 (84.9%)                                    | 97 (90.7%)                                    |
| France (site 5)  | 1/4       | 3/2                        |                                               |                                               |
| France (site 6)  | 0/5       | 1/4                        |                                               |                                               |
| France (site 7)  | 0/5       | 1/4                        |                                               |                                               |
| France (site 8)  | 2/3       | 4/1                        |                                               |                                               |
| UK (site 1)      | 0/5       | 0/5                        |                                               |                                               |
| UK (site 2)      | 0/5       | 0/5                        |                                               |                                               |
| UK (site 3)      | 0/4       | 0/5                        |                                               |                                               |
| UK (site 4)      | 0/5       | 0/5                        |                                               |                                               |
| Ireland (site 1) | 0/5       | 0/5                        | 1/4 (1.4%)                                    | 1/4 (1.4%)                                    |
| Ireland (site 2) | 0/5       | 1/4                        | 8 (8.0%)                                      | 8 (8.0%)                                      |
| Ireland (site 3) | 1/4       | 4/1                        | 102 (102.0%)                                  | 102 (102.0%)                                  |
| Germany (site 1) | 0/5       | 0/5                        | 92 (92.9%)                                    | 92 (92.9%)                                    |
| Germany (site 2) | 1/4       | 0/5                        | 84 (84.9%)                                    | 84 (84.9%)                                    |
| Germany (site 3) | 1/4       | 0/5                        |                                               |                                               |
| Germany (site 4) | 0/5       | 0/5                        |                                               |                                               |
| Belgium (site 1) | 0/5       | 0/4                        |                                               |                                               |
| Belgium (site 2) | 1/4       | 0/5                        |                                               |                                               |

* Number and percentage of QoI-sensitive isolates (sensitive to pyraclostrobin; EC₅₀ < 0.01 mg L⁻¹) and QoI-resistant isolates (resistant to pyraclostrobin; EC₅₀ > 0.05 mg L⁻¹).

on metyltetraprole sensitivity. Nevertheless, our results indicate that the mutations G143A and F129L had very little effect on metyltetraprole sensitivity in all tested species.

The reduction of sensitivity towards metyltetraprole by an MDR phenotype was slightly greater in terms of its magnitude than that conferred by the G143A and F129L mutations in Z. tritici. The median EC₅₀ values of metyltetraprole in MDR isolates were approximately five times bigger than those in non-MDR isolates and thus were almost similar to those of fluxapyroxad and prothioconazole-desthio. In general, there is no or very limited reduction or difference expected for calculated RF values <5 in terms of field efficacy. In fact, we have not observed significant reduction in the effectiveness of metyltetraprole caused by the presence of MDR isolates in field conditions (data not shown). However, these results indicate that the impact of an MDR phenotype on metyltetraprole sensitivity as well as the determination of the selection risk of an MDR phenotype caused by the use of metyltetraprole should be carefully and continuously investigated. This estimation would be also applicable for other newer fungicides that are not or are less affected by any specific mutations in the target sites of the currently available fungicides.

Our results of the cross-resistance studies on the isolates and populations in field conditions confirm the unique position of metyltetraprole as a promising Qol in resistance management strategies. In particular, it is notable that metyltetraprole did not increase the frequency of the G143A mutation (Table 3), although further accumulation of data would be desirable, especially for those cases where the ratio of G143A mutants is still low. On the other hand, a number of previous studies on yeast and other few model microorganisms have been published with respect to Qol-resistant laboratory mutants. Although some studies on plant pathogenic fungi and synthetic Qols, which are used as agricultural fungicides, have reported the risk of the mutations G143A, G143S, and F129V, older studies on natural substances, like streptomycin A and myxothiazol, have reported that other mutations, such as A126T, F129L, Y132C, C133Y, G137S, G137E, G137V, G143A, N256K, N256I, N256Y, L275F, L275S, L275T, and L295F, can cause resistance to those substances in yeast or other model microorganisms. The manner of interaction between these natural substances and CytB protein at the Qo site could be distinct from that between the existing synthetic agricultural Qols and the Qo site because the former does not have a central linking ring, which is assumed to be involved in steric interference with the methyl group of the alanine residue at position 143 in CytB of the G143A mutants. Although the manner of binding of metyltetraprole to the Qo site, owing to the presence of its unique tetrazolinone pharmacophore, would be different from both existing synthetic Qols and natural substances, we should consider the risk of cross-resistance. Some of the above-mentioned or other new mutations could be selected as ‘metyltetraprole-resistant mutations’ in future. The existing synthetic agricultural Qols have led to the selection of only G143A and F129L mutations, as well as of G137R with very low frequency, in the past two
decades, suggesting that 'metyltetraprole-resistant mutants' have not been selected by the existing QoIs. It could therefore be possible to hypothesize that metyltetraprole-resistant mutants resulting from a new mutation might not be highly resistant to the existing synthetic agricultural QoIs. This assumption could be made for major QoIs, such as azoxystrin, pyraclostrobin, and trifloxystrobin, which have been used more frequently than others and have played a major role in the selection of the G143A and F129L mutants. Thus, in cases where the population is still sensitive to the existing QoIs, a mixture of or an alternation between metyltetraprole and major existing QoIs might effectively serve as a resistance management tool for each other. However, these hypotheses need careful evaluation and further research activities.

In the past, diethofencarb was introduced as a special fungicide that controlled carbandazin-resistant strains harboring the mutation E198A or E198G in the target site β-tubulin. However, with time, strains possessing new mutations in the β-tubulin protein and hence resistant to both carbandazin and diethofencarb emerged. Additionally, according to our data, there is a slight difference in the sensitivities between QoI isolates and F129L mutants in P. teres, therefore despite having sufficient activity against F129L mutants, metyltetraprole could increase their frequency. Although its selection strength might not be as high as the existing QoIs owing to the small sensitivity difference between QoI isolates and F129L mutants, the risk of F129L selection by metyltetraprole is the subject of ongoing research in our laboratory. Furthermore, in the case of the pathogen populations in which the G143A mutant isolates are already dominant, a new metyltetraprole-resistant mutant might emerge as a strain that is resistant to both metyltetraprole and existing QoIs because of the stacking of two mutations, the G143A mutation and a new mutation causing metyltetraprole resistance. On the other hand, there is a possibility that the stacking of two mutations might reduce environmental fitness and might have less chance of emerging as a practical problem for metyltetraprole. In fact, the compound mutation of F129L with G143A has not been observed in the field isolates of any plant pathogen, and it could be difficult for such mutations to survive in field conditions. Young et al. described the mode of action of fenpicloram, which is also a novel fungicide for controlling Z. tritici. They showed that fenpicloram inhibited cytochrome bc1 complex at the Qi site, which is a distinct catalytic site from the Qi site. Since mutations in CytB, which confers resistance to fenpicloram, are located at the Qi site, far from the Qi site, it can be inferred that they will not directly affect the sensitivity to metyltetraprole. However, their influence on enzyme efficiency or protein folding could affect the possibility of acquiring novel mutations at the Qi site because further accumulation of mutations in CytB could result in a stronger fitness penalty. The applicability of this hypothesis could be addressed in future studies. Nevertheless, the strategy for metyltetraprole resistance management should be established based on the individual background situation in each crop and region. We hope that our report will help researchers involved in the establishment of fungicide resistance management programs in each crop and region to consider the proper adoption of metyltetraprole in local fungicide application programs.

SUPPORTING INFORMATION
Supporting information may be found in the online version of this article.

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