Research Article

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Microtiter plate test using liquid medium is an alternative method for monitoring metyltetraprole sensitivity in *Cercospora beticola*

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Abstract

BACKGROUND: Metyltetraprole is a new quinone outside inhibitor (QoI) fungicide showing potent activity against QoI-resistant fungi that possess the G143A cytochrome *b* mutation, which confers resistance to existing QoIs such as trifloxystrobin. For its sustainable use, monitoring of metyltetraprole sensitivity is necessary and the establishment of appropriate methodology is important in each pathogen species.

RESULTS: In *Cercospora beticola*, the causal agent of sugar beet leaf spot, some isolates were less sensitive to metyltetraprole (EC_{50} > 1 mg L^{-1}, higher than the saturated concentration) using the common agar plate method, even with 100 mg L^{-1} salicylhydroxamic acid, an alternative oxidase inhibitor. However, microtiter tests (EC_{50} < 0.01 mg L^{-1}), conidial germination tests (EC_{50} < 0.01 mg L^{-1}) and *in planta* tests (>80% control at 75 mg L^{-1} run-off spraying) confirmed that all tested isolates were highly sensitive to metyltetraprole. For trifloxystrobin, G143A mutants were clearly resistant upon microtiter plate tests (median EC_{50} > 2 mg L^{-1}) and distinct from wild-type isolates (median EC_{50} < 0.01 mg L^{-1}). Notably, mycelium fragments were usable for the microtiter plate tests and the test was applicable for isolates that do not form sufficient conidia. Our monitoring study by microtiter plate tests did not indicate the presence of metyltetraprole-resistant *C. beticola* isolates in populations in Hokkaido, Japan.

CONCLUSION: The microtiter tests were revealed to be useful for monitoring the sensitivity of *C. beticola* to metyltetraprole and trifloxystrobin. © 2020 The Authors. *Pest Management Science* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

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

Keywords: metyltetraprole; quinone outside inhibitors; G143A; *Cercospora*

1 INTRODUCTION

Metyltetraprole,[1–3] being developed under the trade name Pavecto®, is a new quinone outside inhibitor (QoI) that overcomes QoI-resistant fungi harboring G143A or F129L mutations in cytochrome *b* (*Cytb*), which are very problematic for disease management in many crops.[4] Although metyltetraprole is very effective against current QoI-resistant fungal populations, new genotypes of metyltetraprole-resistant strains might emerge in the future. Therefore, early detection of such resistant isolates is important to implement a countermeasure before its critical spread. More importantly, the accuracy of any monitoring method is of paramount importance to avoid making decisions on false-positive or false-negative results. As already reported, metyltetraprole shows potent activity against a broad range of species in Ascomycota; its activity against *Cercospora beticola*, the causal agent of sugar beet leaf spot, has not yet been reported.[1] However, some fungal species such as *Cercospora* spp. and *Corynespora* spp. are not highly sensitive to QoI upon agar plate tests, which are commonly used for other fungicide classes such as for sterol biosynthesis inhibitors, even though those fungi are highly QoI-sensitive in *in planta* tests.[5,6] The reason for the low sensitivity in agar plate tests is attributed mainly to the expression of alternative oxidase (AOX), which can regulate the redox balance in mitochondria by oxidizing redundant ubiquinol.[7–12] By contrast, this enzyme plays a minor role in *in planta* QoI sensitivity as it does not generate sufficient ATP to achieve complex and energy-

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consuming host infection processes.[13] In addition, some reports have indicated that plant flavonoids inhibit the induction of *AOX*.[9] Therefore, normal agar plate tests using mycelium inoculum are not always appropriate to confirm QoI sensitivity. In cases where AOX is the main reason for low sensitivity in common agar medium, iron chelating agents such as salicylhydroxamic acid (SHAM) or *n*-propyl gallate have been used successfully to minimize the influence of AOX, as it is an iron-requiring metalloenzyme.[7,11,12,14–16] Nevertheless, according to the literature as well as our laboratory findings, in some *Cercospora* spp., *Colletotrichum* spp. and *Corynespora cassiicola*, depending on the strains, ≈100 mg L^{-1} of SHAM is not always sufficient to recover QoI sensitivity in agar medium, especially if mycelium is used as the inoculum.[10,11,17] To solve this issue, Inada *et al.*[18] used increased concentrations of SHAM and azoxystrobin (flowable formulation) of 1000 and 100 mg L^{-1}, respectively. This approach was successful in identifying G143A-harboring isolates of *Colletotrichum gloeosporioides*. However, metyltetraprole has lower water solubility (0.12 mg L^{-1}) as compared with other QoIs (1 > mg L^{-1} in general) and crystallizes in the medium at oversaturated concentrations when added as an organic solvent solution (e.g. acetone, dimethyl sulfoxide). Such crystallization interrupts evaluation of precise sensitivity owing to its decreased availability to fungal cells. For a more precise monitoring method, metyltetraprole needs to exhibit clear activity against sensitive strains at sufficiently low concentrations so that the distinction from resistant strains becomes obvious. Although we have not found relevant reports which have described such issues, a few QoIs such as trifloxystrobin (water solubility: 0.61 mg L^{-1}) seem to have the same problem when the flowable formulation is not available (Matsuzaki *et al.*, unpublished). Moreover, addressing the sensitivity issues using SHAM or *n*-propyl gallate may not be ideal given that they are not selective AOX inhibitors; instead, they are metal chelating agents that can affect many other enzymes.[19–22] Therefore, AOX inhibitors may have multiple physiological effects on fungi. In fact, 1000 mg L^{-1} SHAM has been shown to be very toxic and it inhibits relative growth of *C. gloeosporioides* by ≈70% with unspecified secondary effects.[18]

An alternative for QoI sensitivity tests is testing conidial germination rate on agar plates instead of determining the radial growth of mycelium. For example, in *Cercospora* spp., inhibition testing of conidial germination is reported to be a suitable method for sensitivity checks because conidial germination is more sensitive to QoIs than mycelial growth on agar plates.[5,15,23–27] This method requires significant counting of conidia under a microscope but some fungal pathogens, depending on the strain, often lose the ability to form conidia after continuous cultivation and storage in medium.[15,28–30] 30 As observed in the study by Kayamori *et al.*,[15] we also encountered difficulty in obtaining a sufficient number of conidia from some Japanese isolates of *C. beticola* for this study, even with the current standard method of forming conidia that is established for USA isolates of this species.[23] This issue affected evaluation of the difference in metyltetraprole sensitivity between current and old isolates collected before the use of QoIs, as the latter no longer formed enough conidia after long-term storage (Matsuzaki *et al.*, unpublished).

Instead of agar plate tests using mycelia or conidia, Spiegel and Stammler[31] reported that the microtiter plate tests with low-nutrient liquid medium were appropriate and SHAM was not necessary for testing QoI sensitivities of *Monilinia* spp. This method also was successful for testing pyraclostrobin sensitivities in *Phyllosticta* spp.[32] and *Leptosphaeria maculans*.[33] Although we previously showed that the microtiter plate test was useful in testing the metyltetraprole sensitivity of some fungal species,[3] we had not adopted this method to investigate *C. beticola*.

In this study, we developed a microtiter plate test system using mycelial fragments as inoculum for monitoring metyltetraprole sensitivity in *C. beticola*. The method was checked to determine if the metyltetraprole sensitivities in it are impacted by the different expression levels of *AOX* in each isolate. In addition, with this methodology, we monitored metyltetraprole sensitivity of *C. beticola* collected in Hokkaido, Japan, where widespread QoI resistance conferred by the G143A mutation in *Cytb* has been reported[15]; this mutation also is prevalent in other sugar beet production areas in the world.[25,34,35]

2 MATERIALS AND METHODS

2.1 Chemical materials

SHAM was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The analytical standard trifloxystrobin was purchased from Sigma-Aldrich (Tokyo, Japan). Metyltetraprole (>99% purity) was synthesized in our laboratory as described previously.[2] For conducting agar medium and liquid medium tests, all chemical compounds were dissolved in dimethyl sulfoxide to make 10 mg mL^{-1} stock solutions, which subsequently were diluted to obtain the desired solutions. For conducting *in planta* tests, 25% trifloxystrobin flowable formulation (Flint, Bayer Cropscience Japan, Tokyo, Japan) was purchased and a 40% suspension concentrate formulation of metyltetraprole was prepared.[36]

2.2 Fungal materials

The *C. beticola* strains were isolated in Japan (Supporting Information, Tables S1 and S2) and kept on potato dextrose agar (PDA) medium (39 g in 1 L water) at 27 °C for normal cultivation and 12 °C for storage. The presence of mutations in *Cytb* in each resistant isolate was checked as previously described.[25] The DNA from mycelia of each isolate was extracted using PrepMan Ultra Reagent (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the *Cytb* gene were performed using the Ex Taq Kit (TaKaRa, Kyoto, Japan) by 40 cycles of 98 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. DNA sequencing was performed using Genetic Analyzer ABI PRISM 3100 (Applied Biosystems) according to the manufacturer's instructions. The primers used in this study are listed in Table S3. Sequences were analyzed using GENETYX v12 (Genetyx Corporation, Tokyo, Japan).

2.3 Agar plate method

Fungi inoculated as a 5-mm diameter mycelium plug, harvested from another PDA medium incubated in the dark at 27 °C for 7–10 days, were cultured on PDA medium supplemented with a series of fungicides at designated concentrations (Table 1). All fungicide-supplemented PDA and fungicide-free reference PDA medium contained SHAM at a final concentration of 100 mg L^{-1}. The medium and the concentration of SHAM were the same as described previously.[35] The mean mycelial radial growth (mm) from inocula, on each agar plate, was measured at designated periods after inoculation (two biological replicates were used). The inhibitory effect of each fungicide was determined from the mean radial growth of the mycelia and compared to that of the fungicide-free medium with SHAM at 100 mg L^{-1}. The values of 50% effective concentrations (EC_{50}) were calculated using the 'Nonlinear regression (curve fit)' function of PRISM

Table 1. Conditions of antifungal tests

Test method	Fungal species	Medium	Incubation	Final concentrations mg L^{-1}
Agar plate	*Cercospora beticola* (Table 2, Fig 1)	PDA + SHAM*	27 °C, 12–17 days	0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10
	Corynespora cassiicola (Fig S2)		27 °C, 4 days	
	Colletotrichum gloeosporioides (Fig S2)		27 °C, 4 days	for all fungicides
Microtiter plate	*Cercospora beticola* (Tables 3 and S2) ‡	YBA	23 °C, 4 days	0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1 for metyltetraprole and trifloxystrobin
	Corynespora cassiicola (Fig S3)		18 °C, 2 days	0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 for azoxystrobin
	Colletotrichum gloeosporioides (Fig S3)		23 °C, 4 days	
Microtiter plate	*Cercospora beticola* (Fig 2)†	YBA	23 °C, 4 days	0.00012, 0.00049, 0.0020, 0.0078, 0.13, 0.031, 0.13, 0.50, 2.0 for metyltetraprole and trifloxystrobin

*Salicylhydroxamic acid (SHAM) was added at the final concentration 100 mg L^{-1}.
† *Cercospora beticola* was tested at different concentrations in microtiter plate tests between Table 3 (see also Table S2) and Fig. 2.

8 (GraphPad Software, San Diego, CA, USA). The resistance factor (RF) was calculated using the following formula:

$$RF = (EC_{50} \text{ for trifloxystrobin–resistant isolate carrying G143A mutation}) / (EC_{50} \text{ for wild–type isolate without mutations in } Cytb).$$

2.4 Microtiter plate method using liquid medium

For *C. beticola*, mycelia were collected from a colony on PDA medium incubated in the dark at 27 °C for 7–10 days, cultured for 17 days in potato dextrose broth (PDB, 24 g in 1 L water) at 23 °C, collected by brief centrifugation, and subjected to fragmentation using a bead mill, Multi-beads shocker (Yasui Kikai, Osaka, Japan). After four rounds of fragmentation (2000 rpm, 30 s each), mycelium fragments that were 30–120 µm long in YBA medium (yeast extract 10 g L^{-1}, peptone 10 g L^{-1} and sodium acetate 20 g L^{-1} in distilled water),[31] reported as a suitable medium for the microtiter plate method, were adjusted to 5×10^3 pieces mm^{-1} as inocula. A 100-fold dilution series of fungicides (0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 mg L^{-1} in dimethyl sulfoxide, corresponding to final concentrations of 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 mg L^{-1} in the medium, respectively) was prepared for each test (Table 1), and aliquots (1 µL) of each fungicide were mixed with the inoculum (99 µL) prepared in the medium. Fungal cultures in 96-well microtiter plates (two biological replicates) were cultured for designated periods at 23 °C on a vertical shaker set at 90 rpm as fungi sometimes form hyphal aggregates on a rotary shaker. After incubating all fungal strains for the incubation periods listed in Table 1, their growth was assessed by measuring the optical density (OD) of each culture solution at a wavelength of 600 nm, using the microplate reader SH-9000 Lab (Corona Electric, Ibaraki, Japan) with a 3 × 3 matrix of scanning points. The OD values were corrected using the value of the blank well without inoculum. The EC_{50} was calculated from the mean of OD values (two replicates) of each fungicide concentration using the 'Nonlinear regression (curve fit)' function of Prism 8. The RF was calculated using the same formula that was used for the agar plate method.

2.5 Expression analysis of *AOX* genes

In order to check the involvement of the *AOX* expression level of each isolate in antifungal tests, the following experiments were performed. For preincubation, mycelia were cultured in 100 mL PDB at 23 °C in the dark for 72 h in a rotary incubator. After preincubation, aliquots (20 µL) of 100 mg L^{-1} metyltetraprole solution or 20 µL dimethyl sulfoxide (control) were mixed with the preincubated inoculum (20 mL) and incubated at 23 °C in the dark for 16 or 24 h in a rotary incubator. Each mycelium sample was filtered and rinsed with distilled water on a hydrophilic nylon membrane (Merck Millipore, Burlington, MA, USA), and immediately frozen in liquid nitrogen. Total RNA was extracted from mycelia using an RNeasy Plant Mini Kit (QIAGEN, Venlo, Netherlands). cDNA was synthesized from 1 µg total RNA with random hexamers using the ReverTra Ace quantitative reverse-transcription (qRT)-PCR kit (Toyobo, Osaka, Japan), following the supplied protocol. Single-strand cDNA was used directly for real-time RT-PCR using SYBR GREEN PCR Master Mix and StepOnePlus System (Applied Biosystems). The primers for qRT-PCR were designed using Primer Express Software (Applied Biosystems) based on the mRNA sequences of *AOX* (GenBank accession number XM_023603643.1) and *α-tubulin* (GenBank accession number XM_023595832.1). The *α-tubulin* was used as a housekeeping gene for normalization purposes. Cycle threshold values were obtained from the exponential phase of PCR amplification, and normalization to gene expression levels was performed using the $2^{-\Delta\Delta CT}$ method.[37]

2.6 *In planta* tests of *C. beticola*

Seedlings of the 'Stout' sugar beet variety at the second true leaf stage were planted into 25-cm diameter plastic pots filled with Echigo-baido soil (Honenagri, Niigata, Japan). One plant was planted per pot. Pots were placed in a greenhouse at an average temperature of 23 °C under natural light conditions. Plants were

Table 2. Range of EC_{50} values for *C. beticola* in agar plate tests

	Genotype of cytochrome *b*	Number of isolates	EC_{50} mg L^{-1}			
			MIN* (isolate)	MAX* (isolate)	Median	MAX/MIN†
Metyltetraprole	Wild-type	11	0.030 (Cb10)	3.926 (Cb15)	0.147	130.9
	G143A	5	0.125 (Cb16)	1.138 (Cb14)	0.841	9.1
Trifloxystrobin	Wild-type	11	0.004 (Cb10)	1.426 (Cb15)	0.039	356.5
	G143A	5	>10 (All isolate)	>10 (All isolate)	>10	N.D.‡

*The lowest (MIN) and the highest (MAX) EC_{50} values.
† Ranges of EC_{50} values, MAX was divided by MIN.
‡ Not calculated because all isolates within the group showed low sensitivity (EC_{50} > 10 mg L^{-1}).

watered as needed, and at three to four weeks after planting, which represents the stage at which six fully expanded leaves were present, plants were used in the experiments.

Dilutions of metyltetraprole (150 or 75 mg active ingredient (AI) L^{-1}, i.e. the expected labeled concentration in Japan for sugar beets or half of that concentration) and trifloxystrobin (167 or 83 mg AI L^{-1}, i.e. the labeled concentration for sugar beets in Japan or half of that concentration) in water with 0.02% v/v spreading agent (Guramin-S, Mitsui-Agro, Tokyo, Japan) were sprayed using a hand sprayer onto sugar beet plants until there was run-off. These plants were kept at 23 °C in a glasshouse until inoculation on the following day, with two replicates for each treatment. Conidia from each isolate (harvested from lesions on sugar beets that were inoculated with mycelia and conidia mixture of each isolate; incubated 20–30 days in the abovementioned glasshouse followed by 3–5 days in a 100% humidity chamber before harvesting conidia) were collected from lesions on sugar beets in the greenhouse, and adjusted to 5×10^3 mL^{-1}. Conidial suspensions were spray-inoculated onto potted sugar beet plants treated with the fungicide previously, and kept in a dew chamber for three days at 23 °C in a glasshouse. After 20–26 days of incubation in a 23 °C glasshouse (maintained at 100% humidity for 4 h per day), the development of *Cercospora* leaf spot disease in each leaf, which had unfolded at the time of spraying, was assessed visually as the percentage area of greenness lost owing to the diseased spot (diseased leaf area). The disease severity for each isolate was calculated as the mean of diseased leaf area without fungicide spraying for each isolate. The efficacy of each treatment was calculated using the following formula:

$$\%\text{Efficacy} = 100 \times [1 - (\text{diseased leaf area of the treated leaf} / \text{mean of diseased leaf area in the untreated plant})]$$

The mean % efficacy was calculated for all assessed leaves with SEs for each fungicide treatment in each *C. beticola* isolate, and was tested between wild-type (WT) isolates ($N = 7$) and G143A isolates ($N = 6$) by the Mann–Whitney *U*-test using BellCurve for Excel (Social Survey Research Information Co., Ltd. Tokyo, Japan).

2.7 *Cercospora beticola* sensitivity monitoring in Hokkaido

Strains of *C. beticola* were isolated from sugar beet leaves in the fields of Hokkaido (Tables S1 and S2). The presence of mutations in each resistant isolate was checked as described in Section 2.2. The isolates were subjected to both the agar plate tests (16 isolates; Table S1) and microtiter plate tests (103 isolates; Table S2). For the test results of the microtiter plate tests, the EC_{50} difference between WT isolates ($N = 72$) and G143A isolates ($N = 31$) was analyzed using the Mann–Whitney *U*-test by BellCurve for Excel. The Smirnov–Grubbs test also was performed by BellCurve for Excel, for a total of 103 *C. beticola* isolates to detect the outliers.

3 RESULTS

3.1 Agar plate tests in *C. beticola*

In *C. beticola*, 10 mg L^{-1} metyltetraprole showed activity against all isolates, regardless of the presence of the G143A mutation. However, the EC_{50} values for metyltetraprole in the most sensitive isolate (Cb10) and in the least sensitive isolate (Cb15) varied by 130.9-fold (Tables 2 and S1). Therefore, Cb15 could be categorized as an isolate that is moderately resistant to metyltetraprole, based on EC_{50} values obtained with this method.

All *cytb* G143A mutation-harboring isolates showed <50% growth inhibition by trifloxystrobin, even at 10 mg L^{-1} in the presence of 100 mg L^{-1} SHAM (Fig. 1). Therefore, separation of WT isolates and G143A mutants was possible at this discriminatory concentration of trifloxystrobin. However, WT isolates without the G143A mutation showed different levels of inhibition by trifloxystrobin, depending on the isolates (Fig. 1). The growth of the Cb10 isolate was completely inhibited by 0.3 mg L^{-1} trifloxystrobin, and the EC_{50} was 0.0041 mg L^{-1} (Tables 2 and S1, Fig. 1). By contrast, the growth of the Cb15 isolate did not show complete inhibition even with 10 mg L^{-1} trifloxystrobin (Fig. 1). The trifloxystrobin EC_{50} values of Cb10 and Cb15 isolates varied by ≈350-fold (Table 2). Therefore, Cb15 could be categorized as an isolate that is resistant to trifloxystrobin, based on the EC_{50} values obtained with this method.

Conidial germination tests[23] were carried out for three representative isolates in each of the WT and G143A-harboring mutants to investigate the possible low QoI sensitivities in the Cb15 isolate. Consequently, the EC_{50} values for the Cb15 isolate (EC_{50} = 0.0026 mg L^{-1} for metyltetraprole and 0.0146 mg L^{-1} for trifloxystrobin) did not differ by >10-fold as compared to those of the most sensitive Cb10 isolate (EC_{50} = 0.0030 mg L^{-1} for metyltetraprole and 0.0021 mg L^{-1} for trifloxystrobin; Table S4). Therefore, the Cb15 isolate was not considered metyltetraprole resistant in conidial germination tests.

3.2 Microtiter plate tests in *C. beticola*

For 16 isolates used for the agar plate tests, metyltetraprole showed potent activity against *C. beticola*, regardless of the

Table 3. Range of EC_{50} values for *Cercospora beticola* in microtiter plate tests*

	Genotype of cytochrome *b*	Number of isolates	EC_{50} mg L^{-1}			
			MIN† (isolate)	MAX† (isolate)	Mean	MAX/MIN‡
Metyltetraprole	Wild-type	11	0.0004 (Cb11)	0.0038 (Cb2)	0.0016	9.5
	G143A	5	0.0024 (Cb14)	0.0067 (Cb18)	0.0036	2.8
Trifloxystrobin	Wild-type	11	0.0003 (Cb9)	0.0025 (Cb10)	0.0010	8.3
	G143A	5	>2 (All isolate)	>2 (All isolate)	>2	N.D.§

*Summary of the 16 isolates that were subjected to agar plate tests (Tables 2 and S1).
† The lowest (MIN) and the highest (MAX) EC_{50} values.
‡ Ranges of EC_{50} values, MAX was divided by MIN.
§ Not calculated because all isolates within the group showed low sensitivity (EC_{50} > 2 mg L^{-1}).

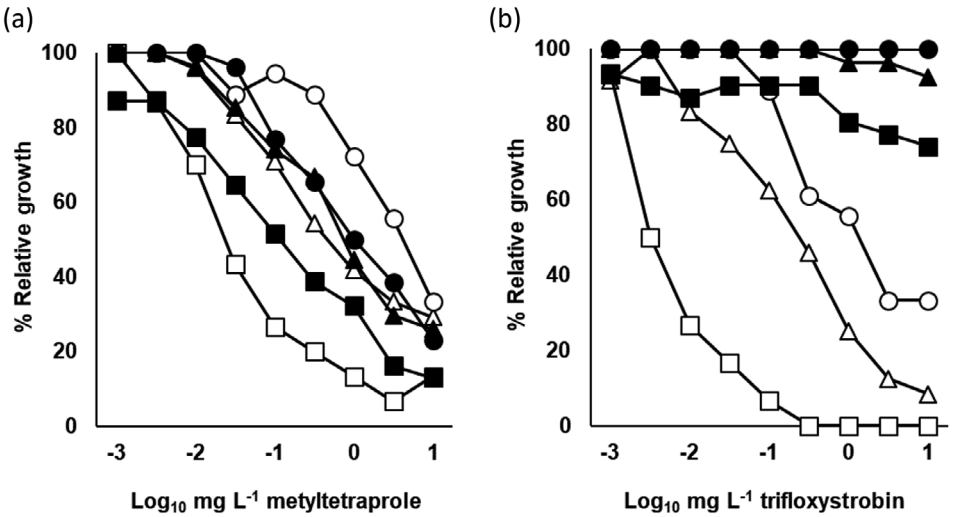

Figure 1. *Cercospora beticola* sensitivity tests using the agar plate method. Dose–response curves for fungal growth in the presence of metyltetraprole (a) and trifloxystrobin (b) in potato dextrose agar plate medium supplemented with 100 mg L^{-1} alternative oxidase inhibitor salicylhydroxamic acid. White squares, triangles and circles represent wild-type Cb10, Cb12 and Cb15 isolates, respectively; black triangles, circles and squares represent G143A-harboring QoI-resistant mutants Cb13, Cb14 and Cb16, respectively. Each point represents the mean of two biological replicates.

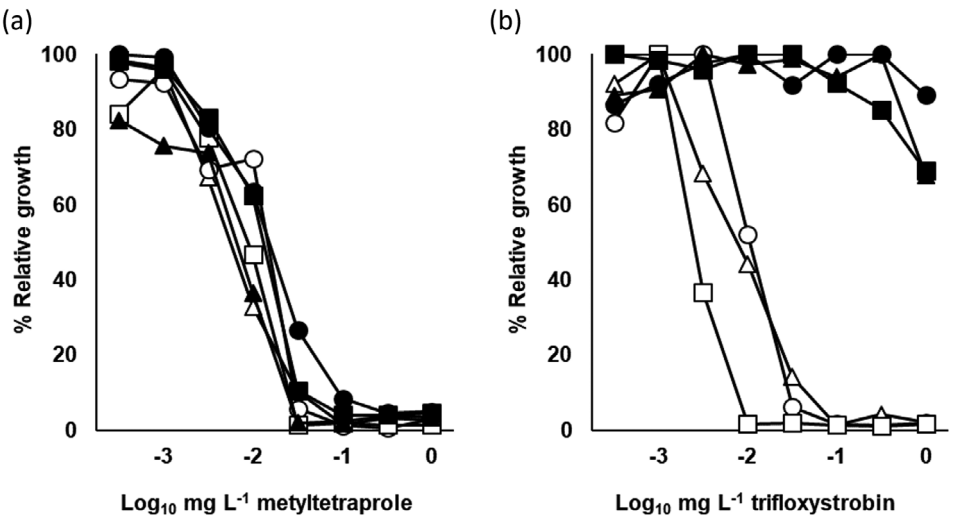

Figure 2. *Cercospora beticola* sensitivity tests using the microtiter plate method. Dose–response curves for fungal growth in the presence of metyltetraprole (a) and trifloxystrobin (b) in YBA (10 g L^{-1} yeast extract, 10 g L^{-1} peptone and 20 g L^{-1} sodium acetate in distilled water). White squares, triangles and circles represent wild-type Cb10, Cb12 and Cb15 isolates, respectively; black triangles, circles and squares represent G143A-harboring QoI-resistant mutants Cb13, Cb14, and Cb16, respectively. Each point represents the mean of two biological replicates.

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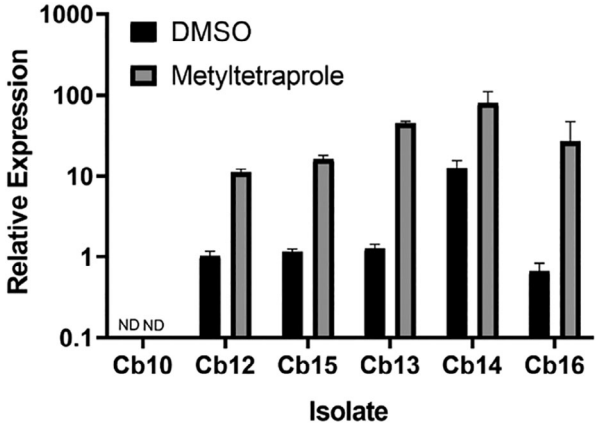

Figure 3. Relative expression of the alternative oxidase (*AOX*) gene in *C. beticola* isolates. Values were normalized relative to the constitutive expression (dimethyl sulfoxide) in the Cb12 isolate. Each bar represents the mean of four replicates and the error bars represent the standard deviations. RNA sampling was performed 60 min after 0.1 mg L^{-1} metyltetraprole treatment. ND, no detectable *AOX* gene expression (<0.3 of relative expression). Cb10, Cb12 and Cb15 are wild-type isolates, whereas Cb13, Cb14 and Cb16 are G143A-harboring isolates.

presence of the G143A mutation, when assessed by the microtiter plate tests (Table 3, Fig. 2). The EC_{50} values ranged from 0.0004 to 0.0067 mg L^{-1}, with less variability than those obtained from the agar plate tests (Tables 2, 3 and S2). All 16 isolates including Cb15 (EC_{50} = 0.0017 mg L^{-1}) were considered sensitive to metyltetraprole with this method, even when using mycelium fragments as inoculum.

With the use of microtiter plate tests, all *Cytb* G143A mutation-harboring isolates grew in medium containing 1 mg L^{-1} trifloxystrobin, whereas all WT isolates were > 90% inhibited by trifloxystrobin at concentrations ranging from 0.01–0.1 mg L^{-1} (Fig. 2). The EC_{50} values for trifloxystorbin were not significantly different between Cb15 and Cb10 isolates (0.0018 and 0.0025 mg L^{-1}, respectively) when using the microtiter plate tests, and thus Cb15 was considered sensitive to this fungicide.

3.3 Expression analysis of the *AOX* gene

Constitutive and inductive expression of *AOX* gene was confirmed in all isolates except for the Cb10 isolate, in which metyltetraprole and trifloxystrobin sensitivities from the agar plate tests were the highest among the six representative isolates (Fig. 3, Table S1). In the isolates with detectable *AOX* gene expression, metyltetraprole increased its expression by approximately 10-fold, and its expression level was similar at 30 and 60 min after metyltetraprole treatment (Figs 3 and S1). The Cb15 isolate showed the highest *AOX* gene expression among the three WT isolates; however, it was not higher than that of the G143A-harboring isolates. G143A-harboring isolates, in general, showed higher *AOX* gene expression in comparison with that in the WT isolates, although the number of tested isolates was small and the trend is not conclusive. The highest *AOX* gene expression among the six isolates was observed in Cb14, which had the highest metyltetraprole EC_{50} value among the G143A mutants upon the agar plate tests (Tables 2 and S1), whereas the metyltetraprole EC_{50} in Cb14 was not higher than that in Cb15.

Table 4. *In planta* sensitivity of *Cercospora beticola* to metyltetraprole and trifloxystrobin

			% Control (SE) of *Cercospora beticola*[a]			
			Metyltetraprole		Trifloxystrobin	
	Isolate	Severity (%)[b]	150 mg L^{-1}	75 mg L^{-1}	166 mg L^{-1}	83 mg L^{-1}
Wild-type (Qol-sensitive)	Cb9	67.0	94.6 (2.1)	94.6 (1.4)	99.5 (0.2)	98.7 (0.5)
	Cb10	29.3	97.2 (1.4)	82.9 (8.5)	98.1 (0.6)	90.3 (5.0)
	Cb12	56.7	92.9 (5.3)	98.2 (0.3)	96.5 (2.3)	99.3 (0.3)
	Cb15	54.1	98.9 (0.5)	88.0 (8.9)	94.2 (1.0)	87.1 (5.3)
	18.32	17.0	96.3 (1.1)	93.3 (2.7)	95.8 (1.1)	97.1 (1.3)
	18.47	25.6	91.5 (1.8)	95.6 (1.6)	97.1 (0.6)	96.6 (0.6)
	18.57	26.1	91.1 (3.7)	87.6 (1.6)	96.2 (0.7)	95.7 (1.1)
	Mean		94.6	91.5	96.8	95.0
G143A (Qol-resistant)	Cb13	100.0	98.6 (0.4)	82.2 (13.1)	48.1 (10.7)	30.8 (9.5)
	Cb14	64.0	98.0 (1.0)	96.6 (1.0)	68.9 (17.1)	40.2 (21.8)
	Cb16	65.0	99.0 (0.4)	94.3 (2.9)	49.6 (17.5)	17.3 (15.1)
	18.25	56.7	98.0 (0.4)	98.7 (0.3)	53.4 (10.4)	36.8 (14.1)
	18.31	62.0	95.9 (1.1)	98.0 (1.0)	48.0 (11.9)	26.4 (11.1)
	18.52	36.9	93.1 (3.1)	95.7 (1.6)	82.5 (5.5)	17.9 (24.8)
	Mean		97.1	94.2	58.4	28.2
	P-value[§]		0.1336	0.2840	0.0034	0.0034

[§] *P*-value of the Mann–Whitney *U*-test between wild-type and G143A isolates.
[a] % efficacy = 100 × [1–(diseased leaf area of the treated leaf/mean of diseased leaf area in the untreated plant)]. The mean of % efficacy was calculated and represented from all assessed leaves with standard errors for each fungicide treatment in each *C. beticola* isolate.
[b] Mean of diseased leaf area without fungicide spraying in each isolate.

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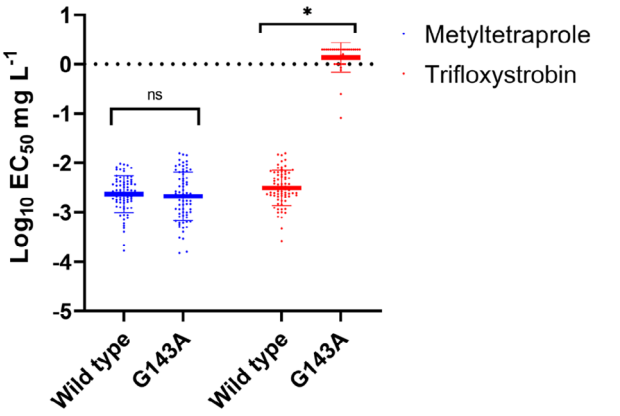

Figure 4. Metyltetraprole (blue) and trifloxystrobin (red) sensitivities of wild-type (WT) isolates ($N = 72$) and G143A mutants ($N = 31$) in the microtiter plate tests. The cut-off value for trifloxystrobin EC_{50} was 2 mg L^{-1}. *, fungicide with $P < 0.01$ in the Mann–Whitney *U*-test between WT and G143A-mutant groups; ns, $P > 0.05$.

3.4 *In planta* tests of *C. beticola*

We next investigated the *in planta* sensitivity of isolates that showed variable sensitivity to metyltetraprole and trifloxystrobin in agar plate tests. As shown in Table 4, all WT isolates, including Cb15, were effectively inhibited by trifloxystrobin even at 83 mg L^{-1}. By contrast, trifloxystrobin did not substantially inhibit disease development by isolates harboring the G143A mutation even at 166 mg L^{-1} (the labeled concentration of trifloxystrobin for sugar beets in Japan). Alternatively, the *in planta* activity of metyltetraprole was not affected by the presence of the G143A mutation. In addition, the Cb15 isolate, which showed lower sensitivity as per the agar plate tests, was effectively suppressed by metyltetraprole, as was observed for the other isolates.

3.5 *Cercospora beticola* sensitivity monitoring in Hokkaido

A total of 103 isolates were collected from Hokkaido, the main sugar beet production area in Japan. Among them, 31 isolates harbored the G143A mutation whereas all others had no mutation in *Cytb* (Fig. 4). Investigation of sequencing charts for these isolates indicated that the position relevant to the G143A mutation showed no evidence of heteroplasmy. The median EC_{50} value for trifloxystrobin in WT isolates was 0.0022 mg L^{-1} and that in the G143A mutants was >2 mg L^{-1} ($P < 0.001$, Mann–Whitney *U*-test). By contrast, the median EC_{50} value of metyltetraprole in WT isolates was 0.0025 mg L^{-1} and that in the G143A mutants was 0.0029 mg L^{-1} ($P = 0.1697$, Mann–Whitney *U*-test). Isolates showing >10-fold higher metyltetraprole EC_{50} values than the median EC_{50} value were not detected. The range of metyltetraprole EC_{50} values was not significantly different between the four old isolates obtained before the use of QoI (Cb1, Cb2, Cb3 and Cb4) and others obtained after the use of QoI on sugar beets of the area (Table S2). Smirnov–Grubbs analysis did not indicate the presence of an outlier at $P = 0.3964$ for metyltetraprole EC_{50} values.

4 DISCUSSION

Fungicide resistance is a growing concern for sustainable agriculture. There is no doubt that early detection of resistant isolates is important to implement prompt countermeasures. As many plant pathogenic fungi can grow in various growth media, *in vitro* tests are widely utilized for monitoring of fungicide sensitivity rather than *in planta* tests owing to higher throughput. However, it is important to confirm whether the *in vitro* test method correlates with the *in planta* tests; otherwise, false-positive or -negative results might cause confusion. Although several methods have been successfully established for examining *C. beticola* sensitivities for existing QoI,[15,23,25,35] they should be checked carefully when applied to metyltetraprole.

The choice of *in vitro* test is critical for the accurate assessment of fungicide effectiveness. For instance, isolates such as Cb15, shown in Fig. 1, could be misidentified as the isolated that are resistant to the fungicides tested, if judged based on the EC_{50} values of the agar plate tests on PDA with 100 mg L^{-1} SHAM. The observed low sensitivity of *C. beticola* by agar plate tests using PDA with 100 mg L^{-1} SHAM might be attributed to constitutive and inductive *AOX* expression. To adopt the agar plate method using mycelia for metyltetraprole sensitivity testing in *C. beticola*, adjustment of the molecule used as an AOX inhibitor and also of its concentration will be required. However, other underlying mechanisms also might be present given the weak correlation between QoI sensitivities on agar plate and AOX expression level (Figs 1 and 3). By contrast, the metyltetraprole and trifloxystrobin sensitivities of the Cb15 isolate were within the range of sensitive isolates in microtiter plate tests and conidial germination tests even without AOX inhibitors. The results of the *in planta* test confirmed that Cb15 is a metyltetraprole- and trifloxystrobin-sensitive isolate. Moreover, in our preventative treatment, Cb15 was successfully suppressed by these two fungicides, which was similar to that observed in other WT isolates for trifloxystrobin or all other isolates for metyltetraprole. The microtiter plate tests also successfully separated WT isolates and G143A mutants with clearly lower *in planta* trifloxystrobin sensitivities.

Cercospora beticola mycelium, similar to that of many other phytopathogenic fungi, needs to grow in apoplasts filled with water, containing some nutrients during the nonsymptomatic early stage of infection.[38–40] Therefore, it would be reasonable to expect a correlation between *in planta* sensitivity and that in liquid medium. In addition, conidia and mycelia can be exposed to fungicide in water droplets formed by rainfall or morning dew. Therefore, the microtiter method using liquid culture would be valid for assessing fungicide sensitivity. Jin *et al.*[11] observed the recovery of fungal oxygen consumption after several hours of azoxystrobin treatment with SHAM in the mycelium of some fungal species/isolates grown in agar medium. Therefore, SHAM was not sufficient to stop oxygen consumption in the mycelium of some fungi, possibly owing to the activation of aerobic glycolysis or other oxidative pathways to generate ATP. In liquid medium, the availability of oxygen is estimated to be low for fungi, and the abovementioned pathways might be insufficient for supporting fast fungal growth. Moreover, as mentioned by Spiegel and Stammler,[31] restriction of nutrients in medium is expected to prevent fungi from gaining energy via alternative pathways such as alternative respiration or anaerobic fermentation. Indeed, QoI fungicides showed potent antifungal activities in microtiter plate tests, even without AOX inhibitors (Table 3, Figs 4 and S3).

Our monitoring of metyltetraprole and trifloxystrobin sensitivity using the microtiter tests did not indicate the presence of metyltetraprole-resistant isolates, but confirmed the spread of G143A-harboring trifloxystrobin-resistant isolates of *C. beticola* in Hokkaido, as already described by Kayamori *et al.*[15]

The microtiter plate tests were not only applicable for assessing metyltetraprole and other QoI fungicide sensitivities in *C. beticola*, but also in *C. gloeosporioides* and *C. cassiicola* (Figs S2 and S3), for which SHAM is reported to be necessary for agar plate tests.[16,18] In our microtiter plate tests without SHAM, both metyltetraprole and azoxystrobin showed satisfactory antifungal activities with lower concentrations than those in agar plate tests, except when azoxystrobin was tested against G143A mutants (Figs S2 and S3). The microtiter plate tests also might be applicable for other fungal species where fungicides show insufficient growth inhibition in the agar plate tests, even at saturating concentrations, or when a high concentration of AOX inhibitor shows adverse effects to the fungi by itself. Although the optimization of the AOX inhibitor and its concentration might improve the accuracy of agar plate tests, use of microtiter plate tests may be a suitable alternative for researchers.

QoI fungicide resistance in fungal species related to *C. beticola*, *C. gloeosporioides* and *C. cassiicola* is a growing concern for the cultivation of many major crops. For example, QoI fungicide resistance, based on the G143A mutation in *Cytb*, also has been detected in *Cercospora sojina*,[27] *Cercospora cf. flagellaris*,[41] *Cercospora nicotianae*,[42,43] *Cercospra kikuchii*,[42,44,45] *Corynespora cassiicola*[17] and *Colletotrichum truncatum*,[45] probably owing to the intensive use of QoIs for soybean production. Therefore, the incorporation of metyltetraprole into the soybean disease protection program could be beneficial for soybean growers. If this does occur, then an effective method for monitoring metyltetraprole sensitivity will be required. In this study, we have demonstrated that microtiter plate testing appears to be a valid option for successful monitoring of the metyltetraprole sensitivity.

For some researchers, the equipment used in this study, such as a microtiter plate reader and a bead mill, might be unavailable. In these cases, this method could be altered by simply growing the mycelial plug in YBA and visually assessing fungal growth. This would be convenient, especially for the simple detection of resistant strains at one discriminatory concentration (e.g. detecting isolates that are less sensitive to metyltetraprole by testing with metyltetraprole at 0.1 mg L^{-1}).

5 CONCLUSIONS

In this study, we established a novel approach, the microtiter plate test, that was effective at monitoring metyltetraprole sensitivity in *C. beticola* and also may be applicable for other QoI fungicides. This research would provide a useful reference for researchers who are involved in fungicide monitoring programs for various crops, worldwide.

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