J. Pestic. Sci., 30(3), 203–208 (2005)

Note

Monitoring of the Sensitivity of *Magnaporthe grisea* to Metominostrobin 2001–2003: No Emergence of Resistant Strains and No Mutations at Codon 143 or 129 of the Cytochrome *b* Gene

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(Received September 21, 2004; Accepted April 11, 2005)

The sensitivity of *Magnaporthe grisea* to Metominostrobin (ORIBRIGHT®, a QoI fungicide) was examined. Six isolates collected in 1999, prior to the introduction of metominostrobin, were tested by conducting *in vivo* assays to establish a baseline for the sensitivity of the fungus. The mean value of the EC_{50} (ranging from 0.65 to 11.8 μg/ml) was 3.3 μg/ml. Ninety isolates were collected in 2001–2003 and 56 isolates were subjected to *in vivo* assays. EC_{50} values ranged from less than 0.1 to 9.4 μg/ml and no significant difference to the baseline was observed. A point mutation at nucleotide position +428 or +387 in the cytochrome *b* gene, resulting in the replacement of glycine 143 with alanine (G143A) or of phenylalanine 129 with leucine (F129L) at the amino acid position of cytochrome *b* known to be the cause of resistance to QoI, was not observed in any of the 96 field isolates including the six collected in 1999. © Pesticide Science Society of Japan

Keywords: metominostrobin, cytochrome *b*, *Magnaporthe grisea*, point mutation.

INTRODUCTION

Strobilurin-based fungicides have proven to be very effective in controlling plant diseases. As their mode of action is the inhibition of the Qo site of the mitochondrial cytochrome bc_1 complex,[1,2] these fungicides are known as Qo inhibitors (QoI). However, after some years on the market, reports of resistance to these compounds by several phytopathogenic fungi including *Blumeria graminis* f. sp. *hordei*,[3] *B. graminis* f. sp. *tritici*,[4] *Mycosphaerella fijiensis*,[5] *Podosphaera fusca*,[6] *Pseudoperonospora cubensis*[6] or *Venturia inaequalis*[7] have emerged. This resistance to QoI is associated with a single point mutation (guanine to cytosine) at nucleotide position +428 in the mitochondrial cytochrome *b* gene resulting in change from glycine (G) to alanine (A) at amino acid position 143, G143A. In 2003, a single point mutation (cytosine to adenine) at nucleotide position +387 in the mitochondrial cytochrome *b* gene of *Pyricularia grisea* (teleomorph, *Magnaporthe grisea*) resulting in an amino acid change from phenylalanine (F) to leucine (L) at position 129, F129L, was identified in perennial ryegrass (*Lolium perenne*) and reported as a cause of lower levels of resistance to QoI fungicides in the USA.[8]

Metominostrobin (ORIBRIGHT®) is a systemic fungicide used to treat rice diseases by water surface application and shows excellent efficacy both for leaf and panicle blast caused by *Magnaporthe grisea*. Metominostrobin and another QoI fungicide, azoxystrobin, were registered in 1998 and both have been used to control rice blast since 1999 in Japan. We have been monitoring the sensitivity to metominostrobin and mutations in the cytochrome *b* gene in field isolates of *M. grisea*. Though these two products have been on the market for some time, they have been used in limited areas and no reduction in control efficacy has been reported so far. Therefore the results for 1999 are considered to be the baseline data.

MATERIALS AND METHODS

1. *Magnaporthe grisea Isolates*

Isolates collected from paddy fields at six locations in Japan in 1999, prior to the introduction of metominostrobin, were tested to establish a baseline for the sensitivity of the fungus. Fifty-six isolates collected from paddy fields in 2001–2003 were tested for sensitivity to metominostrobin. Ninety-six isolates, including the six isolates collected in 1999 and 56 collected in 2001–2003 and assayed for sensitivity to metominostrobin, were subjected to a PCR-RFLP analysis. Ine 86-137, a laboratory strain of *M. grisea* isolated in 1986 and provided by the National Agricultural Research Center, was used as a reference of the sensitivity or in the PCR-RFLP analysis.

M. grisea was isolated from diseased leaves or panicles as follows. Detached leaves or panicles were placed on the inner surface of the lid of a petri dish containing water agar and incubated for 24 to 48 hr at 25°C. A single germinated conidium, which had fallen from the sporulating lesions onto the surface of the water agar, was picked up with a needle under a microscope and maintained on potato dextrose agar (PDA). Details on the isolates collected and used in this study are shown in Tables 1 and 2.

2. *Assessment of Sensitivity to Metominostrobin*

Rice plants in the 3rd to 4th leaf stage (cultivar Aichi-Asahi,

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Table 1. Field isolates of *Magnaporthe grisea* collected in 1999

Isolate no.	Place of isolation	Metominostrobin exposure[a]	Decline of rice blast control[b]	EC_{50} (μg/ml)	Restriction pattern with *Ita*I[c]	Restriction pattern with *Sty*I[d]
99-31-004	Okayama	0	—	3.1	wt	wt
99-31-007	Okayama	0	—	0.65	wt	wt
99-40-021	Fukuoka	0	—	1.8	wt	wt
99-03-047	Iwate	0	—	1.4	wt	wt
99-34-005	Hiroshima	0	—	1.9	wt	wt
99-25-001	Shiga	0	—	11.8	wt	wt

[a] Frequency of application of metominostrobin in the paddy field. [b] Decline of rice blast control on treatment with metominostrobin in the sampling year. [c] Restriction pattern of the internal 879 bp of cytochrome *b* with *Ita*I. [d] Restriction pattern of the internal 879 bp of cytochrome *b* with *Sty*I. —: No application of metominostrobin. wt: wild-type.

Table 2. Field isolates of *Magnaporthe grisea* collected in 2001–2003

Isolate no.	Place of isolation	Metominostrobin exposure[a]	Decline of rice blast control[b]	EC_{50} (μg/ml)	Restriction pattern with *Ita*I[c]	Restriction pattern with *Sty*I[d]
2001						
01-001	Ehime	1 (01)	nd	2.1	wt	wt
01-002	Fukushima	1 (01)	nd	6.3	wt	wt
01-003	Nagano	0	—	2.3	wt	wt
01-005	Iwate	1 (01)	nd	0.13	wt	wt
01-006	Mie	0	—	3.2	wt	wt
01-007	Shiga	0	—	4.1	wt	wt
01-008	Shiga	0	—	2.4	wt	wt
01-009	Shiga	0	—	5.1	wt	wt
01-013	Fukushima	0	—	4.4	wt	wt
01-015	Fukushima	1 (01)	nd	9.4	wt	wt
01-019	Fukuoka	1 (00)	—	0.60	wt	wt
01-020	Fukuoka	0	—	1.1	wt	wt
01-021	Hokkaido	1 (01)	nd	1.2	wt	wt
01-022	Akita	1 (01)	nd	5.3	wt	wt
01-023	Akita	0	—	3.9	wt	wt
01-024	Akita	1 (01)	nd	4.8	wt	wt
01-025	Akita	0	—	5.7	wt	wt
01-026	Nagano	0	—	0.50	wt	wt
01-027	Nagano	0	—	3.7	wt	wt
01-034	Aomori	0	—	1.5	wt	wt
01-036	Aomori	0	—	2.9	wt	wt
01-037	Aomori	1 (00)	—	1.7	wt	wt
01-039	Aomori	0	—	5.7	wt	wt
01-041	Aomori	0	—	4.7	wt	wt
01-045	Fukuoka	0	—	6.3	wt	wt
01-047	Fukuoka	1 (00)	—	1.9	wt	wt
01-050	Iwate	1 (01)	nd	0.60	wt	wt
01-051	Iwate	1 (01)	nd	1.5	wt	wt

Table 2. (Continued)

Isolate no.	Place of isolation	Metominostrobin exposure[a)]	Decline of rice blast control[b)]	EC_{50} (μg/ml)	Restriction pattern with *Ita*I[c)]	Restriction pattern with *Sty*I[d)]
01-052	Iwate	1 (01)	nd	3.1	wt	wt
01-053	Iwate	1 (01)	nd	1.7	wt	wt
01-056	Saitama	0	—	0.40	wt	wt
01-057	Saitama	0	—	0.50	wt	wt
2002						
02-002	Shiga	4 (98, 99, 00, 01)	—	0.16	wt	wt
02-003	Shiga	4 (98, 99, 00, 01)	—	<0.1	wt	wt
02-004	Shiga	4 (98, 99, 00, 01)	—	<0.1	wt	wt
02-005	Shiga	4 (98, 99, 00, 01)	—	0.14	wt	wt
02-006	Shiga	5 (98, 99, 00, 01, 02)	nd	NT	wt	wt
02-007	Shiga	5 (98, 99, 00, 01, 02)	nd	0.12	wt	wt
02-008	Shiga	5 (98, 99, 00, 01, 02)	nd	<0.1	wt	wt
02-009	Shiga	5 (98, 99, 00, 01, 02)	nd	<0.1	wt	wt
02-010	Shiga	5 (98, 99, 00, 01, 02)	nd	0.87	wt	wt
02-011	Fukuoka	1 (02)	nd	0.48	wt	wt
02-012	Fukuoka	1 (02)	nd	<0.1	wt	wt
02-013	Fukuoka	1 (02)	nd	0.21	wt	wt
02-014	Fukuoka	1 (02)	nd	NT	wt	wt
02-015	Fukuoka	1 (02)	nd	0.26	wt	wt
02-016	Fukuoka	1 (02)	nd	NT	wt	wt
02-021	Fukuoka	1 (01)	—	NT	wt	wt
02-022	Fukuoka	1 (01)	—	NT	wt	wt
02-023	Yamagata	1 (02)	nd	NT	wt	wt
02-026	Yamagata	1 (02)	nd	NT	wt	wt
02-027	Yamagata	1 (02)	nd	NT	wt	wt
02-029	Yamagata	2 (99, 00)	—	NT	wt	wt
02-030	Yamagata	2 (99, 00)	—	NT	wt	wt
02-031	Yamagata	2 (99, 00)	—	NT	wt	wt
02-032	Yamagata	2 (99, 00)	—	NT	wt	wt
02-035	Hyogo	0	—	NT	wt	wt
02-036	Hyogo	0	—	NT	wt	wt
02-40	Fukushima	0	—	NT	wt	wt
02-41	Fukushima	0	—	NT	wt	wt
02-42	Fukushima	1 (02)	nd	NT	wt	wt
02-43	Fukushima	0	—	NT	wt	wt
02-44	Fukushima	0	—	NT	wt	wt
02-45	Saga	0	—	NT	wt	wt
02-46	Saga	0	—	NT	wt	wt
02-47	Saga	0	—	NT	wt	wt
02-48	Saga	0	—	NT	wt	wt
2003						
03-002-2	Shiga	5 (98, 99, 00, 01, 02)	—	0.19	wt	wt
03-004-1	Fukui	0	—	NT	wt	wt

Table 2. (Continued)

Isolate no.	Place of isolation	Metominostrobin exposure[a]	Decline of rice blast control[b]	EC_{50} (μg/ml)	Restriction pattern with *Ita*I[c]	Restriction pattern with *Sty*I[d]
03-005-2	Fukui	0	—	NT	wt	wt
03-009-3	Shiga	1 (99)	—	NT	wt	wt
03-010-3	Shiga	1 (03)	nd	NT	wt	wt
03-011-2	Shiga	1 (03)	nd	NT	wt	wt
03-012-3	Shiga	0	—	NT	wt	wt
03-013-3	Shiga	0	—	NT	wt	wt
03-014-3	Akita	0	—	0.10	wt	wt
03-015-1	Akita	0	—	0.13	wt	wt
03-016-2	Saga	0	—	0.12	wt	wt
03-017-1	Saga	1 (03)	nd	0.16	wt	wt
03-018-3	Fukushima	0	—	<0.1	wt	wt
03-019-1	Aomori	1 (03)	nd	0.22	wt	wt
03-020-1	Aomori	0	—	NT	wt	wt
03-021-3	Iwate	1 (03)	nd	<0.1	wt	wt
03-022-1	Iwate	1 (03)	nd	0.14	wt	wt
03-024-2	Iwate	1 (03)	nd	<0.1	wt	wt
03-026-2	Iwate	0	—	<0.1	wt	wt
03-027-3	Iwate	1 (03)	nd	<0.1	wt	wt
03-028-1	Iwate	0	—	NT	wt	wt
03-029-1	Iwate	1 (03)	nd	NT	wt	wt
03-030-2	Miyagi	4 (00, 01, 02, 03)	nd	NT	wt	wt

[a] Frequency of application of metominostrobin in the paddy field. [b] Decline of rice blast control on treatment with metominostrobin in the sampling year. [c] Restriction pattern of the internal 879 bp of cytochrome *b* with *Ita*I. [d] Restriction pattern of the internal 879 bp of cytochrome *b* with *Sty*I. —: No application of metominostrobin. nd: not observed. NT: not tested. wt: wild-type.

cultivated in 8-cm diameter plastic pots) were sprayed with metominostrobin (Bayer CropScience K. K.) at a concentration of 0.5, 2.0, 7.8, 31.3 or 125 μg/ml in each of three replications. Each isolate was cultured for seven days at 25°C on oatmeal agar and aerial hyphae were removed with a paintbrush. Conidia formed after a further five days of incubation under BLB light (FL20S, Toshiba). The inoculation with a conidial suspension ($1.0–3.0\times10^5$ conidia/ml) of each isolate took place one day after the treatment with metominostrobin. The number of lesions that formed on all of the leaves in the three replications was counted seven days after the inoculation and the protective value was calculated according to the formula:

$$\text{Protective value (\%)} = 100 - (\text{lesions on treated leaves}/\text{lesions on untreated leaves}) \times 100$$

These data were plotted against the logarithm of the fungicide concentration, and the EC_{50} value was determined by interpolation of the 50% intercept.

3. *PCR-RFLP Analysis for Mutations in the Cytochrome b Gene*

Ninety-six field isolates and Ine 86-137 were used to screen for a mutation of guanine to cytosine at nucleotide position +428 in the mitochondrial cytochrome *b* gene (resulting in a change from glycine to alanine at amino acid position 143, G143A), or cytosine to adenine at nucleotide position +387 (resulting in a change from phenylalanine to leucine at position 129, F129L) by using the restriction enzymes *Ita*I and *Sty*I.[6,8] DNA was extracted from mycelia with ISOPLANT® (Nippon Gene), and an 879-bp internal fragment of the cytochrome *b* gene was amplified with the Pgcytb_F1 (5′-AGTCCTAGTGTAATG GAAGC-3′) and Pgcytb_R1 (5′-ATCTTCAACGTGTTTAGCACC-3′) primer pair by PCR,[8] and subjected to restriction. The G143A mutation would result in the creation of a third *Ita*I site (G↓C [T/A] GC) in the 879-bp fragment, whereas F129L would cause a loss of the *Sty*I site (C↓C [A/T] [T/A] GG). A pictorial explanation of restriction patterns observed in the wild-type isolates (sensitive) and mutants (resistant) is provided in Fig. 1.

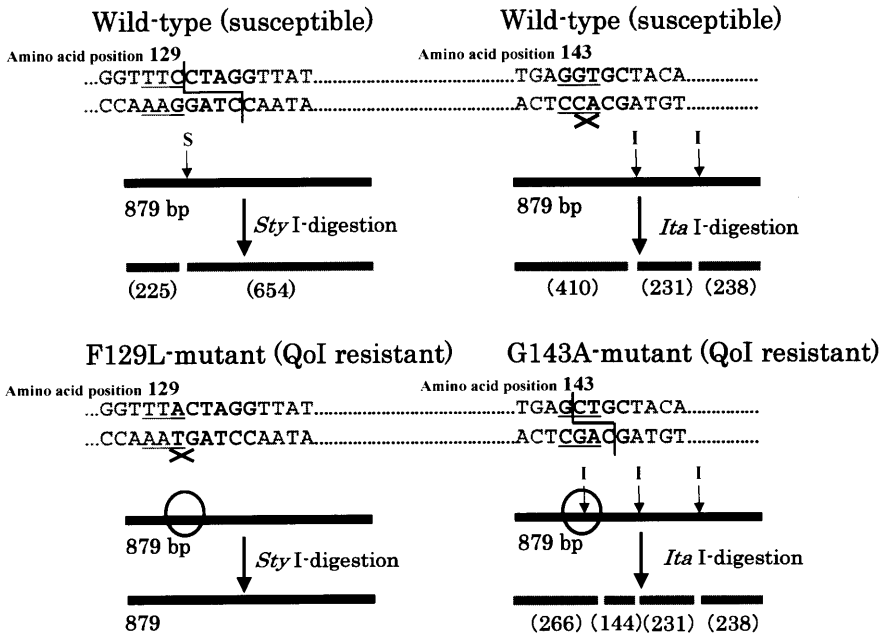

Fig. 1. Cleavage model of the 879-bp internal fragment of the cytochrome *b* gene in the wild-type (susceptible) and mutants (QoI resistant) of *Magnaporthe grisea*. S: *Sty*I site, I: *Ita*I site, circle: site changed.

RESULTS AND DISCUSSION

The baseline for the sensitivity to metominostrobin of *M. grisea* collected from paddy fields at six locations in Japan in 1999 was established as 3.3 μg/ml, the mean value of the EC_{50}, by assessment *in vivo*. The EC_{50} ranged from 0.65 to 11.8 μg/ml (Table 1, Fig. 2). The EC_{50} of Ine 86-137, a laboratory strain of *M. grisea* isolated in 1986, was 0.14 μg/ml.

From 2001 to 2003, metominostrobin provided full control of rice blast in the subjected paddy fields (Table 2). From the results of the assessment *in vivo* of sensitivity to metominostrobin with 56 isolates collected in 2001–2003, the EC_{50} ranged from less than 0.1 to 9.4 μg/ml. There was no significant difference in the range of EC_{50} values between the isolates obtained from the paddy field with a record of metominostrobin treatment, those from the field with no record of treatment and the isolates used to establish the baseline (Fig. 2, Table 2), suggesting that no shift in the sensitivity of the fungus to metominostrobin occurred from 2001 to 2003.

All 96 isolates of *M. grisea* including the six used to establish the baseline and Ine 86-137 showed a wild-type cleavage pattern in the PCR-RFLP analyses using *Ita*I or *Sty*I, revealing that no mutation at amino acid position 143 or 129 in the cytochrome *b* occurred in any of the isolates tested (Tables 1, 2 and Fig. 3).

The resistance to QoI resulting from F129L-mutation, found in cytochrome *b* of *M. grisea* isolated from gray leaf spot on perennial ryegrass, was not as extreme as that due to the G143A-mutation found in several pathogenic fungi.[8] In various plant pathogenic fungi, QoI-resistant isolates without the G143A-mutation play a minor role in the field.[9] The mutation F129L increased resistance to QoI by 30 to 140 fold in terms of the EC_{50} value in comparison to wild-type isolates.[8] The relatively wide range of

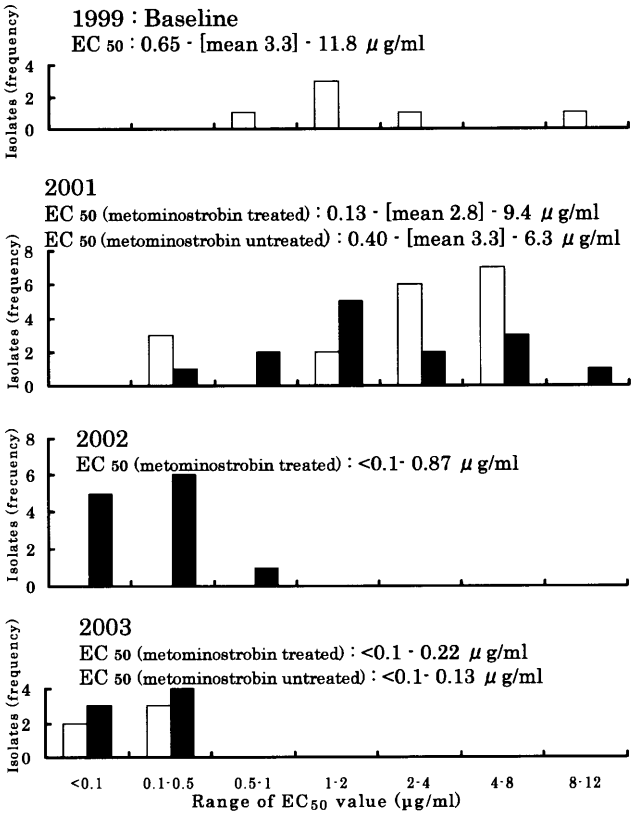

Fig. 2. EC_{50} values for metominostrobin of 62 isolates of *Magnaporthe grisea* collected in 1999 and 2001 through 2003. □: isolates obtained from rice in a paddy field with no record of metominostrobin treatment. ■: isolates obtained from rice in a paddy field with a confirmed record of metominostrobin treatment.

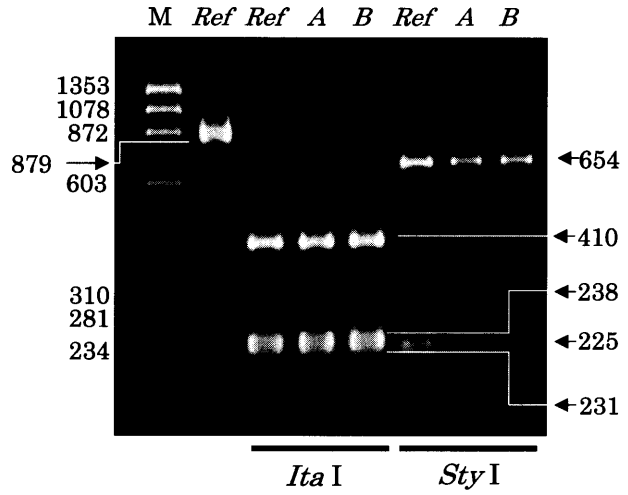

Fig. 3. PCR-RFLP with *Ita*I or *Sty*I in the field isolates of *Magnaporthe grisea* using the 879-bp fragment of the cytochrome *b* gene. M: molecular marker. *Ref*: *M. grisea*, Ine 86-137 (a laboratory stock, race 007, susceptible to metominostrobin, EC_{50} value=0.14 μg/ml). *A*: *M. grisea*, 99-25-001 (a field isolate obtained in 1999, susceptible to metominostrobin, EC_{50} value=11.8 μg/ml). *B*: *M. grisea*, 02-010 (a field isolate obtained in 2002, susceptible to metominostrobin, EC_{50} value=0.87 μg/ml).

EC_{50} values for metominostrobin obtained from our study of *M. grisea* field isolates had been suspected to be the result of the F129L-mutation, but the PCR-RFLP analysis did not support this hypothesis.

The test *in vitro* on sensitivity to metominostrobin was conducted in the presence of SHAM (salycilhydroxamic acid), which suppresses an alternative oxidase,[8] however the EC_{50} values fluctuated between the replications (data not shown). Thus the *in vivo* assessment method discussed here is useful to calculate EC_{50} values for monitoring the sensitivity of rice blast fungus (*M. grisea*) to metominostrobin.

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