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Note

Simple Method for Monitoring the Sensitivity of *Pyricularia oryzae* to Fthalide

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INTRODUCTION

Rice blast caused by *Pyricularia oryzae* is one of the most serious plant diseases in rice production in eastern Asia. Fthalide (4,5,6,7-tetrachlorophthalide, Rabcide®) has been widely used as a foliar fungicide against rice blast disease since 1971. It prevents appressorial penetration by inhibiting melanin biosynthesis in *P. oryzae*[1,2] like tricyclazole[2,3] (5-methyl-1,2,4-triazolo(3,4-*b*)benzothiazole, Beam®) or pyroquilon[3] (1,2,5,6-tetrahydro-4*H*-pyrrolo-(3,2,1-*i,j*)-quinoline-4-one, Coratop®).

While melanin biosynthesis inhibitors (MBIs) have been used as basic rice blast fungicides for nearly 30 years, there have been no reports of insufficient efficacy (relating to the less-sensitive strains) or of the appearance of resistant strains in the fields. Therefore, MBIs are considered a low-risk fungicide in terms of the development of resistance against *P. oryzae*.

As exemplified by tricyclazole action mechanism, the major group of MBIs inhibits the reduction steps from 1,3,6,8-tetrahydroxynaphthalene to scytalone and from 1,3,8-trihydroxynaphthalene to vermelone in the melanin biosynthetic pathway,[2,4-9] whereas carpropamide interferes with the dehydration reactions from scytalone to 1,3,8-trihydroxynaphthalene and from vermelone to 1,8-dihydroxynaphthalene.[10-12] Since MBIs have little or no fungitoxic properties on *P. oryzae*, the minimum inhibitory concentrations, or the effective concentrations, on mycelial growth do not represent the sensitivities of individual strains to MBIs. Thus, the sensitivity of *P. oryzae* to each MBI should be determined by a certain other method. Appressorial penetration tests on cellulose membranes[13] or inoculation tests on rice leaf sheaths[14] or onion epidermis[15] could give the sensitivity profiles of *P. oryzae* for MBIs. But these methods are difficult to use practically when we handle a number of isolates for the tests.

In this paper, a simple method for monitoring the sensitivity of *P. oryzae* to fthalide on cellulose membranes is described, using strains isolated before 1976 and others isolated in the 1990s.

MATERIALS AND METHODS

1. *Chemicals*

A formulation of fthalide 20% flowable (FL) (Kureha Chemical Industry, Tokyo) or a formulation of tricyclazole 20% FL (Takeda Chemical Industries, Tokyo) was directly suspended in distilled water.

2. *Isolates*

The Agricultural Experiment Stations of Niigata, Fukushima, Miyagi, Akita and Hokkaido prefectures kindly supplied 110 field isolates of *P. oryzae*, which were collected from 1995 to 1998. The gene bank of the Japanese Ministry of Agriculture, Forestry and Fisheries provided 11 isolates, which were collected from 1953 to 1976. The standard strain P-2, isolated in 1948,[16] was also used in this experiment.

3. *Cultural Methods*

All the strains were maintained on rice bran agar medium. Four agar disks (4 mm in diameter), cut from the edge of pre-cultured mycelia on an oatmeal-sucrose medium, were placed on a fresh oatmeal-sucrose medium in a petri dish and were incubated at 25°C for 9 days. After removal of the aerial hyphae, the cultures were placed under fluorescent light at 25°C for 4 days. Spores formed were collected and suspended in sterilized distilled water and filtered through bleached cotton cloth. Spore concentrations were adjusted to 1×10^4 to 1×10^5/ml, and the suspension was subjected to the cellulose membrane test.

The mycelial mass of *P. oryzae* from the stock culture was transferred to 30 ml of yeast-glucose liquid medium (yeast extract 20 g/l, glucose 5 g/l) with 10 to 20 glass beads (10 mm in diameter) in a 100-ml Erlenmeyer flask. The flask was incubated at 25°C for 3 days and shaken once a day to disperse the mycelial mass. Then, 2 ml of mycelial suspension was transferred onto the rice bran agar medium in a petri dish and incubated at 25°C for 4 to 10 days under continuous fluorescent light. Spores were harvested and suspended in distilled water. Spore concentrations were adjusted to 1×10^5/ml to 5×10^5/ml, and then the suspension was used for the pot tests.

4. *Determination of Minimum Inhibitory Concentrations of Appressorial Melanization*

The cellulose membrane method was employed to observe the melanization of appressoria, substantially according to the method of Araki and Miyagi,[13] which was modified by

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substituting sterilized water in place of the rice leaf homogenate. The chemical solutions were prepared at the given concentrations for fthalide and tricyclazole with two-fold dilution steps. A sterilized filter paper (Toyo No. 2, 85 mm in diameter) was placed in a petri dish, and 2.5 ml of the chemical solution was added. The cellulose membrane (Seamless Cellulose Tubing, Sanko Junyaku Co. Ltd., Tokyo) was pretreated in boiling distilled water and dried. Cellulose membranes in 10 mm squares were sufficiently immersed in the chemical solution and smoothed out on the filter paper. Then 10 μl of spore suspension was dropped on the cellulose membranes and incubated at 25°C for 2 days. Appressoria on the membranes were observed under a light microscope. The degrees of melanization were assessed according to a scale from "−" (unmelanized) to a "+" (fully melanized), with 20 to 30 appressoria being surveyed from each cellulose membrane piece. Then, the lowest concentration at which the degree of appressorial melanization was "−" was considered as the minimum inhibitory concentration of appressorial melanization (MICAM).

5. Pot Test

The isolates having different MICAM values were inoculated on the rice seedlings treated with fthalide to evaluate the preventive values on plants. Rice (*Oryza sativa* L, cv. Koshihikari) plants were grown in a plastic pot (64 mm×64 mm) to the four-leaf stage. Fthalide was sprayed at 50, 100 or 200 mg/l with a volume of 1000 l/ha. The spore suspension of each isolate, which is chosen from the group of different MICAM values, was sprayed onto the treated plants 3 hr after fthalide application. The plants were kept under a high humidity condition at 25°C for 2 days, and were then maintained in a growth chamber at 25°C for 12 hr (light) and at 20°C for 12 hr (dark). The disease severity index was assessed on 10 rice seedlings 6 days after inoculation. The protective value was calculated on the infected area converted from the severity index.

RESULTS AND DISCUSSION

The ranges of MICAM (minimum inhibitory concentration of appressorial melanization) for the MBIs were evaluated by the cellulose membrane method. Ten out of 12 strains isolated before 1976, and 79 out of 110 strains isolated in the 1990s, formed distinct melanized appressoria on the cellulose membranes. The rest of the tested isolates did not form enough appressoria or did not melanize under these conditions. The degree of melanization in appressoria was assessed visually under a light microscope. Typical examples of melanized appressoria in the control and unmelanized appressoria in the presence of MBI are shown in Fig. 1.

Table 1 The MICAM values of *Pyricularia oryzae* isolates before 1976 to fthalide or tricyclazole.

Isolates	Collected year	MICAM[a] (mg/l)	
		Fthalide	Tricyclazole
MAFF 101109[b]	1976	0.39	0.05
MAFF 101110	1976	1.56	0.20
MAFF 101111	1976	0.39	0.10
MAFF 101113	1976	0.20	0.05
MAFF 101114	1976	0.78	0.20
MAFF 101115	1976	ND[c]	ND
MAFF 101119	1976	ND	ND
MAFF 101122	1976	0.39	0.10
MAFF 101123	1976	0.20	0.10
MAFF 305001	1953	0.39	0.10
MAFF 305473	1968	0.78	0.10
P-2	1948	0.78	0.10

[a] Minimum inhibitory concentration of appressorial melanization on the cellulose membrances. [b] Ministry of Agriculture, Forestry and Fisheries. [c] Not determined.

Control (+) [a]　　Fthalide 0.78 mg/l (−)

Fig. 1 Microscopic photographs of melanized and unmelanized appressoria on the cellulose membranes.
[a] The degree of appressorial melanization: + (fully melanized), − (unmelanized).

Only three strains of *P. oryzae* isolated before the launch of fthalide in 1971 were available for this study. Therefore, these strains together with 9 strains isolated in 1976 were grouped as "not or lesser-exposed" to fthalide. The MICAM values of these isolates before 1976 are shown in Table 1. The sensitivities to fthalide of *P. oryzae* isolates from the 1990s ranged from 0.10 mg/l to 1.56 mg/l, with a peak at 0.39 mg/l (Fig. 2). No significant diversity of sensitivity in the *P. oryzae* isolates to fthalide has been seen, even after nearly 30 years of practical use (Fig. 2).

The MICAM values of the isolates from the 1990s to tricyclazole ranged from 0.01 mg/l to 0.39 mg/l, with a peak at 0.10 mg/l (Fig. 3). Although the MICAM values of the isolates to tricyclazole were lower than those to fthalide, the profiles of the sensitivities to both fungicides were identical. The cross-sensitivity between fthalide and tricyclazole was present with a correlation coefficient of 0.463 ($P<0.01$) (Fig. 4). There was no change in the sensitivity profiles to tricyclazole in *P. oryzae* between the isolates before 1976 and the isolates in the 1990s. As tricyclazole was registered in 1981, the sensitivity profile of the isolates collected before 1976 can be considered as the baseline sensitivity.

Figure 5 shows the box-and-whiskers plots of the MICAM values of the isolates in the 1990s to fthalide in each prefecture. There was no significant difference in the sensitivities of the isolates to fthalide among the regions. The number of applications of fthalide in Akita should be greater than that in Hokkaido, where rice blast fungicides have been used to a lesser extent. These data suggest that the number of application times did not cause much selection pressure or alter the fungal sensitivity to MBIs. Figure 6 shows the MICAM values of isolates to tricyclazole in each region. The mean values of MICAM to tricyclazole in each prefecture are distributed within a narrow range, from 0.07 mg/l to 0.15 mg/l.

In the pot tests, no difference was observed in the preventive values of fthalide at the concentrations of 50, 100 and 200 mg/l to the eight tested strains (Table 2). Strains of MAFF101109, MAFF101110 and A3 formed fewer lesions

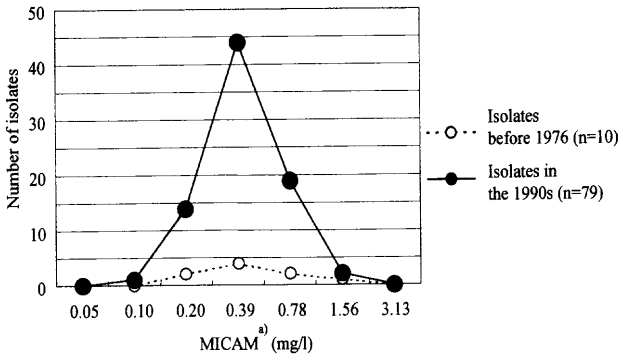

Fig. 2 Sensitivity distribution of *Pyricularia oryzae* isolates to fthalide.
a) MICAM : Minimum inhibitory concentration of appressorial melanization on the cellulose membranes.

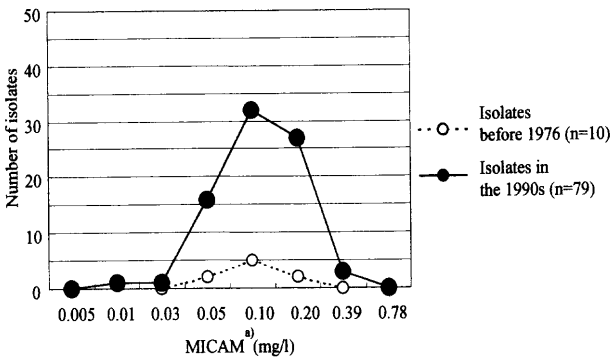

Fig. 3 Sensitivity distribution of *Pyricularia oryzae* isolates to tricyclazole.
a) MICAM : Minimum inhibitory concentration of appressorial melanization on the cellulose membranes.

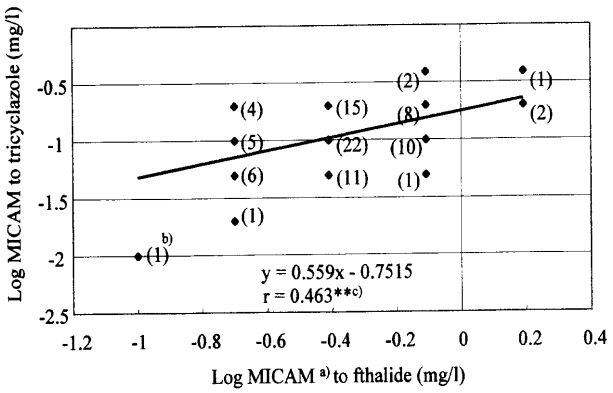

Fig. 4 Cross-sensitivity profile in *Pyricularia oryzae* isolates to fthalide and tricyclazole.
a) MICAM : Minimum inhibitory concentration of appressorial melanization on the cellulose membranes.
b) Figures in parenthesis show the number of isolates.
c) ** : Significance level for correlation, $P<0.01$.

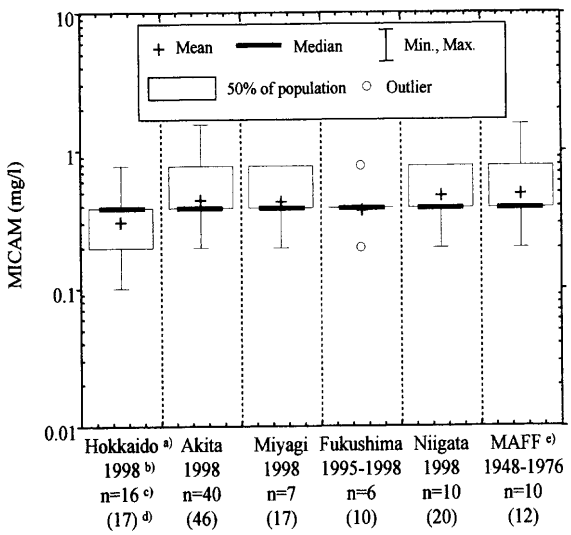

Fig. 5 MICAM values of *Pyricularia oryzae* isolates to fthalide in each prefecture.
a) Origin. b) Collected year. c) Number of isolates forming distinct melanized appressoria on the cellulose membranes. d) Number of tested isolates. e) Ministry of Agriculture, Forestry and Fisheries.

Table 2 Efficacy of fthalide by protective foliar application against rice blast inoculated with field isolates which have different MICAM values.

Isolate[a]	Conc. of spores (counts/ml)	Infected area of untreated control	Preventive value[b] Conc. of fthalide (mg/l) 50	100	200	MICAM[c] (mg/ml)
H15	5×10^5	41.8	100	99.8	100	0.2
MAFF101109	5×10^5	9.6	99	100	100	0.39
P2	5×10^5	31.9	99.7	99.7	100	0.78
N2	5×10^5	44.3	95.1	98.6	99.8	0.78
N11	5×10^5	54.5	91.3	96.7	99.8	0.78
MAFF101110	5×10^5	2.4	100	100	100	1.56
A3	1×10^5	1.8	97.2	99.1	100	1.56
A33	5×10^5	32.9	99.3	99.9	99.9	1.56

[a] H15: Hokkaido (1998), MAFF101109: Nagano (1976), P2: Niigata (1948), N2 and N11: Niigata (1998), MAFF101110: Fukui (1976), A3 and A33: Akita (1998). [b] [1-(mean infected area of treated plant)/(mean infected area of untreated plant)] $\times$ 100. [c] Minimum inhibitory concentration of appressorial melanization on the cellulose membranes.

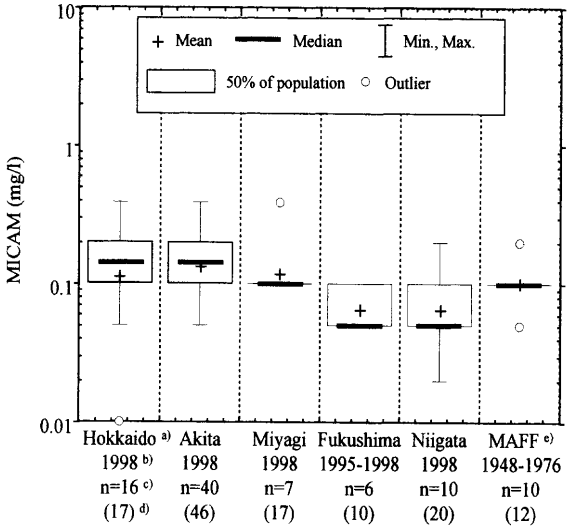

Fig. 6 MICAM values of *Pyricularia oryzae* isolates to tricyclazole in each prefecture.
[a] Origin. [b] Collected year. [c] Number of isolates forming distinct melanized appressoria on the cellulose membranes. [d] Number of tested isolates. [e] Ministry of Agriculture, Forestry and Fisheries.

on the untreated control and seemed less pathogenic than the other strains. N11 caused serious damage to the untreated plants.

MBIs also inhibit mycelial pigmentation of *P. oryzae* in the course of vegetative growth. We attempted to estimate minimum inhibitory concentrations of mycelial melanization to the above-mentioned isolates on agar plates. However, mycelial melanization varied according to the kind or volume of the medium and also according to light conditions (data not shown). Moreover, the diversity of mycelial melanization among untreated isolates made it difficult to draw out the MBI effect on the melanization.

This is the first report to monitor the sensitivity of *P. oryzae* to MBIs by a cellulose membrane method before and after the introduction of MBIs to practical use. We confirmed that there was no significant diversity of sensitivity to fthalide among the strains isolated before 1976 and those in the 1990s, after nearly 30 years of application background. Recently, new MBIs,[17-19] with a different mode of action from that of fthalide or tricyclazole, have been introduced as rice blast fungicides. The approach presented in this paper may be useful for assessing the resistance risk for new MBIs.

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要　　約

イネいもち病菌のフサライドに対する簡易な感受性検定方法

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メラニン生合成阻害剤(MBI)の作用は非殺菌的であることから，イネいもち病に対する感受性検定法が確立していない．セロファン膜上でのイネいもち病菌付着器のメラニン化阻害を指標として，フサライドに対する圃場分離株の感受性分布を調べた．フサライド実用化前後にあたる1976年以前に分離された農水省由来12株中10株と，各県の農業試験場から提供を受けた1990年代の分離菌110株中79株は，セロファン膜上で付着器を形成し，付着器のメラニン化も認められた．これらの菌株に対するフサライドのメラニン化最低阻害濃度（MICAM）は，0.10～1.5 mg/lの範囲で0.39 mg/lを頂点とする一峰性の分布を示し，分離年，分離された地域における違いは認められなかった．トリシクラゾールについても一峰性の分布が得られ，両剤のMICAM値間には正の相関が認めめられた．また，MICAMが異なる菌株をポット接触試験に供したが，これらに対するフサライドの効果に違いは認められなかった．MBI長期使用によっても，イネいもち病菌の感受性に変動は認めめられず，MICAMは，感受性変動を監視する簡便な指標と考えられる．