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Note

Management of melanin biosynthesis dehydratase inhibitor (MBI-D)-resistance in *Pyricularia oryzae* using a non-MBI-D fungicidal application program for nursery boxes and a diclocymet and ferimzone mixture for field foliar applications

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We compared the risk of reselecting melanin biosynthesis dehydratase inhibitor (MBI-D)-resistant *Pyricularia oryzae* isolates between two treatment programs, a nursery box application of diclocymet and a rotational program in which nursery boxes received a non-MBI-D fungicide application and then later, in the field, a foliar application of a diclocymet and ferimzone mixture. Both were effective against panicle blast. However, the latter prevented the reselection of MBI-D-resistant isolates more effectively than the former. © Pesticide Science Society of Japan

Keywords: MBI-D, resistance management, diclocymet, ferimzone, rice blast.

Melanin biosynthesis inhibitors (MBIs) are important fungicides that control rice blast. There are three known groups of MBIs at present. Scytalone dehydratase (SH) inhibitors, including melanin biosynthesis dehydratase inhibitors (MBI-Ds), form one group of MBIs. The MBI-Ds include carpropamid,[1–3] diclocymet and fenoxanil. Both carpropamid and diclocymet are mainly applied in nursery boxes to control rice blast. The risk of *P. oryzae* developing resistance to MBIs was assumed to be very low because MBIs, without fungicidal activities, inhibit the pathogen's infection process in rice leaves. However, *P. oryzae* is classified as a pathogen that has a high risk of developing fungicidal resistance as assessed by the Fungicide Resistance Action Committee.[4] *P. oryzae* isolates resistant to carpropamid were first detected in 2001 in Saga Prefecture, Japan.[5] These resistant isolates also showed cross-resistance to diclocymet and fenoxanil. Suzuki *et al.* (2010) monitored the emergence frequency of resistant isolates from fields in Kyushu after MBI-D applications were stopped and showed that the frequency declined.[6] Kimura and Fujimoto (2015) reported that the emergence frequency of resistant isolates decreased rapidly without the presence of MBI-Ds in laboratory and field tests.[7] Kimura and Fujimoto (2017) also reported that the resistant SH activity was lower than the sensitive SH activity.[8] Previous reports suggested that *P. oryzae* isolates resistant to MBI-Ds paid a fitness penalty to acquire the resistance. However, MBI-D reuse has not been implemented in the area in which resistant isolates were widely found because there are no good resistance management practices to prevent the re-emergence of MBI-D-resistant isolates. In general, an application program using several fungicides with different modes of action, or using a mixture of single-site and multisite fungicides, is recommended as the management strategy for fungicide resistance.[9] However, management strategies for MBI-D-resistance have rarely been thoroughly investigated. There was only one report regarding MBI-D-resistance management by Kimura and Fukuchi, which focused on the contribution of benomyl to the control of seed-borne *P. oryzae*.[10] There are three ways to control rice blast during the pathogen's life cycle: controlling seed-borne rice blast, controlling leaf blast in paddy fields, and controlling panicle blast. In a previous report, we focused on seed-borne rice blast treatments to manage the risk of MBI-D resistance. We indicated that benomyl was a prominent candidate to control seed-borne rice blast and that its use resulted in a reduced emergence risk of MBI-D-resistant leaf blast isolates in a paddy field.[10]

In this study, we focused on controlling panicle blast and managing the emergence risk of MBI-D-resistant panicle blast isolates. We investigated resistance management practices using application programs that incorporated fungicides with different modes of action and those using a mixture of diclocymet and ferimzone, after which resistant isolates have never been detected. We also compared the risk of the re-emergence of MBI-D-resistant isolates between a nursery box application of an MBI-D fungicide and a foliar application of an MBI-D-related product. In general, foliar applications of fungicides have shorter efficacy periods than nursery box applications because the formulations for the latter usually have a controlled-release mechanism. We assumed that the risk of re-emergent resistant isolates after a foliar application of a fungicide is lower than after a nursery box application of a fungicide. Thus, we compared the re-emergence risk of *P. oryzae* resistant to MBI-D between a nursery box ap-

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Table 1. Products used for the Ehime trial

Common name	Target pest	Content (%)	Formulation	Note
Ferimzone	Blast	2	Dust	Diclocymet is an MBI-D.
Diclocymet	Blast	0.2		
Clothianidin	Insect	0.15		
Ferimzone	Blast	2	Dust	No MBI-Ds
Phthalide	Blast	1.5		
Clothianidin	Insect	0.15		
Diclocymet	Blast	3	Granule	Diclocymet is an MBI-D.
Furametpyr	Sheath blight	4		
Fipronil	Insect	1		
Orysastrobin	Blast	7	Granule	No MBI-Ds
Clothianidin	Insect	1.5		
Tiadinil	Blast	6	Granule	No MBI-Ds
Furametpyr	Sheath blight	4		
Fipronil	Insect	1		

plication of diclocymet and a foliar application of a mixture of diclocymet and ferimzone, and we evaluated the efficacy rates of an application program that incorporated a nursery box application of a non-MBI-D fungicide and the field application of a mixture of diclocymet and ferimzone.

We used two rice fields in this study. One rice field in Ehime Prefecture was used in 2008. This field had been used in a previous report.[10] The frequency of MBI-D-resistant isolates in this area was −100% in 2003. MBI-D applications were halted in 2004. Thereafter, the frequency of MBI-D-resistant isolates declined annually, and by 2006, the frequency was almost 0%.[11] The other rice field in Saga Prefecture was used in 2008 and 2009 for this study. The frequency of MBI-D-resistant isolates in the field was −100% in 2001. MBI-D applications were halted in 2002. Thereafter, the frequency of MBI-D-resistant isolates declined annually, and by 2004, the frequency was approximately 1%.[7]

Pyricularia oryzae isolates were collected from panicle blast lesions in a field using a previously published method.[7] For the Ehime trial, the chemicals used are shown in Table 1 and were purchased from an agricultural cooperative. All granule products were applied in nursery boxes at planting time. The application rate was 50 g of formulation per nursery box (registered dosage in Japan). All dust products were applied on leaves and panicles at 4 kg of formulation per 10 ares (registered dosage in Japan) at the end of panicle emergence (BBCH scale: 59). Seedlings were transplanted into the paddy field using a commercial mechanical transplanter (Kubota). Each treatment occupied a plot containing 78 m^2 (6 m×13 m). To evaluate the efficacy of our application program on panicle blast, we assessed three different points in each plot 31 days after foliar application. The rice cultivar used was Kinuhikari.

Our trial application program for the Saga trials was included in field trials, described as renrakushiken in Japanese, to evaluate the novel application program. These trials were coordinated by the Saga Agricultural Research Center. For the Saga trials, the chemicals used are shown in Table 2 and were purchased from an agricultural cooperative. All granule products were applied in nursery boxes at planting time. The application rate was 50 g of formulation per nursery box (registered dosage in Japan). All dust products were applied on leaves and panicles at 4 kg of formulation per 10 ares (registered dosage in Japan). One flowable product was diluted to $1\times1{,}000^{-1}$ in water

Table 2. Products used for the Saga trials

Common name	Target pest	Content (%)	Formulation	Note
Ferimzone	Blast	15	Flowable	Diclocymet is an MBI-D.
Diclocymet	Blast	3.5		
Ferimzone	Blast	2	Dust	No MBI-Ds
Tricyclazole	Blast	0.5		
Tricyclazole	Blast	4	Granule	No MBI-Ds
Thifluzamide	Sheath blight	3		
Imidacloprid	Insect	2		
Spinosad	Insect	0.75		

(registered concentration in Japan). These products were applied twice to maximize the emergence risk of resistant isolates by diclocymet+ferimzone when 20% of panicles had emerged (BBCH scale: 52) and at the end of panicle emergence (BBCH scale: 59). Seedlings were transplanted into the paddy field using a commercial mechanical transplanter (Yanmar). Three replications for each treatment were designed for the studies. Each plot not receiving a granule product application in a replication occupied 9 m^2 (1.5 m×6 m) and each plot receiving a granule product application occupied 24 m^2 (4 m×6 m) in 2008 and 18 m^2 (3 m×6 m) in 2009. To evaluate the efficacy of our application program on panicle blast, we assessed three different points in each plot 24 days after the last foliar application in 2008 and 17 days after the last foliar application in 2009. The rice cultivar used was Hinohikari.

Lesions of panicle blast were collected from each plot. *P. oryzae* was isolated as mentioned above. The frequency of the resistant isolates in each plot was analyzed using primer-introduced restriction enzyme analysis-based PCR, following the method of Kaku *et al.*[12)]

In the Ehime trial, the assessment of efficacy was conducted 31 days after foliar application. Incidences of panicle blast (% of diseased panicles) were assessed at three different points in each plot. In total, 500 panicles (25 plants at 20 panicles/plant) were assessed at one point. Thus, in each plot, 1,500 panicles were assessed. A χ^2 test ($p<0.05$) was conducted based on the percentage of diseased panicles (incidence). The disease incidence in the untreated plot (UTC, none/none program (nursery/foliar application)) was 16.5. The disease pressure was acceptable for evaluating the fungicides' efficacy levels. Disease incidences under the none/ferimzone+phthalide and none/diclocymet+ferimzone programs were 7.7 and 3.9, respectively. These incidence levels were statistically lower than that of the UTC. These mixtures showed good efficacy levels against panicle blast. All fungicide programs with nursery box and foliar applications showed a very low disease incidence in this field, regardless of the fungicide's mode of action. Disease incidence levels for diclocymet/none and diclocymet/diclocymet+ferimzone programs were 1.0 and 1.1, respectively, which indicated that the performance of diclocymet recovered in the field. Disease incidence levels under orysastrobin/ferimzone+diclocymet and tiadinil/ferimzone+diclocymet programs were 0.5 and 0.7, respectively, which were comparable with those of the orysastrobin/ferimzone+phthalide and tiadinil/ferimzone+phthalide programs. This indicated that the diclocymet and ferimzone mixture did not negatively affect the efficacy levels of application programs against panicle blast (Table 3).

In the UTC plot, the frequency of resistant isolates from panicle blast was 0. However, in the MBI-D only program (diclocymet/none) and MBI-D/MBI-D mixture program (diclocymet/ferimzone+diclocymet), the frequencies of resistant isolates were 20.0 and 47.4, respectively. The frequencies of resistant isolates in both the MBI-D only and MBI-D/MBI-D mixture programs were statistically different from that in the UTC plot according to Fisher's exact test ($p<0.05$). However, in the none/diclocymet+ferimzone program, the frequency of resistant isolates was 9.4. The frequency of resistant isolates in the none/MBI-D mixture program was not statistically different from that in the UTC plot according to Fisher's exact test ($p<0.05$). Thus, the foliar application of diclocymet+ferimzone resulted in a lower risk of the re-emergence *P. oryzae* resistant to MBI-Ds than nursery box applications of MBI-Ds. The frequency of resistant isolates in the QoI fungicide (orysastrobin)/diclocymet+ferimzone program was 14.3. The frequency of resistant isolates in the systemic acquired resistant (SAR) fungicide (tiadinil)/diclocymet+ferimzone program was 0. The frequencies of resistant isolates in all plots treated with non MBI-D fungicide/diclocymet+ferimzone programs were also lower than that in the MBI-D program (diclocymet/diclocymet+ferimzone). These frequencies were not statistically different from that in the UTC plot according to Fisher's exact test ($p<0.05$). These results indicated that non-MBI-D/diclocymet+ferimzone programs had lower risks of the re-emergence of MBI-D-resistant *P. oryzae* than nursery box applications of MBI-Ds. In particular, in the SAR fungicide (tiadinil)/diclocymet+ferimzone program, no *P. oryzae* isolates resistant to MBI-Ds were detected in this study. Thus, the SAR/diclocymet+ferimzone and UTC programs had comparable risks of the re-emergence of resistant isolates (Table 4).

In the Saga trial in 2008, the disease incidence in the UTC plot (none/none/none program (nursery/1st foliar/2nd foliar application)) was 50.3. The disease pressure was high enough for evaluating the fungicides' efficacy levels. Disease incidences under the none/diclocymet+ferimzone/diclocymet+ferimzone and none/ferimzone+tricyclazole/ferimzone+tricyclazole programs were 14.0 and 14.1, respectively. These incidence levels

Table 3. Panicle blast incidence in each plot in Ehime[a)]

Products for blast control[b)]		Disease incidence (%)[c)]
Nursery box	Foliar application	
Orysastrobin	Ferimzone+diclocymet	0.5 a
Orysastrobin	Ferimzone+phthalide	1.0 a
Orysastrobin	None	2.2 a#
Tiadinil	Ferimzone+diclocymet	0.7 a
Tiadinil	Ferimzone+phthalide	1.4 a
Tiadinil	None	2.0 a#
Diclocymet	Ferimzone+diclocymet	1.1 a
Diclocymet	Ferimzone+phthalide	1.3 a
Diclocymet	None	1.0 a
None	Ferimzone+diclocymet	3.9 a#
None	Ferimzone+phthalide	7.7 a#
None	None	16.5#

[a)] Application date: May 20 (planting), June 12 (flowering). Assessment date: September 17. [b)] Type of application. [c)] "a" Statistically different from the none–none plot according to a χ^2 test ($p<0.05$). "#" Statistically different from the orysastrobin–ferimzone+phthalide plot based on a χ^2 test ($p<0.05$).

Table 4. Frequency of resistant isolates to MBI-Ds in each plot in Ehime[a)]

Products for blast control		No. of tested isolates[c)]	No. of resistant isolates[d)]	% of resistant isolates[e)]
Nursery box[b)]	Foliar application[b)]			
Orysastrobin	Ferimzone+diclocymet	14	2	14.3
Orysastrobin	Ferimzone+phthalide	10	1	10
Orysastrobin	None	16	0	0
Tiadinil	Ferimzone+diclocymet	19	0	0
Tiadinil	Ferimzone+phthalide	17	0	0
Tiadinil	None	19	0	0
Diclocymet	Ferimzone+diclocymet	19	9	47.4#
Diclocymet	Ferimzone+phthalide	13	1	7.7
Diclocymet	None	20	4	20.0#
None	Ferimzone+diclocymet	32	3	9.4
None	Ferimzone+phthalide	33	0	0
None	None	39	0	0

[a)] Application date: May 20 (planting), June 12 (flowering) Sampling date: September 17. [b)] Type of application. [c)] *P. oryzae* isolates were isolated from panicle blast. [d)] Sensitivities were analyzed by primer-introduced restriction enzyme analysis-based PCR. [e)] "#" Statistically different from the none–none plot according to Fisher's exact test ($p<0.05$).

were statistically lower than that of the UTC. These mixtures showed good efficacy levels against panicle blast. All fungicide programs with nursery box and foliar applications showed very low disease incidence levels in this field, regardless of the fungicide's mode of action. For example, the disease incidence level for the tricyclazole/diclocymet+ferimzone/diclocymet+ferimzone program was 5.8, which was comparable with that of the reference program (tricyclazole/ferimzone+tricyclazole/ferimzone+tricyclazole). This indicated that the diclocymet and ferimzone mixture did not negatively affect the efficacy levels of application programs against panicle blast (Table 5).

In the UTC (none/none/none program) and tricyclazole plots (tricyclazole/none/none program), the frequency levels of resistant isolates from panicle blast were 2.2 and 2.7, respectively. In the MBI-D-related product only program (none/diclocymet+ferimzone/diclocymet+ferimzone), the frequency of resistant isolate emergence was 5.9, which was not statistically different from those in the UTC and tricyclazole plots. Thus, a foliar application of diclocymet+ferimzone did not induce the re-emergence of resistant isolates in this field. Additionally, the frequency of resistant isolates in the tricyclazole/diclocymet+ferimzone/diclocymet+ferimzone program was 0. In this trial, one *P. oryzae* isolate resistant to MBI-Ds was detected from the UTC. However, no *P. oryzae* isolates resistant to MBI-Ds were detected from the tricyclazole/diclocymet+ferimzone/diclocymet+ferimzone program. Thus, a program using nursery box applications of a non-MBI-D product reduced the risk of resistant isolate re-emergence as compared with the foliar application of an MBI-D-related product (Table 6).

The results of the 2009 Saga trial were very similar to those of 2008. All of the fungicide programs with nursery box and foliar applications showed very low disease incidences and no *P. oryzae* isolates resistant to MBI-Ds were detected from the trial in 2009, regardless of the fungicide's mode of action (Tables 5 and 6).

Table 5. Panicle blast incidence in each plot in Saga in 2008 and 2009[a)]

Products for blast control			Disease incidence (%)[c)]	
Nursery box[b)]	1st foliar application[b)]	2nd foliar application[b)]	2008	2009
Tricyclazole	Ferimzone+diclocymet	Ferimzone+diclocymet	5.8a#	2.0a
Tricyclazole	Ferimzone+diclocymet	Ferimzone+tricyclazole	6.8a	1.8a
Tricyclazole	Ferimzone+tricyclazole	Ferimzone+tricyclazole	7.8a	1.5a
Tricyclazole	None	None	32.1a#	Not tested
None	Ferimzone+diclocymet	Ferimzone+diclocymet	14.0a#	Not tested
None	Ferimzone+diclocymet	Ferimzone+tricyclazole	13.0a#	Not tested
None	Ferimzone+tricyclazole	Ferimzone+tricyclazole	14.1a#	Not tested
None	None	None	50.3#	6.8#

[a)] Application date: June 17 (planting), August 21 and 28 in 2008; and June 18 (planting), August 20 and 30 in 2009. Assessment date: September 21 in 2008 and September 16 in 2009. [b)] Type of application. [c)] "a" Statistically different from the none–none–none plot according to a χ^2 test ($p<0.05$). "#" Statistically different from the tricyclazole–ferimzone+tricyclazole–ferimzone+tricyclazole plot accurding to a χ^2 test ($p<0.05$).

Table 6. Frequency of resistant isolates to MBI-Ds in each plot in Saga in 2008 and 2009[a]

Products for blast control			% of resistant[c] (No. of tested isolates[d])	
Nursery box[b]	1st foliar application[b]	2nd foliar application[b]	2008	2009
Tricyclazole	Ferimzone+diclocymet	Ferimzone+diclocymet	0 (31)	0 (30)
Tricyclazole	Ferimzone+diclocymet	Ferimzone+tricyclazole	0 (40)	0 (30)
Tricyclazole	Ferimzone+tricyclazole	Ferimzone+tricyclazole	0 (37)	0 (30)
Tricyclazole	None	None	2.7 (37)	not tested
None	Ferimzone+diclocymet	Ferimzone+diclocymet	5.9 (34)	not tested
None	Ferimzone+diclocymet	Ferimzone+tricyclazole	0 (39)	not tested
None	Ferimzone+tricyclazole	Ferimzone+tricyclazole	0 (38)	not tested
None	None	None	2.2 (45)	0 (30)

[a]Application date: June 17 (planting), August 21 and 28 in 2008; and June 18 (planting), August 20 and 30 in 2009. Sampling date: September 21 in 2008 and September 16 in 2009. [b] Type of application. [c] Sensitivities were analyzed by primer-introduced restriction enzyme analysis-based PCR. [d] *P. oryzae* isolates were isolated from panicle blast.

In the two Saga trials, the tricyclazole/diclocymet+ferimzone/diclocymet+ferimzone program showed an excellent rate of panicle blast control, and no *P. oryzae* isolates resistant to MBI-Ds were detected from the program plots even after two applications of diclocymet+ferimzone to maximize the emergence risk of resistant isolates. However, from the perspective of resistance-risk management, we do not recommend two applications of diclocymet+ferimzone per season.

In the Ehime field, diclocymet and diclocymet+ferimzone showed high rates of panicle blast control (Table 3). However, more resistant isolates were reselected in some of programs than in the UTC of the Ehime trial (Table 4). Thus, successive applications of MBI-Ds, year after year, without the inclusion of other active ingredients with different modes of action, led to the re-emergence of resistant isolates. Therefore, it is very important to establish sound resistance management strategies for areas in which sensitive isolates are likely to again become dominant. Non-MBI-D/diclocymet+ferimzone programs not only showed high abilities to control panicle blast but also maintained lower frequencies of resistant isolates (Tables 3–6). In particular, under the SAR fungicide (tiadinil) and diclocymet+ferimzone program, no MBI-D-resistant *P. oryzae* isolates were detected in the Ehime field (Tables 3 and 4). Currently, SAR products (for example, isotianil, probenazole and tiadinil) are mainly used for nursery box applications in Japan. Additionally, from 2015 to 2016, in the Kyushu area, an application program using a SAR product for the nursery box application and diclocymet+ferimzone for the foliar application on panicles was evaluated in five fields to assess the novel application program described as renrakusiken, in accordance with our trial application program. In these trials, the application program showed high efficacy against rice blast in fields, and no MBI-D-resistant isolates were detected among the 120 isolates selected from the application program's plots over two seasons (data not shown). Thus, we believe that a program combining a SAR product (for nursery box application) plus a diclocymet+ferimzone mixture (for foliar application) represents a good MBI-D-resistance management strategy for fields where MBI-D performance has recovered. Under a tricyclazole and diclocymet+ferimzone program, no MBI-D-resistant *P. oryzae* isolates were detected during the two years of the Saga trials (Table 6). Tricyclazole is an MBI fungicide that inhibits the reductase in melanin biosynthesis (MBI-R). Resistant *P. oryzae* isolates have not been detected for more than 30 years, since the launching of MBI-Rs. The MBI-R-resistance risk is thus assumed to be low. Therefore, tricyclazole would be a candidate for use in a resistance management strategy. However, in the 2008 Saga trial, the frequency of resistant isolates in the none/diclocymet+ferimzone/diclocymet+ferimzone program was not statistically different from that in the UTC. Thus, we hypothesized that the risk of resistant-isolate re-emergence in the Saga field was lower than that in the Ehime field. In a further study, a tricyclazole/diclocymet+ferimzone program should be evaluated in a field similar to that in Ehime. Thus, it is necessary to conduct additional field trials to confirm that the tricyclazole/diclocymet+ferimzone application program is efficient in preventing the re-emergence of MBI-D-resistant isolates.

We believed that foliar application of diclocymet+ferimzone was a candidate for MBI-D-resistance management. The application program, consisting of a nursery box application of a non-MBI-D fungicide and a field foliar application of a mixture of MBI-D fungicides, was one of the best programs for MBI-D-resistance management in fields where MBI-D performance had recovered. We considered that the resistance risk of fungicides, which would be applied to a nursery box and/or in a mixture with diclocymet in this study, is low. SARs, MBI-Rs and ferimzone have been used in paddy fields for decades. No *P. oryzae* isolates resistant to these chemical classes have been isolated. Thus, the resistance risks of these chemical classes are assumed to be low, making them good candidates for resistance management strategies. Recently, some new fungicides, such as tolprocarb[13] and tebufloquin, were registered to control blast. These chemicals could also be candidates for resistance management strategies. However, the resistance risks of these two chemicals are unknown because of their short history of use in paddy fields. Thus, their resistance risks also need to be evaluated.

This is the first report that describes a sound resistance management strategy using a non-MBI-D fungicide for a nursery box application and a mixture of diclocymet and ferimzone for the foliar application. Unfortunately, *P. oryzae* isolates resistant to QoI fungicides have been detected in many areas of Japan.[14,15] This study also shows the possibility of the re-use of fungicides (preferably mixtures of fungicides with different modes of action) that have had their nursery box applications halted because of resistance issues. This study also provides important insights that can be applied to the management of resistance to other fungicides, such as QoI.

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