Note

A method for monitoring the sensitivity of *Botrytis cinerea* to pyribencarb

Makiichi TAKAGAKI,* Satoshi KATAOKA,
Kenichi KIDA, Koichiro KAKU and
Tsutomu SHIMIZU

Life Science Research Institute, Kumiai Chemical Industry Co., Ltd.,
3360 Kamo, Kikugawa, Shizuoka 439–0031, Japan

(Received September 29, 2010; Accepted January 6, 2011)

Pyribencarb is a novel benzylcarbamate-type fungicide having excellent activity against a wide range of plant pathogenic fungi, especially gray mold diseases caused by *Botrytis cinerea*. The primary target site of pyribencarb is the Qo site of cytochrome *b* in the electron transfer system of the respiratory chain. The inhibitory effects of pyribencarb to cytochrome *b* enzyme on the transfer system of a QoI-resistant strain of *B. cinerea* are stronger than those of other QoI fungicides. Pyribencarb exhibited adequate control in greenhouse trials when QoI-resistant strains were inoculated. We have developed a practical method for evaluating the sensitivity of *B. cinerea* to pyribencarb. The final concentrations of pyribencarb in the PDA medium were set at 1 and 100 mg a.i. per liter with 1 mM salicylhydroxamic acid (SHAM). The isolates that were completely inhibited at 1 mg a.i. per liter of pyribencarb were “sensitive strains,” and the isolates that were inhibited by more than 80% at 100 mg a.i. per liter of pyribencarb were “weakly resistant strains.” In our assays so far, no strains with mycelial growth inhibition rates of less than 80% at 100 mg per liter of pyribencarb have ever been found. In the event that such strains should be found in the future, it will be necessary to monitor their sensitivity to pyribencarb using an *in vivo* assay. © Pesticide Science Society of Japan

Keywords: benzylcarbamate fungicide, pyribencarb, *Botrytis cinerea*, monitoring method, baseline, Qo inhibitor, Ool.

Introduction

Pyribencarb, methyl {2-chloro-5-[(*E*)-1-(6-methyl-2-pyridylmethoxyimino) ethyl]benzyl}carbamate (experimental code number, KIF-7767 or KUF-1204) is a novel benzylcarbamate-type fungicide.¹⁾ This fungicide is active against a wide range of economically important fungal pathogens and is especially efficacious against gray mold diseases caused by *Botrytis cinerea* and

stem rot diseases caused by *Sclerotinia sclerotiorum*.^{2,3)} Pyribencarb inhibits mitochondrial respiration by binding at the ubiquinol oxidation center (Qo) site of the cytochrome *b* enzyme complex, similar to strobilurin fungicides (QoIs).⁴⁾ Because of their site-specific mode of action, the risk for developing resistance to QoI fungicides is considered high,^{5,6)} and several fungal pathogens have developed resistance to QoI fungicides.^{7–10)}

The development of resistant *B. cinerea* to conventional fungicides has been widely recognized, posing increasingly serious problems. It is therefore important to set up resistance management programs to reduce the potential risks of resistance to respective classes of fungicides. A useful, reliable and practical test for evaluating fungal sensitivity is therefore needed for resistance monitoring programs.

Resistance to QoI fungicides has been found in field isolates of *B. cinerea* in Japan.¹¹⁾ These isolates change from glycine to alanine at position 143 (G143A) in the cytochrome *b* gene.¹²⁾ On the other hand, inhibitory effects of pyribencarb to cytochrome *b* enzyme on the transfer system of a QoI-resistant strain of *B. cinerea* were stronger than those of other QoI fungicides.⁴⁾ In other words, the binding site of pyribencarb to cytochrome *b* was assumed to be a little different from that of other QoI fungicides. Pyribencarb exhibited adequate control in the field with QoI-resistant strains.¹³⁾ Consequently, we have attempted to establish a practical and efficient method of evaluating the sensitivity of *B. cinerea* to pyribencarb for use in resistance monitoring.

Materials and Methods

1. Chemicals

Pyribencarb was synthesized by Ihara Chemical Industry Co., Ltd. and formulated as a 40% WG. Azoxystrobin (20% SC) was purchased from commercial sources.

2. Sample collection of *B. cinerea*

Three hundred eighteen single spore isolates were obtained from a wide range of crops in different locations in Japan where QoI fungicides had not been applied during 1995–1997. These isolates were cultured on potato-dextrose agar (PDA Difco) plates at 20°C in the dark for 3 days in an incubator and then stored at 4°C. The mycelia were used to set a sensitivity baseline of wild types to pyribencarb and a preliminary monitoring study.

3. Reference isolates

Nine isolates (Yoshi-7, -14, -20, M09149, M09152, M09161, M09163, M09182 and M09212) of *B. cinerea* were tested as resistant strains (G143A mutants) to azoxystrobin. Yoshi-7, Yoshi-14 and Yoshi-20 were collected from citrus plants in Wakayama Prefecture, Japan.^{11,12)} M09149, M09152, M09161, M09163, M09182 and M09212 were collected from tomatoes in Mie Prefecture, Japan. The sensitive strains KU-26-1, KU-59 and KU-76, maintained in the Life Science Research Institute of Kumiai, were used as the standard. Mycelia of this reference isolate were

* To whom correspondence should be addressed.

E-mail: m-takagaki@kumiai-chem.co.jp

Published online May 7, 2011

© Pesticide Science Society of Japan

prepared in the same manner as the wild types. For the *in vivo* assay, spores of these isolates were produced by illuminating with near ultraviolet light for 2–3 days after 3-day incubation on PDA plates at 20°C.

4. Mycelial growth assay

4.1. Preparation of basal medium

After autoclaving at 121°C for 10 min, 20 ml each of the PDA medium (Difco) was placed in plastic Petri dishes with a diameter of 9 cm. Fantasista (40% pyribencarb) WG was used to prepare a series of concentrations of pyribencarb in PDA medium. The final concentrations in PDA medium were set at 0.01, 0.1, 1, 10 and 100 mg a.i. per liter. At the same time, PDA medium was supplemented with 1 mM salicylhydroxamic acid (SHAM) to inhibit cyanide-insensitive alternative oxidase (AOX).

4.2. Measurement of mycelial growth

The fungal sensitivities to pyribencarb were determined as follows. Mycelial discs (6 mm in diameter) were cut from the growing edge of fungal cultures and then placed on plates containing pyribencarb at different concentrations. The plates were incubated at 20°C for 3 days. The colony diameter in each plate was measured and the percentages of the average diameters relative to that of the untreated control (100%) were calculated.

5. In vivo assay, cucumber gray mold

Cucumber seedlings (*Cucumis sativus* cv. Sagami-hanjiro; Asahinohen, Japan) were grown in 6 cm plastic pots filled with fertilized granulated soil (Kureha Corporation, Tokyo, Japan) under non-sterile conditions to the cotyledon stage. Seedlings were each sprayed with 10 ml per pot at 100 mg, a.i. per liter of pyribencarb and azoxystrobin or distilled water (control) supplemented with 0.02% spreader (Kumiten®; Kumiai Chemical Industry Co., Ltd.). Spraying was performed 3 hr prior to inoculation for the preventive effect assay. For inoculation, conidial spores of *B. cinerea* were suspended in medium containing 0.3% yeast extract and 2% glucose,^[14,15] and paper discs (6 mm in diameter, thick type; Toyo Roshi Kaisya, Ltd.) dipped in the spore suspension (1×10^5 spores/ml) were placed in the center of cotyledons. The inoculated plants were incubated in a chamber with high humidity at 20°C. Three days after inoculation, the lesion diameter was measured and the disease control effect of pyribencarb was assessed as a percentage of reduced lesion size in comparison with untreated plants. These tests were conducted with three replications.

Control value (%) = $(1 - \text{developed lesion length of fungicide-treated leaf} / \text{developed length of untreated leaf}) \times 100$

6. Genetic diagnosis of *QoI*-resistant *B. cinerea* with PCR-RFLP

6.1. DNA extraction from *B. cinerea*

Genomic DNA was isolated from mycelium powders using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was detected by 1% agarose

gel electrophoresis and its concentration was measured with the NanoDrop 2000 spectrometer (Thermo Fisher Scientific).

6.2. PCR experiments

PCR reaction mixture consisted of total DNA, a set of forward and reverse primers (1 µl for each, 25 pmol/µl), 0.2 µl Takara EX Taq™, 2.5 µl 10×EX Taq Buffer (with Mg²⁺), and 2 µl dNTPs (2.5 mM for each) and was made up to 25 µl with sterile water.

To amplify cytochrome *b* gene fragments from total DNA, the following PCR primers were used: BOTO-CYTB-K1F (forward), 5'-ATATAAAAGGTCGCGACAGA-3'; BOTO-CYTB-K1R (reverse), 5'-GGCCTTAGAACTAACTCCAA-3'. These two primers were designed with the complete sequence of the cytochrome *b* gene from *B. cinerea* (DDBJ/EMBL/Genbank Acc. No. AB262969), sequenced by Kumiai Chemical Industry Co., Ltd.⁴⁾ The DNA in the PCR reaction mixture was initially denatured for 2.5 min at 94°C, followed by 35 cycles of 0.5 min denaturing at 94°C, 1 min annealing at 52°C, 1.5 min extension at 72°C, and a final extension for 8.5 min at 72°C before cooling down to 4°C.

6.3. Restriction fragment length polymorphism (RFLP) analysis
PCR products were digested with restriction enzyme *Fnu4HI* (New England Biolabs, Beverly, MA), an isoschizomere of *ItaI* with the recognition sequence 5'-GCNGC-3', according to the manufacturer's protocol. Digestion products were analyzed on 1.5% agarose gels with 1×TAE and stained with ethidium bromide.

Results

1. Mycelial growth assay

Using the mycelial growth assay on PDA, preliminary monitoring tests were conducted utilizing 318 isolates sampled from several fields in Japan where no *QoI* fungicides had ever been applied. MIC values were distributed in a range from 0.01 to 1 mg a.i. per liter; namely 45.6% of the isolates for 0.01 mg, 47.5% for 0.1 mg and 6.9% for 1 mg, a.i. per liter (Fig. 1). Based on these results, it

Fig. 1. Distribution of isolates of *B. cinerea* from Japan based on sensitivity to pyribencarb in a mycelial growth test on PDA medium with 1 mM SHAM. A total of 318 isolates were collected from several fields in Japan where strobilurin fungicides had never been applied and tested by the mycelial growth method. MIC values were in a range from 0.01 to 10 mg a.i. per liter; namely 45.6% of the isolates for 0.01 mg, 47.5% for 0.1 mg and 6.9% for 1 mg a.i. per liter.

Table 1. Mycelial growth inhibition of *B. cinerea* by azoxystrobin and pyribencarb on the PDA medium

Strain No.	Inhibition of mycelial growth (%)					EC ₅₀ values	Inhibition of mycelial growth (%)					EC ₅₀ values
	Azoxystrobin (mg/l)						Pyribencarb (mg/l)					
	0.01	0.1	1	10	100		0.01	0.1	1	10	100	
Yoshi-7	0	0	0	7.6	30.4	100<	0	11.2	38.8	80.5	100	1.61
Yoshi-14	0	0	0	12.3	41.5	100<	0	3.9	28.6	75.9	87.3	4.18
Yoshi-20	0	0	0	15.9	35.8	100<	0	16.7	42.2	100	100	0.34
M09149	2.7	5.9	5.2	13.6	24.3	100<	2.0	6.8	29.6	73.9	100	2.71
M09152	0	0	1.6	14.2	24.2	100<	0.5	5.8	62.2	100	100	0.41
M09161	1.1	3.3	3.0	14.8	23.6	100<	1.4	11.1	32.9	100	100	0.43
M09163	0	6.6	5.1	10.4	16.1	100<	0	0	26.4	100	100	0.63
M09182	0	0	6.2	17.6	22.5	100<	0	0	49.7	63.8	100	1.27
M09212	1.9	0	11.6	6.9	20.4	100<	0	7.2	28.4	100	100	0.46
KU-26-1	13.4	47.4	100	100	100	0.04	39.3	100	100	100	100	0.01
KU-59	7.7	36.1	100	100	100	0.04	30.5	83.2	100	100	100	0.02
KU-76	13.1	55.5	100	100	100	0.03	64.1	100	100	100	100	0.01

Table 2. EC₅₀ values and MIC values of azoxystrobin and pyribencarb calculated from data shown in Table 1

Compound	QoI-sensitive strains		QoI-resistant strains	
	EC ₅₀ values (mg/l)	MIC values (mg/l)	EC ₅₀ values (mg/l)	MIC values (mg/l)
Azoxystrobin	0.03–0.04	1.0	100<	100<
Pyribencarb	0.01–0.02	0.1–1.0	0.34–4.18	10<

was concluded that the baseline sensitivity to pyribencarb should be set at 1 mg a.i. per liter in wild-type isolates of *B. cinerea*.

For reference isolates, EC₅₀ values and MIC values of azoxystrobin to QoI-sensitive strains were 0.03–0.04 mg, a.i. per liter and 1 mg, a.i. per liter, respectively. Those of pyribencarb to QoI-sensitive strains were 0.01–0.02 mg, a.i. per liter and 0.1–1 mg, a.i. per liter, respectively. The inhibition rate of mycelial growth by azoxystrobin in QoI-resistant strains was 16.1–41.5% for 100 mg, a.i. per liter, whereas that for pyribencarb was 87.3–100% at the same concentration (Table 1). EC₅₀ values and MIC values of azoxystrobin for QoI-resistant strains were more than 100 mg, a.i. per liter. Those of pyribencarb for QoI-resistant strains were

0.34–4.18 mg, a.i. per liter and more than 10 mg, a.i. per liter, respectively (Table 2).

2. In vivo assay, cucumber gray mold

An *in vivo* assay using cucumber was carried out to find out whether the three isolates from Wakayama Prefecture are resistant to pyribencarb. Pyribencarb and azoxystrobin showed good efficacy, more than 90% control of sensitive strains at 100 mg a.i. per liter (practical use). On the other hand, for QoI-resistant strains, the control value of azoxystrobin at 100 mg a.i. per liter was 21.8–39.5%, and that of pyribencarb at the same concentration was 63.4–69.9% (Table 3).

Using the cucumber assay, preliminary monitoring tests were conducted utilizing 318 isolates sampled from several fields in Japan where no QoI fungicides had ever been applied. When these isolates were inoculated, pyribencarb at 100 mg a.i. per liter showed good efficacy, more than 90% control (data not shown).

3. Genetic diagnosis of QoI-resistant *B. cinerea* with PCR-RFLP

It is well known that PCR-restriction fragment length polymorphism (RFLP) is a simple method to diagnose a point mutation on condition that the region of interest contains a restriction en-

Table 3. Effect of azoxystrobin and pyribencarb on cucumber gray mold

Compound	Concentration mg/l	Control (%)					
		Yoshi-7	Yoshi-14	Yoshi-20	KU-26-1	KU-59	KU-76
Azoxystrobin	100	39.5	38.4	21.8	93.4	95.5	100
Pyribencarb	100	63.4	69.9	65.1	97.7	100	94.1

Fig. 2. PCR-RFLP method for detecting cytochrome *b* gene of *B. cinerea*. (A) Primer map for PCR analysis of cytochrome *b* gene. Primers were designed from cytochrome *b* gene of *B. cinerea* (accession no. AB262969). (B,C) PCR-RFLP using the reference isolated both sensitive and resistant strains (G143A). PCR products were amplified before (B), or after digestion with *Fnu*4HI (C), and separated on 1.5% agarose gel. M: 100 bp ladder, 1: KU-26-1, 2: KU-59, 3: KU-76, 4: Yoshi-7, 5: Yoshi-14, 6: Yoshi-20

zyme site. It was previously reported that QoI-resistant *B. cinerea* isolated in Wakayama Prefecture bears a single point mutation (G143A) in the cytochrome *b* gene.¹²⁾ Furthermore, it could be shown that the restriction enzyme *Fnu*4HI specifically digested the resistant allele of a PCR fragment covering the point mutation of the cytochrome *b* gene, hence allowing the discrimination of resistant and sensitive strains on a molecular level. The concentration of total DNA extracted from *B. cinerea* was 2.5 mg/ml. The fragments of the cytochrome *b* gene containing amino acid

codon 143 were amplified using a primer set of BOTO-CYTB-K1F and BOTO-CYTB-K1R. The length of the PCR product was 1031 bp. PCR-RFLP analysis was carried out by digesting amplified PCR products with *Fnu*4HI restriction enzyme, and then splitting into two fragments of 220 bp and 811 bp in resistant strains, whereas the same PCR fragment from sensitive strains was not cut (Fig. 2). This result clearly demonstrates that the PCR-RFLP method is effective to diagnose the strain of *B. cinerea* resistant to QoI fungicides.

Discussion

As a method for monitoring the sensitivity of *B. cinerea* to pyribencarb, the mycelial growth assay on PDA medium associated with an *in vivo* assay is preferable. We think that the mycelial growth assay can be regarded in general as the most simple, quick and inexpensive means for evaluating the sensitivity of fungicides and for monitoring. From the results of our preliminary monitoring studies, the sensitivity baseline to pyribencarb was determined as 1 mg a.i. per liter in wild type isolates of *B. cinerea* (Fig. 1).

The inhibition rate of mycelial growth for azoxystrobin to QoI-resistant strains was 16.1–41.5% for 100 mg a.i. per liter, whereas that for pyribencarb was 87.3–100% at the same concentration (Table 1). EC₅₀ and MIC values of azoxystrobin for QoI-resistant strains were more than 100 mg, a.i. per liter, and those of pyribencarb for QoI-resistant strains were 0.34–4.18 mg, a.i. per liter and more than 10 mg, a.i. per liter, respectively (Table 2). Additionally, the control value of azoxystrobin at 100 mg a.i. per liter was 21.8–39.5% and that of pyribencarb at the same concentration was 63.4–69.9% in an *in vivo* assay performed on cucumber cotyledons (Table 3). Pyribencarb therefore exhibited adequate control of QoI-resistant strains, with an MIC value of more than 1 mg a.i. per liter in mycelial growth assay. The inhibitory effects of pyribencarb on the electron transfer system and mycelial growth of a QoI-resistant strain of *B. cinerea* (G143A) are stronger than those of other QoI fungicides.⁴⁾ Furthermore, pyribencarb exhibited adequate control in the greenhouse trial and field trial where resistant strains are dominant.¹³⁾ This indicated that, although QoI-resistant strains also develop cross-resistance against pyribencarb, the extent of sensitivity reduction in QoI-resistant strains to pyribencarb was clearly lower than other QoI fungicides.

Based on the above results, we have prepared a practical method of evaluating the sensitivity of *B. cinerea* to pyribencarb. The final concentrations of pyribencarb in the PDA medium were set at 1 and 100 mg a.i. per liter with 1 mM salicylhydroxamic acid (SHAM). The isolates that are completely inhibited at 1 mg a.i. per liter of pyribencarb are “sensitive strains”. The EC₅₀ value of pyribencarb for QoI-resistant strains was low, 0.34–4.18 mg, a.i. per liter, and pyribencarb exhibited adequate control of QoI-resistant strains, with an EC₅₀ value from 0.34 to 4.18 mg, a.i. per liter in the mycelial growth assay; therefore, the isolates that are inhibited more than 80% at 100 mg a.i. per liter of pyribencarb are “weakly resistant strains”. We think that this classification is a suitable standard for monitoring the sensitivity of *Botrytis cinerea* to pyribencarb; however, in our assays so far, no strains with mycelial growth inhibition rates of less than 80% at 100 mg per liter of pyribencarb have been found. In the event that such strains are found in the future, it will be necessary to monitor their sensitivity to pyribencarb using an *in vivo* assay.

Additionally, restriction fragment length polymorphism (RFLP) analysis of PCR products with *Fnu4HI* was performed and partial digestion was observed (Fig. 2). This method is suitable for detecting QoI-resistant *B. cinerea* (G143A mutant); however, this isolate (G143A mutant) is a “weakly resistant strain” to

pyribencarb. When a resistant isolate to pyribencarb is generated in the future, it will not be detected, although this method is suitable to detect QoI-resistant *B. cinerea* (G143A mutant); therefore, it was found that this method is not able to assess sensitivity to pyribencarb.

We think that it is important to develop a method for monitoring the sensitivity of *Botrytis cinerea* before pyribencarb is used in the field. In our group, a monitoring study will be conducted over a large area in the future, and more detailed examination is underway.

Acknowledgements

The authors express their deepest thanks to Mr. Masanori Kansako of Wakayama Research Center of Agriculture, Forestry and Fisheries, Fruit Tree Experimental Station, Japan and Mr. Hirofumi Suzuki of Mie Prefecture Agricultural Research Institute, Japan for providing QoI-resistant strains of *B. cinerea*. The authors also deeply thank Dr. Hideo Ishii of National Institute for Agro-Environmental Science, Japan for his advice and support during the early period of this work.

References

- 1) M. Ozaki, S. Fukumoto, R. Tamai, N. Yonekura, K. Ikegaya, T. Kawashima, J. Sakai, N. Muramatsu, M. Takagaki and K. Nagayama (Kumiai Chemical Industry Co., Ltd. and Ihara Chemical Industry Co. Ltd.): WO 01/10825 A1 (2001).
- 2) M. Takagaki, M. Kawata, S. Fukumoto and I. Miura: *Jpn. J. Phytopathol.* **71**, 256 (2005) (in Japanese)
- 3) M. Takagaki, S. Kataoka, K. Kida, I. Miura, S. Fukumoto and R. Tamai: *J. Pestic. Sci.* **35**, 10–14 (2010).
- 4) S. Kataoka, M. Takagaki, K. Kaku and T. Shimizu: *J. Pestic. Sci.* **35**, 99–106 (2010).
- 5) Fungicide Resistance Action Committee. FRAC Code List: Fungicides sorted by mode of action. <http://www.frac.info> (accessed on 1 September 2010).
- 6) A. N. Markoglou, A. A. Malandrakis, A. G. Vitoratos and B. N. Ziogas: *Eur. J. Plant Pathol.* **115**, 149–162 (2006).
- 7) Fungicide Resistance Action Committee. FRAC Code List: Pathogens with field resistance towards QoI fungicides. <http://www.frac.info> (accessed on 1 September 2010).
- 8) H. Sierotzki, J. Wullschleger and U. Gisi: *Pestic. Biochem. Physiol.* **68**, 107–112 (2000).
- 9) M. Inada, H. Ishii, W. H. Chung, T. Yamada, J. Yamaguchi and A. Furuta: *Jpn. J. Phytopathol.* **74**, 114–117 (2008).
- 10) J. Jiang, L. Ding, T. J. Michailides, H. Li and Z. Ma: *Pestic. Biochem. Physiol.* **93**, 72–76 (2009).
- 11) M. Kansako, Y. Yoneda, K. Shimadu and H. Ishii: *Jpn. J. Phytopathol.* **71**: 249 (2005) (in Japanese).
- 12) H. Ishii, J. Fontaine, W. H. Chung, M. Kansako, K. Nishimura, K. Takahashi and M. Oshima: *Pest Manag. Sci.* **65**, 916–922 (2009).
- 13) M. Takagaki, S. Kataoka, S. Fukumoto, H. Ishii, J. Yamaguchi, M. Inada, M. Kansako and K. Nozaki: *Jpn. J. Phytopathol.* **72**: 274 (2006) (in Japanese).
- 14) K. Ko, K. Akutsu, Y. Kobayashi, Y., Om, T. Watanabe and T. Misato: *Ann. Phytopathol. Soc. Jpn.* **47**, 228–233 (1981).
- 15) K. Akutsu, Y. Kobayashi, Y. Matsuzawa, T. Watanabe, K. Ko and T. Misato: *Ann. Phytopathol. Soc. Jpn.* **47**, 234–243 (1981).