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Original Article

Sensitivity monitoring of powdery mildew pathogens to cyflufenamid and the evaluation of resistance risk†

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Sensitivity monitoring studies for a novel fungicide, cyflufenamid, (*Z*)-*N*-[α-(cyclopropylmethoxyimino)-2,3-difluoro-6-(trifluoromethyl)benzyl]-2-phenylacetamide, were performed on various pathogens causing powdery mildew. The mean EC_{50} value for *Blumeria graminis* f. sp. *tritici* was 0.029 ppm by pot assay and that for *Sphaerotheca cucurbitae* was 0.0019 ppm by leaf disk assay in Japan. The mean EC_{50} values for *B. graminis* f. sp. *tritici* were between 0.0022 ppm and 0.0111 ppm and those for *B. graminis* f. sp. *hordei* were between 0.0249 ppm and 0.0457 ppm in 2000 to 2004 by leaf segment assay in Europe. The EC_{50} values of each strain in these pathogens were distributed within a narrow range, and no classes less sensitive to cyflufenamid were found. No significant change in the sensitivity of *B. graminis* f. sp. *tritici* to cyflufenamid was observed throughout selection pressure tests in the greenhouse and field. Cross-resistance between cyflufenamid and other commercial fungicides was not observed in *S. cucurbitae*. © Pesticide Science Society of Japan

Keywords: cyflufenamid, powdery mildew, baseline sensitivity.

Introduction

Powdery mildew is one of the most serious diseases in agricultural production worldwide. A novel fungicide, cyflufenamid, (*Z*)-*N*-[α-(cyclopropylmethoxyimino)-2,3-difluoro-6-(trifluoromethyl)benzyl]-2-phenylacetamide (Code Name: NF-149, Pancho®),[1] belongs to an amidoxime group that represents a new class of chemicals associated with the control of powdery mildew.

Cyflufenamid shows excellent control activities against powdery mildew in various crops and brown rot in stone fruits.[2–6] In the life cycle of pathogens causing powdery mildew in wheat, cyflufenamid did not affect infection before the formation of appressoria, but significantly inhibited the formation of haustoria, colonies, and spores. The biochemical mode of action of cyflufenamid has not been clarified yet.

Many chemicals have been developed as commercial fungicides for the control of powdery mildew. However, the appearance of resistant strains in pathogens causing powdery mildew to some commercial fungicides, such as benzimidazole (BI) fungicides, sterol demethylation-inhibiting (DMI) fungicides and strobilurin (QoI) fungicides, has caused poor disease control.[7–13] In order to avoid early resistance development, evaluation of the resistance risk for a new fungicide and establishment of a resistance management strategy is very important.[8,9,11]

In this paper, we describe the baseline sensitivity of powdery mildew on wheat (*B. graminis* f. sp. *tritici*), barley (*B. graminis* f. sp. *hordei*) and cucumber (*Sphaerotheca cucurbitae*) towards cyflufenamid in Japan and/or Europe, using the results of selection pressure test using cyflufenamid against *B. graminis* f. sp. *tritici* in the greenhouse and field. Cross-resistance studies between cyflufenamid and other commercial fungicides on *S. cucurbitae* were also carried out.

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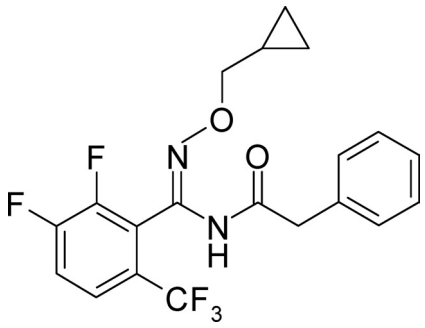

Fig. 1. Chemical structure of cyflufenamid.

Materials and Methods

1. Chemicals and formulations

Cyflufenamid was prepared as a 10% (w/w) wettable granule (WG) and a 50 g/liter emulsion in water (EW) formulation as described previously.[1] Kresoxim-methyl [41.5% suspension concentrate (SC)], triflumizole [30% wettable powder (WP)], thiophanate-methyl (70% WP) were purchased from a commercial source, and used as reference fungicides in the following experiments.

2. Sensitivity monitoring for cyflufenamid

2.1. Monitoring studies in Japan

Sixty-three strains of *B. graminis* f. sp. *tritici* were collected and isolated as single-colony isolates from various districts of Japan (63 fields in Hokkaido, Akita, Fukushima, Kanagawa, Shizuoka and Saga) in 1995 and 1996. These strains were assayed individually for their sensitivity to cyflufenamid by the following method (pot assay). Ten-day-old wheat (*Triticum aestivum*, cv. Chihoku) seedlings were sprayed with the solutions (0, 0.01, 0.05, 0.2, 0.8, 3.1 ppm cyflufenamid) including 0.05% Tween 20. After the solutions were air-dried, the treated seedlings were inoculated with spore dust of each strain of *B. graminis* f. sp. *tritici* individually, and incubated at 20°C (12 hr light/12 hr dark) for 7 days. Fungicidal activity against each strain was evaluated by observing the area of visible lesions and was expressed as the percentage of diseased leaf area (0 to 100%). The control value (CV) was calculated from the following equation.

$$CV=(1-T/C)\times 100$$

T represents the percentage of diseased leaf area in the treated seedlings and *C* represents the percentage of diseased leaf area in the non-treated seedlings. The EC_{50} value of each strain was calculated by Probit analysis individually, and also the geometric mean of EC_{50} (MEC_{50}) value was calculated.

Ninety-four strains of *S. cucurbitae* were collected and isolated as single-colony isolates from various districts of Japan (94 fields in Hokkaido, Fukushima, Niigata, Gunma, Saitama, Ibaragi, Kanagawa, Shizuoka, Kyoto, Hyogo, Kochi, Fukuoka, Nagasaki, Miyazaki, Kumamoto and Okinawa) from 1999 to 2001. These strains were assayed individually for their sensitivity to cyflufenamid using the leaf disk assay.[14] Leaf disks were cut from the first leaf of cucumber (*Cucumis sativus*, cv. Hikari 3P) seedlings and floated on the solutions (0, 0.0001, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1 ppm cyflufenamid). The floated leaf disks were inoculated with spore dust of each strain of *S. cucurbitae* individually, and incubated at 20°C for 5 days under 12 hr light/12 hr dark. Fungicidal activity against each strain was determined by observing the area of visual lesions relative to the untreated control and EC_{50} value was calculated by Probit analysis as described above.

2.2. Monitoring studies in Europe

Monitoring studies of sensitivity to cyflufenamid of *B. graminis* f. sp. *tritici* and *B. graminis* f. sp. *hordei* in various areas of Europe (Tables 1 and 2) were performed from 2000 to 2004 using the leaf segment pre-sprayed assay.[15,16] Random air samples of spores of *B. graminis* f. sp. *tritici* or *B. graminis* f. sp. *hordei* were collected using a car-mounted jet spore trap. The trapping distance within each area was approximately 100 km. Collected spores were transferred onto leaf segments of wheat (cv. Kanzler) or barley (*Hordeum vulgare*, cv. Igri), and kept as single-colony isolates on water agar (6 g/liter agar and 35 ppm benzimidazole) in Petri dishes for storage and multiplication before testing. Ten-day-old wheat or barley seedlings were sprayed with the solutions (0, 0.00125, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32 ppm cyflufenamid). One day after spraying, the leaves were cut into 3 cm long leaf segments and inoculated with spore dust of each strain of *B. graminis* f. sp. *tritici* or *B. graminis* f. sp. *hordei* individually, and incubated on water agar in Petri dishes at 18°C (continuous light) for 10 days. Fungicidal activity against each strain was determined by observing the lesion area visually relative to the untreated control, and EC_{50} value was calculated by Probit analysis as described in Section 2.1. The standard (wild-type) strains were included in the sensitivity tests. They were obtained from the field in the 1970s, before modern fungicides were commercialized, and therefore represent the sensitivity of fungi in original, unselected populations. In order to characterize the respective population and describe the quantitative shift of sensitivity to cyflufenamid of the test strains obtained from each area, the mean resistance factor (MRF) was calculated by the following equation.

$$MRF=MEC_{50}\text{ for test strains}/MEC_{50}\text{ for standard strains}$$

3. Selection pressure tests

3.1. Selection pressure test in the greenhouse

Thirty-two strains of *B. graminis* f. sp. *tritici* were used in these experiments, and formed part of the collected strains in Section 2.1. The spores of each strain were mixed equally, and used for the selection pressure test as inocula.

Wheat (cv. Chihoku) was cultivated in the greenhouse at

Bandai Agricultural Research Station, Nippon Soda Co., Ltd. (Nisso), Fukushima, Japan. The test field was 2 m×10 m (20 m^2), and the field was divided into two plots (A and B, 1 m×10 m). Initially, wheat seeds were sown only in plot-A, and the first inoculation was performed when the wheat seedlings were grown at BBCH[17] (Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und Chemische Industrie) 15 (5 leaves unfolded). After confirmation of lesion formation by *B. graminis* f. sp. *tritici* on wheat in plot-A, wheat seeds were sown in plot-B. When lesions developed (10–20% infected leaves) in plot-A, the first application of cyflufenamid (6.25 g a.i./ha) for selection pressure to plot-A was applied using a CO_2-pressurized sprayer (Trigger spray gun with Teejet 8004EVS, 1.5 atm). After confirmation of lesion formation by *B. graminis* f. sp. *tritici* again in plot-A and plot-B, the wheat plants in plot-A were removed and wheat seeds were re-sown in the same plot. A second application of cyflufenamid to plot-B was applied by spraying at the same dosage when lesions developed (10–20% infected leaves) in plot-B. Thereafter, cyflufenamid applications to wheat seedings in plot-A and -B were done alternately. The application of cyflufenamid against *B. graminis* f. sp. *tritici* was repeated 16 times (about 30-day intervals) from 1995 to 1997.

Just before each application, spores of *B. graminis* f. sp. *tritici* on the wheat leaves were collected from the test field, and were used to monitor the sensitivity to cyflufenamid. For the sensitivity monitoring test, the leaf segment floating assay[18] was used. Leaf segments (10 mm length) were cut from the second leaf of wheat (cv. Chihoku) seedlings. Each five leaf segments were floated on the solution (0, 0.000625, 0.00125, 0.0025, 0.005, 0.01 ppm cyflufenamid) in the Petri dish (4.5 cm diameter), respectively. The leaf segments were inoculated with spore dust collected from the test field. After 7-day incubation at 20°C under 12 hr light/12 hr dark, fungicidal activity was determined by observing the lesion area visually relative to the untreated control, and MEC_{50} value from 3 replications was calculated as described in Section 2.1.

3.2. Selection pressure test in the field

Selection pressure test was performed in the wheat (cv. Chihoku) field in Shinshinotsu, Hokkaido, Japan from 1998 to 2000. In the wheat field, the same 100 m^2 plots were used for 3 years. Every year, wheat plants were treated with cyflufenamid (25 g a.i./ha) two times at BBCH 30 (beginning of stem elongation) and 37 (flag leaf just visible) by spraying the plants with a knapsack type power sprayer. After symptoms of wheat powdery mildew were observed in the cyflufenamid-treated plot, spores of *B. graminis* f. sp. *tritici* were collected and isolated as single-colony isolates. These strains were assayed individually for cyflufenamid sensitivity by the pot assay as described in Section 2.1.

4. Cross-resistance study

Kresoxim-methyl (KM) -resistant (R) (SFKMR-1), thiophanate-methyl (TM) -R (SFTMR-1), triflumizole (TF) -R (SFTFR-1) and wild-type (SFS-1) strains of *S. cucurbitae* maintained in our laboratory (Odawara, Japan) were used in this study. Twenty-day-old seedlings of cucumber (cv. Sagamihanjiro, 1.2 leaf stage) were grown in pots under standard conditions in the greenhouse. The test plants were sprayed with the test chemical solutions (at a range of concentrations) included 0.01% Tween 20. After the solutions were air-dried, the treated plants were inoculated with spore dust of each strain of *S. cucurbitae* individually and incubated at 20°C for 10 days under 12 hr light/12 hr dark. The percentage of disease control was assessed by visually measuring the diseased leaf area as described in Section 2.1, and the minimum inhibitory concentration (MIC) of each fungicide for lesion formation was determined.

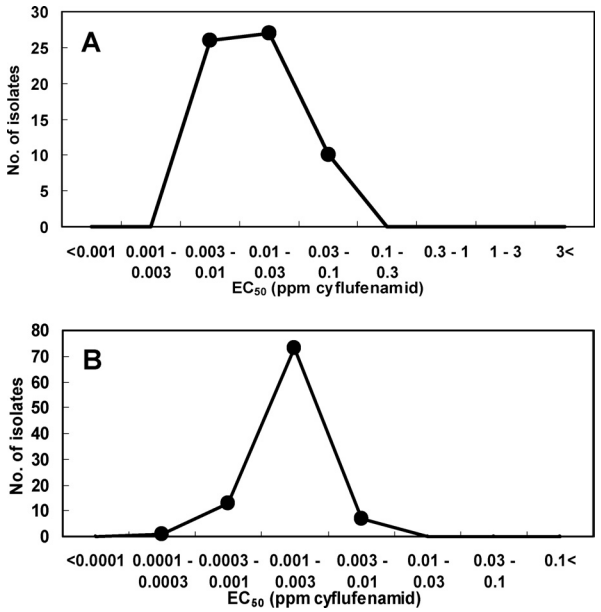

Fig. 2. Sensitivity monitoring result of powdery mildew pathogens to cyflufenamid in Japan. A: *Blumeria graminis* f. sp. *tritici* by pot assay (n=63, 1995 and 1996) and B: *Sphaerotheca cucurbitae* by leaf disk assay (n=94, 1999 to 2001).

Results

1. Sensitivity monitoring for cyflufenamid

1.1. Monitoring studies in Japan

In the case of *B. graminis* f. sp. *tritici*, the distribution ranges of the EC_{50} values of cyflufenamid for 63 strains isolated from various districts of Japan were between 0.005 and 0.090 ppm by pot assay. The MEC_{50} value was 0.029 ppm (Fig. 2-A). In this assay, MIC values of these strains were between 0.2 and 0.8 ppm (data not shown).

On the other hand, the EC_{50} value of cyflufenamid for 94 strains of *S. cucurbitae* isolated from various districts of Japan was distributed between 0.00028 and 0.0033 ppm by leaf disk assay. The MEC_{50} value was 0.0019 ppm (Fig. 2-B). Distribution ranges of MIC values of these strains were between 0.001 and 0.01 ppm (data not shown).

Table 1. Mean EC_{50} (MEC_{50}) and mean resistance factors (MRF)[a] of airborne samples of *Blumeria graminis* f. sp. *tritici* to cyflufenamid by leaf segment assay in Europe between 2000 and 2004

Country and region of collection	n[b]	2000		2001		2002		2003		2004	
		MEC_{50} (+SD, −SD)[c]	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF
United Kingdom											
Edinburgh-Grantshouse	10	0.0067 (0.0055, 0.0082)	0.8	0.0041 (0.0033, 0.0052)	0.8	0.0028 (0.0022, 0.0034)	1.1	0.0090 (0.0066, 0.0122)	1.2	0.0048 (0.0038, 0.0060)	0.9
East Anglia (North)	10	0.0064 (0.0051, 0.0080)	0.8	0.0045 (0.0035, 0.0052)	0.9	0.0024 (0.0018, 0.0033)	0.9	0.0072 (0.0061, 0.0084)	1.0	0.0043 (0.0036, 0.0051)	0.8
Belgium											
Brüssel-Aachen	10	0.0062 (0.0056, 0.0070)	0.8	0.0042 (0.0031, 0.0056)	0.8	0.0024 (0.0018, 0.0030)	0.9	0.0084 (0.0065, 0.0108)	1.1	0.0043 (0.0032, 0.0058)	0.8
France											
Paris-Reims	10	0.0065 (0.0055, 0.0077)	0.8	0.0049 (0.0041, 0.0059)	1.0	0.0026 (0.0019, 0.0035)	1.0	0.0065 (0.0055, 0.0076)	0.9	0.0048 (0.0038, 0.0059)	0.9
Auch-Toulouse	10	0.0060 (0.0053, 0.0067)	0.8	0.0049 (0.0041, 0.0058)	1.0	0.0022 (0.0017, 0.0029)	0.8	ND[d]	ND	ND	ND
Denmark											
Nyborg-Kopenhagen	10	0.0096 (0.0066, 0.0141)	1.2	0.0045 (0.0035, 0.0059)	0.9	0.0024 (0.0018, 0.0031)	0.9	0.0077 (0.0060, 0.0100)	1.0	0.0050 (0.0043, 0.0058)	1.0
Germany											
Neustadt-Hamburg	10	0.0072 (0.0056, 0.0093)	0.9	0.0047 (0.0036, 0.0063)	0.9	0.0027 (0.0019, 0.0038)	1.0	0.0077 (0.0060, 0.0097)	1.0	0.0045 (0.0036, 0.0057)	0.9
Hannover-Kassel	10	0.0111 (0.0089, 0.0139)	1.4	0.0037 (0.0032, 0.0043)	0.7	0.0025 (0.0018, 0.0034)	1.0	0.0073 (0.0063, 0.0085)	1.0	0.0043 (0.0035, 0.0053)	0.8
Magdeburg-Halle	10	0.0109 (0.0097, 0.0123)	1.4	0.0061 (0.0045, 0.0084)	1.2	0.0027 (0.0020, 0.0035)	1.0	0.0094 (0.0077, 0.0155)	1.3	0.0052 (0.0043, 0.0062)	1.0
Nürnberg-Freising	10	0.0073 (0.0057, 0.0092)	0.9	0.0066 (0.0058, 0.0075)	1.3	0.0025 (0.0021, 0.0030)	1.0	0.0084 (0.0068, 0.0103)	1.1	0.0049 (0.0039, 0.0062)	0.9
Austria											
Marchfeld near Vienna	10	0.0091 (0.0071, 0.0117)	1.1	0.0070 (0.0052, 0.0093)	1.4	0.0027 (0.0023, 0.0032)	1.0	0.0074 (0.0053, 0.0102)	1.0	0.0053 (0.0044, 0.0064)	1.0

Table 1. Continued

Country and region of collection	$n^{b)}$	2000		2001		2002		2003		2004	
		MEC_{50} (+SD, −SD)[c)]	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF
Italy											
Verona-Brescia	10	0.0079 (0.0066, 0.0095)	1.0	0.0052 (0.0036, 0.0076)	1.0	0.0031 (0.0025, 0.0037)	1.2	0.0083 (0.0069, 0.0100)	1.1	0.0049 (0.0040, 0.0061)	0.9
Standard strains[e)]											
Benno, Sappo and W72	3×3[f)]	0.0080 (0.0066, 0.0098)		0.0049 ND		0.0026 (0.0021, 0.0033)		0.0074 (0.0068, 0.0082)		0.0052 (0.0048, 0.0056)	

[a)] MEC_{50} for test strains/MEC_{50} for standard strains. [b)] Number of strains tested. [c)] Geometric standard deviation. [d)] Not determined. [e)] The wild-type reference strains included as standard were Benno, Sappo and W72. [f)] Replicated 3 times.

No classes less sensitive to cyflufenamid were observed in both pathogens.

1.2. Monitoring studies in Europe

The results of monitoring the sensitivity of *B. graminis* f. sp. *tritici* to cyflufenamid by leaf segment pre-sprayed assay across Europe (UK, Belgium, France, Denmark, Germany, Austria and Italy) between 2000 and 2004 are shown in Table 1. MEC_{50} values of cyflufenamid by year were: 2000, 0.0060 to 0.0111 ppm; 2001, 0.0037 to 0.0070 ppm; 2002, 0.0022 to 0.0031 ppm; 2003, 0.0065 to 0.0094 ppm and 2004, 0.0043 to 0.0053 ppm. Values of MRF in 2000 were: 0.8 to 1.4; in 2001: 0.7 to 1.4; in 2002: 0.8 to 1.2; in 2003: 0.9 to 1.3 and in 2004: 0.8 to 1.0.

Results of the sensitivity of *B. graminis* f. sp. *hordei* to cyflufenamid by sprayed leaf segment assay across Europe (UK, France, Germany, Austria and Italy) between 2000 and 2004 are shown in Table 2. MEC_{50} values of cyflufenamid by year were: 2000, 0.0249 to 0.0313 ppm; 2001, 0.0264 to 0.0322 ppm; 2002, 0.0266 to 0.0325 ppm; 2003, 0.0281 to 0.0355 ppm and 2004, 0.0323 to 0.0457 ppm. Values of MRF in 2000 were: 0.9 to 1.2; in 2001: 0.9 to 1.2; in 2002: 1.0 to 1.2; in 2003: 1.0 to 1.3 and in 2004: 0.8 to 1.1.

No significant change was observed in MEC_{50} and MRF values, and no less sensitive classes of strains were found throughout the sensitivity monitoring program in both pathogens.

2. Selection pressure tests

2.1. Selection pressure test in the greenhouse

The change in sensitivity of *B. graminis* f. sp. *tritici* strains to cyflufenamid by 16 applications of selection pressure in the greenhouse was investigated. Just before the first application of cyflufenamid as selection pressure, the MEC_{50} value of cyflufenamid was 0.0011 ppm by leaf segment floating assay. During the 16 applications (cyflufenamid 6.25 g a.i./ha), the MEC_{50} value of cyflufenamid slightly fluctuated between 0.00088 ppm and 0.0018 ppm, but no significant change of sensitivity of *B. graminis* f. sp. *tritici* strains to cyflufenamid was observed (Fig. 3).

2.2. Selection pressure test in the field

The change of sensitivity of *B. graminis* f. sp. *tritici* strains to cyflufenamid by 6 applications of selection pressure (cyflufenamid 25 g a.i./ha, 2 times per year) in the field for 3 years was investigated. At the first application (BBCH 30), the infection degree (% infected leaves) of *B. graminis* f. sp. *tritici* on wheat in the test field in 1998 was 10%; in 1999, 20% and in 2000, 5%. The EC_{50} values of cyflufenamid for strains isolated from both the cyflufenamid-treated plot and untreated control plot were equally distributed between 0.007 and 0.097 ppm by pot assay. There was no significant change of sensitivity of *B. graminis* f. sp. *tritici* strains to cyflufenamid over the 3 years (Fig. 4).

Table 2. Mean EC_{50} (MEC_{50}) and mean resistance factors (MRF)[a] of airborne samples of *Blumeria graminis* f. sp. *hordei* to cyflufenamid by leaf segment assay in Europe between 2000 and 2004

Country and region of collection	n[b]	2000		2001		2002		2003		2004	
		MEC_{50} (+SD, −SD)[c]	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF	MEC_{50} (+SD, −SD)	MRF
United Kingdom											
Edinburgh-Grantshouse	10	0.0276 (0.0223, 0.0342)	1.0	0.0289 (0.0253, 0.0330)	1.0	0.0325 (0.0259, 0.0407)	1.2	0.0355 (0.0230, 0.0421)	1.3	0.0457 (0.0328, 0.0637)	1.1
France											
Paris-Reims	10	0.0249 (0.0204, 0.0304)	0.9	0.0266 (0.0246, 0.0287)	1.0	0.0318 (0.0277, 0.0365)	1.1	0.0351 (0.0300, 0.0411)	1.3	0.0323 (0.0254, 0.0411)	0.8
Germany											
Neustadt-Hamburg	10	0.0256 (0.0226, 0.0288)	1.0	0.0264 (0.0232, 0.0300)	0.9	0.0277 (0.0245, 0.0312)	1.0	0.0328 (0.0285, 0.0377)	1.2	0.0349 (0.0301, 0.0406)	0.9
Hannover-Kassel	10	0.0296 (0.0243, 0.0360)	1.1	0.0270 (0.0206, 0.0352)	1.0	0.0315 (0.0252, 0.0393)	1.1	0.0287 (0.0251, 0.0328)	1.1	0.0364 (0.0311, 0.0426)	0.9
Magdeburg-Halle	10	0.0275 (0.0226, 0.0335)	1.0	0.0308 (0.0266, 0.0355)	1.1	0.0307 (0.0240, 0.0394)	1.1	0.0281 (0.0228, 0.0345)	1.0	0.0359 (0.0288, 0.0447)	0.9
Austria											
Marchfeld near Vienna	10	0.0313 (0.0269, 0.0363)	1.2	0.0294 (0.0257, 0.0336)	1.1	0.0270 (0.0237, 0.0369)	1.0	0.0287 (0.0230, 0.0357)	1.1	0.0397 (0.0328, 0.0480)	1.0
Italy											
Verona-Brescia	10	0.0280 (0.0220, 0.0357)	1.1	0.0322 (0.0280, 0.0370)	1.2	0.0266 (0.0218, 0.0324)	1.0	0.0351 (0.0320, 0.0386)	1.3	0.0381 (0.0301, 0.0482)	0.9
Standard strains[d]											
Gi1, Tr2, Al1 and Ru3	4	0.0269 (0.0232, 0.0313)		0.0280 (0.0239, 0.0327)		0.0280 (0.0240, 0.0327)		0.0272 (0.0234, 0.0311)		0.0404 (0.0326, 0.0499)	

[a] MEC50 for test strains/MEC_{50} for standard strains. [b] Number of strains tested. [c] Geometric standard deviation. [d] The wild-type reference strains included as standard were Gi1, Tr2, Al1 and Ru3.

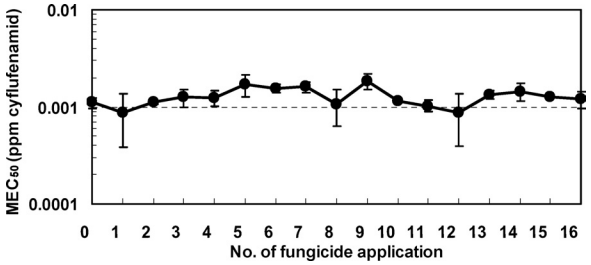

Fig. 3. Sensitivity change of *Blumeria graminis* f. sp. *tritici* to cyflufenamid during the selection pressure test in the greenhouse. The test was done using 16 applications of cyflufenamid at 6.25 g a.i./ha. The sensitivity of each strain was evaluated by leaf segment floating assay. Bars indicate geometric standard deviations from the mean (n=3).

3. Cross-resistance study

To clarify the absence of cross-resistance between cyflufenamid and other commercial fungicides, the differences of sensitivity to cyflufenamid in KM-R (SFKMR-1), TM-R (SFTMR-1), TF-R (SFTFR-1) and wild-type (SFS-1) strains of *S. cucurbitae* were examined. As shown in Table 3, the MIC value of KM was 0.8 ppm in the wild-type strain and higher than 100 ppm in the KM-R strain. The MIC value of TM was 1 ppm in the wild-type strain and higher than 100 ppm in the TM-R strain. In the case of TF, the MIC value was 0.1 ppm in the wild-type strain and 5 ppm in the TF-R strain, respectively. In contrast, all strains of *S. cucurbitae* used in this study were equally sensitive to cyflufenamid at low concentrations (MIC=0.8 ppm).

Discussion

In order to establish a resistance management strategy for a new fungicide, evaluation of the resistance risk is an essential requirement.[8,11] In the monograph of FRAC (Fungicide Resistance Action Committee), it was discussed how the factors determining the probability of resistance development against a new fungicide can be assessed as risk indicators, and the extent to which they can be combined into an overall estimation of resistance risk.[9] In this study, factors concerning the fungicide-associated resistance risk of cyflufenamid were investigated, including the baseline sensitivity of the pathogens causing powdery mildew, selection pressure of cyflufenamid against *B. graminis* f. sp. *tritici* in the greenhouse and the field, and cross-resistance between cyflufenamid and other commercial fungicides on *S. cucurbitae*.

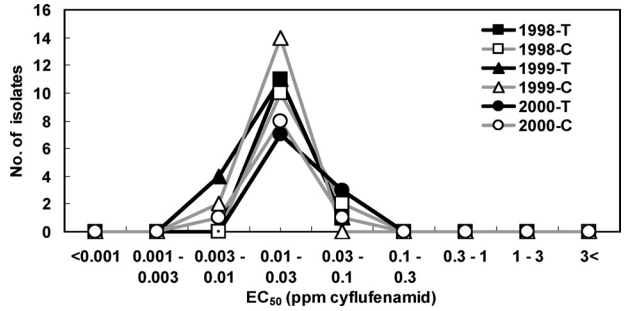

Fig. 4. Sensitivity change of *Blumeria graminis* f. sp. *tritici* to cyflufenamid during the selection pressure test in the field. The test was done by applying cyflufenamid (25 g a.i./ha, 2 times/year) for three years in a wheat field in Hokkaido, Japan. The sensitivity of each strain was evaluated by pot assay. 1998-T: Treated with cyflufenamid in 1998, 1998-C: Untreated control in 1998, 1999-T: Treated with cyflufenamid in 1999, 1999-C: Untreated control in 1999, 2000-T: Treated with cyflufenamid in 2000, and 2000-C: Untreated control in 2000.

Table 3. Effect of cyflufenamid against strains of *Sphaerotheca cucurbitae* resistant to other commercial fungicides

Chemical	MIC (ppm)[a]			
	SFS-1[b]	SFKMR-1[c]	SFTMR-1[d]	SFTFR-1[e]
Cyflufenamid	0.8	0.8	0.8	0.8
Kresoxim-methyl	0.8	>100	ND[f]	ND
Thiophanate-methyl	1	ND	>100	ND
Triflumizole	0.1	ND	ND	5

[a] Minimum inhibitory concentration. [b] Wild-type strain. [c] Resistant strain to kresoxim-methyl. [d] Resistant strain to thiophanate-methyl. [e] Resistant strain to triflumizole. [f] Not determined.

To obtain baseline sensitivity data, monitoring studies of cyflufenamid for pathogens causing powdery mildew were performed on wheat, barley and cucumber in Japan and/or Europe. In Japan, the MEC_{50} value for *B. graminis* f. sp. *tritici* was 0.029 ppm by pot assay and that for *S. cucurbitae* was 0.0019 ppm by leaf disk assay (Fig. 2). The EC_{50} value of each strain was distributed within a narrow range, and no less sensitive classes to cyflufenamid were found in both pathogens. Similar results were obtained in sensitivity monitoring studies in Europe (Tables 1 and 2). The MEC_{50} value for *B. graminis* f. sp. *tritici* was between 0.0022 and 0.0111 ppm (Table 1) and that for *B. graminis* f. sp. *hordei* was between 0.0249 and 0.0457 ppm (Table 2) in 2000 to 2004 by sprayed leaf segment assay. No significant change was observed in the MRF values, and no less sensitive classes of strains were found throughout the sensitivity monitoring program in both pathogens from 2000 to 2004 (Tables 1 and 2). These results of sensitivity monitoring studies provide no indication about the presence of mutant strains with very low cyflufenamid sensitivity. This positive aspect, however, does not rule out the possibility of resistance evolution (quantitative or qualitative), which might occur under selection pressure in the field.

To answer this question and evaluate further the resistance risk to cyflufenamid, selection pressure tests of cyflufenamid against *B. graminis* f. sp. *tritici* were conducted under green-

house and practical field conditions. During the 16 applications of cyflufenamid in the greenhouse (Fig. 3) or 6 applications (2 times per year) in 3 years in the field (Fig. 4), no significant changes of the sensitivity of *B. graminis* f. sp. *tritici* to cyflufenamid were observed in both experiments. These results may indicate that the basic risk of selecting cyflufenamid-resistant strains is low.

In cross-resistance experiments, all strains (sensitive or resistant to KM, TM and TF) of *S. cucurbitae* were equally sensitive to cyflufenamid at low concentrations although the sensitivity of each strain to KM, TM and TF was totally different (Table 3). These results suggest that cross-resistance does not exist between cyflufenamid and QoI, BI and DMI fungicides. We have already reported that the fungicidal spectrum and morphological effects of cyflufenamid on pathogens were different from those of QoI, BI and DMI fungicides.[2,4,5)] The results of this cross-resistance study also suggest that the biochemical mode of action of cyflufenamid differs from QoI, BI and DMI fungicides.

In this study, we could not obtain any data which indicate high fungicide-associated resistance risk for cyflufenamid. Cyflufenamid belongs to a new fungicide class, amidoxime.[1)] Although the biochemical mode of action of cyflufenamid has not yet been clarified, it is unique and different from those of commercial fungicides such as QoI, BI and DMI.[2,4,5)] However, the disease-associated resistance risk of powdery mildew pathogens was classified as high because of the occurrence of many short generations per season and abundant sporulation.[8,9)] Therefore, it is apparent that there is a high risk of resistance development in powdery mildew pathogens against cyflufenamid. The sensitivity monitoring study will be continued to assess the long-term efficacy of the resistance management strategies described below.

i) The use of cyflufenamid should be restricted to no more than 2 applications per crop.
ii) Cyflufenamid should be applied in preventive conditions before disease development.
iii) Cyflufenamid should only be applied at the label recommended rate.
iv) Cyflufenamid should be applied as a mixture with fungicides with different modes of action (‘partner’ compounds), or as one component in a rotation or alternation of different fungicide treatments. Even if cyflufenamid is applied as a mixture, the continuous application of not only cyflufenamid but also the ‘partner’ compounds should be avoided.

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References

1) I. Kasahara, H. Ooka, S. Sano, H. Hosokawa and H. Yamanaka (Nippon Soda Co., Ltd.): *PCT Int. Appl.* WO96-19442 (1996) (in Japanese).
2) S. Sano, H. Yamanaka, H. Hosokawa, M. Haramoto and H. Hamamura: *Abstr. 26th Annu. Meeting Pestic. Sci. Soc. Jpn.*, p. 151, 2001 (in Japanese).
3) M. Haramoto, H. Yamanaka, H. Hosokawa, H. Hamamura and S. Sano: *Abstr. 26th Annu. Meeting Pestic. Sci. Soc. Jpn.*, p. 152, 2001 (in Japanese).
4) M. Haramoto: *Abstr. 12th Symposium of Research Committee on Fungicide Resistance, Phytopathol. Soc. Jpn.*, pp. 19–24, 2002 (in Japanese with English summary).
5) M. Haramoto, H. Yamanaka, H. Hosokawa, H. Sano, H. Hamamura and S. Sano: *Abstr. 10th IUPAC Int. Congress Chem. Crop Protection*, Vol. 1, p. 76 (2002).
6) C. Yokota: *Agrochem. Jpn.* **84**, 12–14 (2004).
7) K. J. Brent: “Fungicide Resistance in Crop Protection,” ed. by J. Dekker and S. G. Georgopoulos, Pudoc, Wageningen, pp. 219–230, 1982.
8) K. J. Brent (ed.): “Fungicide Resistance in Crop Pathogens: How Can It Be Managed?,” GIFAP, Brussels, pp. 1–48, 1995.
9) K. J. Brent and D. W. Hollomon (eds.): “Fungicide Resistance: The Assessment of Risk,” GCPF, Brussels, pp. 1–47, 1998.
10) H. Ishii, T. Sugiyama, K. Nishimura and Y. Ishikawa: “Modern Fungicides and Antifungal Compounds III,” ed. by H.-W. Dehe, U. Gisi, K. H. Kuch, P. E. Russell and H. Lyr, AgroConcept, Bonn, pp. 149–159, 2002.
11) H. Ishii: “Environmental Fate and Safety Management of Agrochemicals,” ed. by J. M. Clark and H. Ohkawa, Amer. Chem. Soc., Washington DC, pp. 280–288, 2005.
12) M. A. de Waard: “Fungicide Resistance,” ed. by S. Heaney, D. Slawson, D. W. Hollomon, M. Smith, P. E. Russell and D. W. Parry, British Crop Protection Council, Farnham, Surrey, pp. 3–10, 1994.
13) C. M. Smith: “Fungicide Resistance in North America,” ed. by C. J. Delp, Amer. Phytopathol. Soc., St. Paul, pp. 23–24, 1988.
14) H. T. A. M. Schepers: *Neth. J. Pl. Path.* **90**, 165–171 (1984).
15) F. G. Felsenstein, C. Steden and J. Speich: *Brighton Crop Protection Conference—Pests and Diseases*, 4c-12, 475–480 (1994).
16) F. G. Felsenstein and K. H. Kuck: *PflanzenschutzNachr.* **51**, 5–24 (1998).
17) P. D. Lancashire, H. Bleiholder, T. van den Boom, P. Langelüddeke, R. Stauss, E. Weber and A. Witzenberger: *Ann. Appl. Biol.* **119**, 561–601 (1991).
18) Y. Nakazawa, N. Ohtsuka and M. Yamada: *Ann. Phytopathol. Soc. Jpn.* **58**, 99 (1992) (abstr. in Japanese).