Original Article

Fungicidal activities of cyflufenamid against various plant-pathogenic fungi*

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The activity of a novel fungicide, cyflufenamid, (*Z*)-*N*-[α -(cyclopropylmethoxyimino)-2,3-difluoro-6-(trifluoromethyl)benzyl]-2-phenylacetamide, against various fungi pathogenic to plants was investigated. In pot tests, cyflufenamid showed excellent control of powdery mildew caused by various pathogens at 0.8 to 1.6 ppm. In culture tests, several species of Ascomycetes and Deuteromycetes were sensitive to cyflufenamid. Notably, *Monilinia fructicola* was affected at a concentration of just 0.01 ppm. In the life cycle of pathogens causing powdery mildew in wheat, cyflufenamid did not affect infections before the formation of the appressoria, but significantly inhibited the formation of haustoria, colonies, and spores. It also affected the elongation of germ tubes after the germination of spores in *M. fructicola*. In ultrastructural experiments, cyflufenamid induced a reduction in the amount of highly electron-dense material in the vacuoles and immature septa in *M. fructicola*. © Pesticide Science Society of Japan

Keywords: cyflufenamid, fungitoxic spectrum, powdery mildew.

Introduction

Powdery mildew is one of the most serious disease groups in agricultural production. Many chemicals have been developed as commercial fungicides for the control of powdery mildew. However, the appearance of strains resistant to commercial fungicides among the pathogens causing powdery mildew has led to poor disease control.^{1–4)}

A novel fungicide, cyflufenamid, (*Z*)-*N*-[α -(cyclopropylmethoxyimino)-2,3-difluoro-6-(trifluoromethyl)benzyl]-2-phenylacetamide (Code Name: NF-149, Pancho[®])⁵⁾ (Fig. 1), shows excellent control of powdery mildew in various plants.^{6–9)} Cyflufenamid belongs to an amidoxime group that represents a new class of chemicals associated with the control of powdery mildew.

In this paper, we describe the fungicidal activities including

the fungitoxic spectra *in vivo* and *in vitro* of cyflufenamid, and the mode of action of cyflufenamid against pathogens.

Materials and Methods

1. Chemicals and formulations

Cyflufenamid was prepared as a 10% wettable granule (WG) formulation as described previously.⁵⁾ The formulated chemical was used in this study.

Fig. 1. Chemical structure of cyflufenamid.

* Studies on a novel fungicide, cyflufenamid (Part 1)

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2. Fungitoxic spectrum *in vivo*

The fungitoxic spectrum of cyflufenamid was evaluated based on efficacy to control powdery mildew on wheat (*Triticum aestivum*, cv. Chihoku) caused by *Blumeria graminis* f. sp. *tritici*, grey mold on kidney bean (*Phaseolus vulgaris*, cv. Nagazura) caused by *Botrytis cinerea*, late blight on tomato (*Lycopersicon esculentum*, cv. Regina) caused by *Phytophthora infestans*, downy mildew on grape (*Vitis vinifera*, cv. Kousyu) caused by *Plasmopara viticola* and scab on apple (*Malus pumila* var. *domestica*, cv. Rall's Janett) caused by *Venturia inaequalis*.

Control efficacy against powdery mildew, late blight, downy mildew and scab was determined in pot tests. Plants at the 2–3 leaf stage were used in this experiment. The test chemical solutions containing 0.01% Tween 20 were sprayed onto young seedlings. After the solutions had air-dried, the plants were artificially inoculated with either the spore suspension or spore dust depending on the test pathogen, and incubated at 20°C (12 hr light/12 hr dark) for a given period. Percent disease control was assessed 5–10 days after the inoculation by visually measuring the diseased leaf area.

Control efficacy against grey mold was determined in a flower test. Flowers of kidney bean plants were detached and immersed in the chemical solution. After air-drying, the flowers were inoculated with the spore suspension of *B. cinerea* and then placed on untreated detached leaves of kidney bean in a Petri dish. After incubation for 5 days at 20°C (12 hr light/12 hr dark), the diameter of lesions that developed on the leaves was measured and % disease control was calculated based on the mean of lesion sizes.

Fungitoxic activities *in vivo* of cyflufenamid against 7 pathogens causing powdery mildew listed in Table 2 were evaluated based on pot tests. The young seedlings or rooted cutting plants were treated by spraying with the test chemical solutions containing 0.01% Tween 20. After air-drying, the plants were artificially inoculated with spore dust of each pathogen and incubated at 20°C (12 hr light/12 hr dark) for a given period. Percent disease control was assessed 7–10 days after the inoculation by visually measuring the diseased leaf area, and the minimum inhibitory concentration (MIC) was determined.

3. Fungitoxic spectrum *in vitro*

The fungitoxic spectrum *in vitro* of cyflufenamid was evaluated by culture tests using the agar dilution method. In this experiment, 37 fungal species listed in Table 3 maintained on potato sucrose agar (PSA) in our laboratory (Odawara, Japan) were used. The test chemical dissolved in sterilized deionized water was added to PSA media held at 45–50°C to obtain the given concentration. Mycelial agar discs (6 mm in diameter) of test fungi were placed at the center of agar plates (9 cm in diameter) and incubated at their optimum temperatures. The diameters of mycelial colonies of the fungi were measured after 3–20 days and EC₅₀ values were calculated.

4. Light microscopic observations

The effect of cyflufenamid on the life cycle of *B. graminis* f. sp. *tritici* on wheat (cv. Chihoku) was evaluated. Wheat seedlings were sprayed with the test chemical solutions containing 0.01% Tween 20. After air-drying, the treated plants were artificially inoculated with spore dust of *B. graminis* f. sp. *tritici*. The inoculated pots were incubated at 20°C (12 hr light/12 hr dark) for a given period. Wheat leaves were detached, and the epidermal tissues were peeled off and stained with 0.1% cotton blue. The stained samples were observed with a light microscope (BH-2, Olympus Optical Co., Ltd., Japan).

The effect of cyflufenamid on the spore germination stage of *Monilinia fructicola* was also observed. Spores of *M. fructicola* were suspended in potato sucrose yeast extract (PSY) liquid medium. The spore suspension was mixed with the test chemical solutions and put on a 96-well microplate. The microplate was incubated at 20°C under high humidity in the dark for 2, 8, 16 and 24 hr. Spore germination and germ tube elongation were observed light-microscopically.

5. Transmission electron microscopic observations

Spores of *M. fructicola* were suspended in PSY liquid medium. The spore suspension was mixed with the test chemical solutions, and put on a 96-well microplate containing a thin layer of PSY agar medium. The microplate was incubated at 20°C under high humidity in the dark. After incubation for 24 hr, the small agar blocks containing the spores were removed from wells and pre-fixed with 2% glutaraldehyde (0.05 M phosphate buffer pH 7.0) at 4°C for 24 hr. After 3 rinses with the same buffer, the blocks were post-fixed with 1% osmium tetroxide, dehydrated in ethyl alcohol, and then embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. The stained ultrathin sections were observed with a transmission electron microscope (HS-9, HITACHI Ltd., Japan).

Results

1. Fungitoxic spectrum *in vivo*

Cyflufenamid at 200 ppm exhibited no fungitoxic activity against *B. cinerea* on kidney bean, *P. infestans* on tomato, *P. viticola* on grape or *V. inaequalis* on apple. In contrast, cyflufenamid showed excellent fungitoxic activity against *B. graminis* f. sp. *tritici* on wheat even at 0.8 ppm (Table 1).

The fungitoxic activity of cyflufenamid among various pathogens causing powdery mildew on plants was investigated. Cyflufenamid exhibited complete control of powdery mildew on cucumber, strawberry, sweet pepper and wheat at 0.8 ppm, and on apple, grape and rose at 1.6 ppm (Table 2).

2. Fungitoxic spectrum *in vitro*

The fungitoxic spectrum *in vitro* of cyflufenamid against 37 species of fungi is shown in Table 3. Some pathogens of Ascomycetes and Deuteromycetes such as *Botryosphaeria* sp.,

Table 1. Efficacy of cyflufenamid against diseases caused by various pathogens

Cyflufenamid concentration (ppm)	Control efficacy (%) ^{a)} of disease by				
	<i>Blumeria graminis</i>	<i>Botrytis cinerea</i>	<i>Phytophthora infestans</i>	<i>Plasmopara viticola</i>	<i>Venturia inaequalis</i>
200	100	0	0	0	0
50	100	NT ^{b)}	NT	NT	NT
12.5	100	NT	NT	NT	NT
3.1	100	NT	NT	NT	NT
0.8	100	NT	NT	NT	NT
0.2	66	NT	NT	NT	NT
0.05	30	NT	NT	NT	NT
0.01	6	NT	NT	NT	NT

^{a)} $n=3$. ^{b)} Not tested.

Cercospora beticola, *Fusarium nivale*, *Monilinia fructicola* and *Rosellinia necatrix* were sensitive to cyflufenamid at concentrations of 0.01 to 0.7 ppm (EC_{50}). Notably, *M. fructicola* was highly sensitive to cyflufenamid, the EC_{50} being 0.01 ppm. In contrast, pathogens of Phycomycetes and Basidiomycetes were insensitive to cyflufenamid even at 100 ppm.

3. Light microscopic observations

3.1. Effect of cyflufenamid on the life cycle of *B. graminis* f. sp. *tritici*

To elucidate the point at which cyflufenamid acts in the life cycle of *B. graminis* f. sp. *tritici* on wheat, light microscopic observation was performed. Cyflufenamid at 12.5 ppm did not affect the germination of spores or formation of appressoria (Tables 4 and 5). However, the formation of haustoria, colonies and spores were all strongly inhibited by cyflufenamid at 0.2 ppm (Tables 6 and 7).

Table 2. Efficacy of cyflufenamid against powdery mildew caused by various pathogens

Plant	Cultiver	Pathogen	MIC ^{a)} (ppm)
Cucumber	Sagamihanjiro	<i>Sphaerotheca cucurbitae</i>	<0.8
Strawberry	Nyohou	<i>Sphaerotheca aphanis</i> var. <i>aphanis</i>	<0.8
Sweet pepper	Tosakatura	<i>Oidiopsis sicula</i>	<0.8
Wheat	Chihoku	<i>Blumeria graminis</i> f. sp. <i>tritici</i>	<0.8
Apple	Rall's Janett	<i>Podosphaera leucotricha</i>	<1.6
Grape	Kousyu	<i>Uncinula necator</i>	<1.6
Rose	Red planet	<i>Sphaerotheca pannosa</i>	<1.6

^{a)} Minimum inhibitory concentration, ($n=3$).

3.2. Effect of cyflufenamid on the spore germination and germ tube elongation of *M. fructicola*

The effect of cyflufenamid on the germination of spores and elongation of germ tubes in *M. fructicola* was observed to elucidate the site of action of cyflufenamid. Spores of *M. fructicola* in cyflufenamid and water solutions started to germinate equally within 1 to 2 hr, and germ tubes elongated continuously. Cyflufenamid at 1 ppm affected neither the germination nor the elongation until 8 hr after treatment (Fig. 2). Moreover, cyflufenamid at a high concentration (100 ppm) had no effect at this stage (data not shown). However, at 16 hr after the treatment with cyflufenamid, the elongation of germ tubes stopped (Fig. 2). Swelling at the tip of germ tubes, and vacuolation and rupturing of the cytoplasm in germ tubes were observed 24 hr after treatment (Fig. 3).

4. Transmission electron microscopic observations

The effect of cyflufenamid on the hyphal growth of *M. fructicola* was observed under a transmission electron microscope. In untreated control hyphae, many vacuoles filled with highly electron-dense materials were observed in cytoplasm. The septa were thicker than the cell walls, and small pores were observed in the center of septa (Fig. 4-1). In the hyphae treated with cyflufenamid at 1 ppm, although vacuoles existed in the cytoplasm, highly electron-dense materials in the vacuoles were not detected (Figs. 4-2 and 4-3). The septa were thinner and smaller than in the untreated control, and septal pores were large (Figs. 4-1 and 4-2). There were no differences in the number and shape of mitochondria and nuclei between cyflufenamid-treated and untreated hyphae (Figs. 4-1 and 4-2). Ruptured hyphae were detected frequently following treatment with cyflufenamid and vesicles of various sizes which originated from organelle were observed away from the ruptured sites (Fig. 4-3). Vacuolated hyphae also appeared on cyflufenamid treatment, and contained extremely immature septa (Fig. 4-4).

Table 3. Fungitoxic activity of cyflufenamid against various pathogens in culture (Agar dilution method)

Pathogen	EC ₅₀ (ppm)
<i>Alternaria alternata</i> apple pathotype	>100
<i>Botrytis cinerea</i>	>100
<i>Botrytis squamosa</i>	100
<i>Botryosphaeria</i> sp.	0.07
<i>Cercospora beticola</i>	0.3
<i>Cercospora kikuchii</i>	1
<i>Cercospora theae</i>	>100
<i>Cladosporium cucumerinum</i>	>100
<i>Cochliobolus miyabeanus</i>	>100
<i>Colletotrichum lagenarium</i>	>100
<i>Colletotrichum theae-sinensis</i>	>100
<i>Diaporthe citri</i>	6
<i>Elsinoe ampelina</i>	5
<i>Fulvia fulva</i>	>100
<i>Fusarium nivale</i>	0.3
<i>Fusarium oxysporum</i>	>100
<i>Fusarium roseum</i>	>100
<i>Gibberella zeae</i>	>100
<i>Glomerella cingulata</i>	>100
<i>Helicobasidium mompa</i>	>100
<i>Monilinia fructicola</i>	0.01
<i>Penicillium italicum</i>	7
<i>Pestalotia longiseta</i>	>100
<i>Phomopsis</i> sp.	>100
<i>Phytophthora infestans</i>	>100
<i>Pseudocercospora herpotrichoides</i>	>100
<i>Pyricularia oryzae</i>	3
<i>Pythium aphanidermatum</i>	>100
<i>Rhizoctonia solani</i>	>100
<i>Rosellinia necatrix</i>	0.7
<i>Sclerotinia sclerotiorum</i>	1
<i>Septoria nodurum</i>	>100
<i>Taphrina deformans</i>	3
<i>Trichoderma viride</i>	>100
<i>Valsa ceratosperma</i>	8
<i>Venturia inaequalis</i>	>100
<i>Verticillium dahliae</i>	>100

Table 4. Effect of cyflufenamid at 12.5 ppm on spore germination of *Blumeria graminis* f. sp. *tritici* on wheat leaves

Chemical	Rate of spore germination (%) ^{a)} ±SD ^{b)}			
	Time after treatment			
	3 hr	6 hr	12 hr	24 hr
Cyflufenamid	19.8±6.9	47.6±14.3	60.7±12.3	61.5±12.2
Untreated control	22.5±7.6	49.8±17.3	67.4±8.6	72.2±4.2

^{a)} n=2. ^{b)} Standard deviation.

Discussion

To elucidate the fungitoxic spectrum *in vivo* of cyflufenamid, fungitoxic activities against pathogens causing powdery mildew on wheat, grey mold on kidney bean, late blight on tomato, downy mildew on grape, and scab on apple were investigated. As shown in Table 1, cyflufenamid achieved excellent control of powdery mildew on wheat at 0.8 ppm, but exhibited no activity against the other four diseases even at 100

Table 5. Effect of cyflufenamid at 12.5 ppm on appressorium formation of *Blumeria graminis* f. sp. *tritici* on wheat leaves

Chemical	Rate of appressorium formation (%) ^{a)} ±SD ^{b)}			
	Time after treatment			
	3 hr	6 hr	12 hr	24 hr
Cyflufenamid	0.3±0.1	0.4±0.1	28.1±7.5	87.9±3.7
Untreated control	1.7±1.4	3.5±2.5	22.3±10.2	89.7±4.0

^{a)} n=2. ^{b)} Standard deviation.**Table 6.** Effect of cyflufenamid at various concentrations on haustorium formation of *Blumeria graminis* f. sp. *tritici* in wheat leaves

Cyflufenamid concentration (ppm)	Inhibitory rate of haustorium formation (%) ^{a)}	
	Time after treatment	
	76 hr	168 hr
12.5	100	100
3.1	99.4	99.9
0.8	96.4	99.4
0.2	77.6	80.1

^{a)} n=2.**Table 7.** Effect of cyflufenamid at various concentrations on colony and spore formation of *Blumeria graminis* f. sp. *tritici* on wheat leaves

Cyflufenamid concentration (ppm)	Inhibitory rate (%)	
	Colony formation ^{a)}	Spore formation ^{b)}
12.5	100	100
3.1	100	100
0.8	96.9	100
0.2	81.2	97

^{a)} Observed 5 days after cyflufenamid treatment, (n=2). ^{b)} Observed 7 days after cyflufenamid treatment, (n=2).

Fig. 2. Effect of cyflufenamid on spore germination of *Monilinia fructicola*. Observations under a light microscope were made at 2, 8 and 16 hr after cyflufenamid (1 ppm) treatment. Scale bar=30 μ m.

ppm. Pot tests of cyflufenamid against powdery mildew on plants caused by various pathogens revealed excellent control at 0.8 to 1.6 ppm (Table 2). These results suggest that cyflufenamid has strong fungicidal activity against many genera in Erysiphaceae.

We also performed culture tests using the agar dilution method to evaluate the fungitoxic spectrum *in vitro* of cyflufenamid. Cyflufenamid was active against limited numbers of fungi classified into Ascomycetes and Deuteromycetes (Table 3). The pattern of fungitoxic activity *in vitro* was clearly dif-

ferent from that of broad-spectrum fungicides such as benzimidazole (BI) fungicides¹⁰⁾ and demethylation-inhibitor (DMI) fungicides.¹¹⁾

In the light microscopic experiments, cyflufenamid at 0.2 ppm strongly inhibited the formation of haustoria, colonies and spores in *B. graminis* f. sp. *tritici* (Tables 6 and 7). However, it did not affect the germination of spores or formation of appressoria even at 12.5 ppm (Tables 4 and 5). In the case of *M. fructicola*, cyflufenamid at 1 ppm did not affect the germination of spores or elongation of germ tubes until 8 hr after

Fig. 3. Effect of cyflufenamid on hyphal growth of *Monilinia fructicola*. Observations under a light microscope were made at 24 hr after cyflufenamid (1 ppm) treatment. Scale bar=30 μ m. R: Rupturing, V: Vacuolation.

treatment (Fig. 2). An inhibitory effect of cyflufenamid on *M. fructicola* was observed at 16 hr after treatment; a cessation of germ tube elongation (Fig. 2). After 24 hr, swelling at the tip of germ tubes, and vacuolation and rupturing of germ tubes were observed (Fig. 3). In the transmission electron microscopic experiments, treatment with cyflufenamid at 1 ppm for

24 hr in *M. fructicola* induced ultrastructural changes such as a reduction in highly electron-dense materials considered to be polyphosphoric acid¹²⁻¹⁴) in the vacuoles, and the appearance of immature of septa (Figs. 4-1, 4-2, 4-3 and 4-4). These morphological changes clearly differ from those caused by BI fungicides,¹⁰) DMI fungicides¹¹) and strobilurin fungicides.¹⁵)

Fig. 4. Ultrastructural effect of cyflufenamid on hyphal growth of *Monilinia fructicola*. Observations under a transmission electron microscope were made at 24 hr after cyflufenamid (1 ppm) treatment. Scale bar=2 μ m. 1: Hypha of control, 2: Hypha treated with cyflufenamid, 3: Ruptured hypha caused by cyflufenamid treatment, 4: Vacuolated hypha caused by cyflufenamid treatment, M: Mitochondrion, N: Nucleus, S: Septum, V: Vacuole.

Thus, a novel fungicide, cyflufenamid, has excellent fungicidal activity against various pathogens causing powdery mildew and some other fungi such as *M. fructicola*. The results also suggest that the biochemical mode of action of cyflufenamid is different from that of commercial fungicides such as BI, DMI and strobilurin. We are now conducting experiments to clarify the site of action of cyflufenamid.

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