Regular Article

Aminopyrifin, a novel 2-aminonicotinate fungicide with a unique effect and broad-spectrum activity against plant pathogenic fungi

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S Supplementary material

Aminopyrifin is a novel 2-aminonicotinate fungicide with unique chemistry and a novel mode of action. The fungicide showed high antifungal activity mainly against Ascomycetes and its related anamorphic fungi under *in vitro* and pot conditions (EC_{50} values: 0.0039–0.23 mg/L and 1.2–12 mg/L, respectively). The active ingredient strongly inhibited germ-tube elongation of *Botrytis cinerea* below 0.1 mg/L and invasion into a plant. The compound exhibited no cross-resistance to commercial fungicides in *B. cinerea*. The antifungal agent showed high preventive efficacy and translaminar action. In the field, aminopyrifin controlled gray mold and powdery mildew at 150 mg/L. Our findings suggest that aminopyrifin is useful for protecting crops from various plant pathogens.

Keywords: aminopyrifin, 2-aminonicotinate fungicide, gray mold, GPI, GWT-1 inhibitor.

Introduction

Many kinds of fungicides with different modes of action have been utilized for crop protection. Target site-specific inhibitors such as benzimidazoles, dicarboximides, ergosterol biosynthesis inhibitors (EBIs), quinone outside inhibitors (QoIs), and succinate dehydrogenase inhibitors (SDHIs) have been used frequently. However, several plant pathogens have developed resistance to these fungicides in various crops, which causes serious yield loss.^{1,2)} Therefore, discovering and developing a new fungicide with a novel mode of action is a mission assigned to the agrochemical industry for sustainable crop production.³⁾

Through our pesticide screening program, we discovered a

novel 2-aminonicotinate derivative as the active compound against gray mold and powdery mildew. As a result of compound optimization, aminopyrifin was selected as one of the most effective compounds against various pathogens. This compound is a novel 2-aminonicotinate fungicide with a unique chemical structure (Fig. 1), and our previous report suggests that aminopyrifin has a novel mode of action.⁴⁾

In this study, we showed the antifungal spectrum against various plant pathogenic fungi. The biological characteristics of aminopyrifin against *Botrytis cinerea* were also examined in laboratory and greenhouse conditions. In addition, the field performances of aminopyrifin against gray mold and powdery mildew were investigated.

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Fig. 1. Chemical structure of aminopyrifin.

Materials and methods

1. Fungicides

Aminopyrifin and 2-amino-*N*-[[5-(phenylmethyl)-2-thienyl]-methyl]-3-pyridinecarboxamide (M006) was synthesized by AGRO-KANESHO Co., Ltd.^{5,6)} For laboratory experiments, aminopyrifin were dissolved in acetone and suspended in distilled water for a predetermined concentration. The final concentration of acetone in each solution is below 1%. For greenhouse tests, aminopyrifin SC was prepared⁷⁾ and diluted in deionized water with 200 mg/L of Tween 20; for field trials, the fungicide SC was diluted in water. Conventional fungicides were purchased from commercial sources.

2. Plants

Cucumbers (*Cucumis sativus* cv. Sagamihanjiro-fushinari, first-leaf or cotyledon stage) were planted in plastic pots (40 mm in diameter, one plant/pot). Tomatoes (*Solanum lycopersicum* cv. Kyoryoku-Beiju, second-leaf stage) were potted in plastic pots (40 mm in diameter, one plant/pot). Wheats (*Triticum aestivum* cv. Nohrin-61, first-leaf stage) were grown in plastic pots (60 mm in diameter, three plants/pot for wheat rust, 5 plants/pot for wheat powdery mildew). Rice (*Oryza sativa* cv. Koshihikari, second-leaf stage) were planted in plastic pots (60 mm in diameter, 5 plants/pot).

3. Antifungal spectrum

3.1. In vitro experiments

Potato dextrose agar medium containing fungicide was prepared within a Petri dish 90 mm in diameter. Each pathogen was pre-cultured in appropriate conditions. The mycelial disc (4 mm in diameter) was placed on the medium. After incubation at 20°C 2–10 days for the pathogens, with the exception of *Venturia inaequalis*, which was incubated at 13°C for 24–43 days under lighted condition, the diameter of the radial mycelial growth was measured. Inhibitory activity was calculated as the percent of inhibition as compared with the untreated plot. Tested fungi and pseudofungus were *Botrytis cinerea* (strain name: ma4-3), *Colletotrichum acutatum* (strain name: An8), *Fusarium oxysporum* f. sp. *spinaciae* (strain name: M4), *Glomerella cingulata* (strain name: An1), *Monilinia fructicola* (strain name: F9), *Sclerotium rolfsii* (strain name: M28), *Sclerotinia sclerotiorum* (strain name: SR4), *Venturia inaequalis* (strain name: F20), *Verticillium dahliae* (strain name: MAFF731077), *Rhizoctonia solani* AG-1 IA (strain name: Rs0508), and *Pythium aphanidermatum* (strain name: MAFF725006). Tests were repeated 2 or 3 times.

3.2. In vivo pot tests

3.2.1. Cucumber diseases

Solutions of tested fungicides were applied to whole plants using a spray gun. One day after treatment, conidial suspensions (1.0×10^6 spores/mL) of *Colletotrichum orbiculare* (strain name: Dai) or sporangial suspensions (1.0×10^5 sporangia/mL) of *Pseudoperonospora cubensis* (strain name: AK) were sprayed onto the abaxial surface of leaves at 0.5 mL per pot using a spray gun. In-

oculated plants of *C. orbiculare* or *P. cubensis* were kept at 20°C in the dark in a humid chamber for 48 hr or 24 hr, respectively. Those plants were transferred to a greenhouse. Disease severity was assessed at 7 days after inoculation based on criteria shown in 3.2.5. Tests were repeated 2 or 3 times.

Tested fungicides were applied to whole plants. One day after treatment, conidial suspensions (1.0×10^5 spores/mL) of *Podosphaera xanthii* (strain name: AK) were inoculated onto the adaxial surface of leaves at 0.5 mL per pot using a spray gun. Inoculated plants were kept in the greenhouse, and disease severity was assessed 10 days after inoculation based on criteria shown in 3.2.5. Tests were repeated 3 times.

3.2.2. Tomato disease

Fungicide solutions were applied to the whole plants. One day after treatment, sporangial suspensions (1.0×10^5 sporangia/mL) of *Phytophthora infestans* (strain name: PiN5-2) were sprayed onto the adaxial surface of leaves at 0.5 mL per pot using a spray gun. Inoculated plants were kept at 20°C in a humid chamber for 24 hr. After incubation, plants were transferred to the greenhouse. Disease severity was assessed 7 days after inoculation based on criteria shown in 3.2.5. Tests were repeated 2 times.

3.2.3. Wheat diseases

Diluted solutions of tested fungicides were sprayed on whole plants at a rate of 1000 L/ha using an atomizer. One day after treatment, the first leaf was fragmented into 30 mm lengths (5 fragments per pot), and the fragments were placed on wet paper in a plastic box ($235 \times 325 \times 50$ mm³). Infected leaves with large amounts of conidia of *Blumeria graminis* f. sp. *tritici* (strain name: AK) were tapped from 300 mm above the box. After inoculation, the fragments were transferred to 1.5% agar medium in a 90 mm Petri dish and incubated in a growth chamber under conditions of 20°C, 50% relative humidity, and 5000 lx (16 hr/day). Disease severity was assessed 7 days after treatment based on criteria shown in 3.2.5. Tests were repeated 3 times.

Solutions of tested fungicides were applied to seedlings at a rate of 1000 L/ha using an atomizer. One day after treatment, conidial suspensions (1.0×10^5 spores/mL) of *Puccinia recondita* (strain name: AK) were sprayed onto whole plants using a spray gun. Inoculated plants were kept at 20°C in a humid chamber for 48 hr. After incubation, those plants were transferred to the greenhouse. Disease severity was assessed 7 days after inoculation based on criteria shown in 3.2.5. Tests were repeated 3 times.

3.2.4. Rice disease

Fungicide solutions were applied to whole plants. One day after treatment, conidial suspensions (1.0×10^5 spores/mL) of *Pyricularia oryzae* (strain name: 05044) were inoculated onto the leaves using a spray gun. After inoculation, tested plants were kept at 20°C in a humid chamber for 48 hr. The plants were transferred to the greenhouse after incubation. The number of lesions on secondary leaves was counted 7 days after inoculation. Efficacy was calculated by comparing the number of lesions of the treated pot with that of the untreated pot. Tests were repeated 2 times.

3.2.5. Evaluation of disease severity and calculation of control value

Disease severity and efficacy were evaluated as follows.

Rate of lesion area (disease severity): No infection was observed (0), very slight infection was observed (6.3), 6.3–12.5% of leaf surfaces (12.5), 12.5–25% of leaf surfaces (25), 25–50% of leaf surfaces (50), and 50–100% of leaf surfaces (100) were infected.

Mean of disease severity

$$= \frac{\sum (\text{disease severity of each leaf})}{\text{number of evaluated leaves}}$$

Control value

$$= 100 \times (1 - \text{mean of disease severity in treated pot} / \text{mean of disease severity in untreated pot})$$

4. Effect on the initial stage of the life cycle of *Botrytis cinerea*

4.1. Effect on spore germination and germ-tube elongation

Ten microliters of conidia (1.0×10^5 spores/mL) suspended in potato sucrose liquid medium was dropped on 3% agar medium amended with fungicide. Fungicide-treated spores were incubated at 20°C under light conditions in the laboratory. Investigation was performed 20 hr after treatment. One hundred conidia per plot were observed under light microscope, and germ-tubes below 50 µm were counted. Tests were conducted 3 times, consisting of two replicates per plot. Percent inhibitions of conidial germination and germ-tube elongation were calculated.

4.2. Inhibition of invasion into cucumber epidermal cells

Effect of aminopyrifin on infection process of *B. cinerea* on cucumber cotyledons was examined. Solutions of aminopyrifin SC and M006 were sprayed on the whole plant. One day after treatment, 5 µL of conidial suspensions (1.0×10^5 spores/mL) were dropped onto the upper side of cucumber cotyledons, and inoculated plants were kept in a humid condition of 20°C. The inoculated part of the cucumber cotyledon was cut into 5 mm squares 6, 24, and 48 hr after inoculation. The tissue was fixed and decolorized by immersing into a formalin-acetic acid-alcohol solution (36.0–38.0% formaldehyde: 99.7% acetic acid: 99% ethanol = 1:1:1). The observation was conducted under a light microscope after staining with 0.1% methyl blue.

5. Cross-resistance among commercial fungicides in *B. cinerea*

Four strains—ma4-3, 22, to0405, and 24—of *B. cinerea* were used in this test. The strain ma4-3 was used as the standard strain. Strain 22 has an E198K mutation in the *BenA/Mbc1* gene and a Q369P mutation in the *BcOS-1/Daf1* gene. Strain to0405 has an E198V mutation in the *BenA/Mbc1* gene and a G143A mutation in the *cytB* gene. Strain 24 has an E198A mutation in the *BenA/Mbc1* gene, a I365S mutation in the *BcOS-1/Daf1* gene, and an H272V mutation in the *sdhB* gene. These mutations were identified by DNA sequencing of the corresponding genes in accordance with previous reports.^{8–10} A paper disc 8 mm in diameter immersed in 50 µL of spore suspension (1.0×10^6 spores/mL) was placed on the upper side of each cu-

cumber cotyledon. Then, 50 µL of fungicide solution was applied onto each paper disc. The treated plants were kept at 20°C under high humidity and light conditions for 72 hr. Each plot contained two cotyledons. Tests were repeated 2 or 3 times. The severity of symptoms was assessed by the following criteria.

Lesion size (disease index): No lesion (0), very slight lesion (6.3), 5–8 mm inside the paper disc (13), within 2 mm around the paper disc (25), 5–10 mm around the paper disc (50), and not less than 10 mm around the paper disc (100)

Mean of disease severity

$$= \frac{\sum (\text{disease index} \times \text{leaf number belonging to each disease index})}{\text{the total number of evaluated leaves}}$$

Control value

$$= 100 \times (1 - \text{mean of disease severity in treated plot} / \text{mean of disease severity in untreated plot})$$

6. Biological properties against cucumber gray mold in pots

Cucumber cotyledons were used for tests of preventive and curative activity and translaminar action. For a preventive test, the solutions of aminopyrifin SC were applied to the seedlings using a spray gun. Spore suspensions (1.0×10^6 spores/mL; strain name: ma-4-3) were inoculated onto the abaxial surface of the leaves one day or 8 days after treatment. In the test of translaminar action, fungicide solutions were sprayed on the adaxial surface of the leaves. The spore suspensions were inoculated onto the abaxial surface of the leaves one day after application. For a curative test, the spore suspensions were inoculated onto the abaxial surface of the leaves. Fungicide solutions were sprayed onto the whole plants 21.5 hr after inoculation. Evaluation was performed based on criteria shown in 3.2.5. Tests were repeated 2 or 3 times.

7. Field trials against gray mold and powdery mildew

Efficacies against gray mold of eggplant and powdery mildew of cucumber were evaluated in Daisenbo field of AGRO-KANESHO Co., Ltd. in Japan.

Eggplant seedlings (cv. Senryo 2go) were planted in late January 2020. The first symptoms of gray mold were observed on April 2, 2020. Tested fungicides were sprayed 5 times at rates of 2190–3130 L/ha from April 3, 2020, at 7-day intervals using a backpack sprayer. Each treatment consisted of four replicates. The investigation was performed from April 16 to May 8 2020. Efficacy was calculated using the following formula.

$$\text{Infected fruit rate (\%)} = 100 \times \frac{\text{total infected fruits}}{\text{total evaluated fruits}}$$

Efficacy % control

$$= 100 \times (1 - \text{mean of infected fruit rate in the treated plot} / \text{mean of infected fruit rate in the untreated plot})$$

Cucumber seedlings (cv. SHARP-1) were planted in early February 2017. Tested fungicides were sprayed 4 times from April

17 at about 7-day intervals using a backpack sprayer. Application volumes were 1670–2930 L/ha. As disease symptoms of powdery mildew were observed at the time of first application, all diseased leaves were removed, and then the fungicide treatment was started. Each fungicide treatment and untreated control consisted of three and four replicates, respectively. The evaluation was carried out 7 days after the fourth application using the following formula.

Rate of lesion area (disease index): No infection was observed (0), 0–6.3% of leaf surfaces (1), 6.3–13% of leaf surfaces (2), 13–25% of leaf surfaces (3), 25–50% of leaf surfaces (4), and 50–100% of leaf surfaces (5) were infected.

Disease severity

$$= \left(\frac{\sum (\text{Number of leaves belonging to each disease index} \times \text{each disease index})}{5 \times \text{total evaluated leaves}} \right) \times 100$$

Control value

$$= 100 \times (1 - \text{mean of disease severity in the treated plot} / \text{mean of disease severity in the untreated plot})$$

Results

1. Antifungal spectrum

Aminopyrifin, (4-phenoxy)phenylmethyl 2-amino-6-methylpyridine-3-carboxylate, has a novel structure with 2-aminonicotinate (Fig. 1). First, we evaluated the antifungal activities of this new active ingredient against necrotrophs, hemibiotrophs, and biotrophs in laboratory and greenhouse tests. On potato dextrose agar medium containing aminopyrifin, this fungicide showed high activity against Ascomycetes and its related anamorphic fungi: *Botrytis cinerea*, *Colletotrichum acutatum*, *Fusarium oxysporum* f. sp. *lycopersici*, *Glomerella cingulata*, *Monilinia fructicola*, *Sclerotinia sclerotiorum*, *Venturia inaequalis*, and *Verticillium dahliae* (EC_{50} : 0.0039–0.23 mg/L, Table 1). Regarding Basidiomycetes, while aminopyrifin was highly effective against *Rhizoctonia solani* AG-1 IA (EC_{50} : 0.029 mg/L), it showed low activity against *Sclerotium rolfii* (EC_{50} : 5.9 mg/L). In contrast, this fungicide at 10 mg/L did not exhibit activity against pseudo-

Table 1. Antifungal spectrum of aminopyrifin *in-vitro*

Pathogen	EC_{50} value (mg/L)
<i>Botrytis cinerea</i>	0.0039
<i>Monilinia fructicola</i>	0.0055
<i>Fusarium oxysporum</i> f.sp. <i>spinaciae</i>	0.0056
<i>Verticillium dahliae</i>	0.0065
<i>Colletotrichum acutatum</i>	0.016
<i>Rhizoctonia solani</i> AG-1 IA	0.029
<i>Sclerotinia sclerotiorum</i>	0.058
<i>Glomerella cingulata</i>	0.16
<i>Venturia inaequalis</i>	0.23
<i>Sclerotium rolfii</i>	5.9
<i>Pythium aphanidermatum</i>	>10

Table 2. Antifungal spectrum of aminopyrifin on potted plants

Plant disease (Pathogen)	EC_{50} value (mg/L)
Wheat powdery mildew (<i>Blumeria graminis</i> f.sp. <i>tritici</i>)	4.3
Wheat rust (<i>Puccinia recondita</i>)	4.8
Cucumber powdery mildew (<i>Podosphaera xanthii</i>)	12
Cucumber downy mildew (<i>Pseudoperonospora cubensis</i>)	>400

fungi, e.g., *Pythium aphanidermatum*, which belongs to Oomycetes. In potted tests, the fungicide had no activity against tomato late blight (*Phytophthora infestans*), but it exhibited activity against cucumber anthracnose (*Colletotrichum orbiculare*) and rice blast (*Pyricularia oryzae*) at EC_{50} 3.6 mg/L and 1.2 mg/L, respectively. In tests of preventive activities against biotrophs using plant seedlings, the antifungal agent at 400 mg/L showed no activity against cucumber downy mildew (*Pseudoperonospora cubensis*). On the other hand, the agent was effective against cucumber powdery mildew (*Podosphaera xanthii*), wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*), and wheat rust (*Puccinia recondita*) (EC_{50} : 4.3–12 mg/L, Table 2). These results indicated that aminopyrifin showed high activity mainly against Ascomycetes and its related anamorphic fungi, to which many plant pathogens belong. It was suggested that the extent of activity against Basidiomycetes depends on the genus or species.

2. Effect on the initial stage of the life cycle of *Botrytis cinerea*

The effect of aminopyrifin on the initial stage of the life cycle of *B. cinerea*, which is highly sensitive to aminopyrifin (Table 1), was examined on 3% agar medium containing this fungicide. Untreated spores started to germinate and elongate; the length of germ-tubes at 20 hr was greater than 200 μ m. Although aminopyrifin exhibited almost no inhibitory activity on spore germination, even at 10 mg/L 20 hr after treatment, germ-tube elongation was strongly inhibited at a low concentration (EC_{50} : 0.035 mg/L, Table 3). Aminopyrifin-treated germ-tubes were swollen, and they were less than 50 μ m long (Fig. 2(A)). Moreover, the effect of the antifungal on cucumber epidermal cells was investigated using potted cucumber seedlings. Spore suspensions were inoculated one day after treatment. In untreated plot, spores germinated, and germ-tubes of about 50 μ m were observed 6 hr after inoculation (Fig. 2(B)g). Twenty-four hr after inoculation, lesions were detected visually, and superficial hyphae and secondary appressoria were formed (Fig. 2(B)h). On the contrary, aminopyrifin retarded spore germination 6 hr after inoculation (Fig. 2(B)a). Lesions were not observed in aminopyrifin-treated plot 24 and 48 hr after inoculation. At those times, germ-tubes were hyperbranched and swollen. The appressoria formed in untreated plot were not observed at the tips of germ-tubes (Fig. 2(B)bc). In addition, M006, a pyridine amide derivative, was used as the marker of GPI (Glycosylphosphatidylinositol)-anchored wall transfer protein 1 (GWT-1) inhibitor in this morphological experiment (Supplemental Fig. S1). Nakamoto *et al.* (2010) reported that pyridine

Fig. 2. Effect of aminopyrifin on germ-tubes of *B. cinerea* *in vitro* and *in vivo*. (A) The effect of the fungicides on spores was investigated on 3% agar medium amended with the fungicides. Ten microliters of spore suspensions was spotted on the medium. Spores were observed under light microscope 20 hr after treatment. Left: aminopyrifin 0.1 mg/L; right: untreated control. Co: conidia; Gt: germ-tube. Scale bar indicates 50 μm. (B) The effect of aminopyrifin on cucumber epidermal cells was investigated. Solutions of the antifungals at 100 mg/L were sprayed onto cucumber cotyledons. Spore suspensions were inoculated one day after treatment. The tissues were fixed and stained with 0.1% methyl blue 6, 24, and 48 hr after inoculation. Spores and germ-tubes were observed under light microscope. Co: conidia; Gt: germ tube; Sh: superficial hypha; Sa: secondary appressorium. Scale bar indicates 50 μm.

amide derivatives including M006 show activity against *Candida albicans* and *Aspergillus fumigatus* via the inhibition of the Gwt1 protein in an early step of the GPI biosynthetic pathway.⁶⁾ In the case of this compound, the same phenomena as those in aminopyrifin-treated spores and germ-tubes were observed in the infection process from spore germination to germ-tube elonga-

tion (Fig. 2(B)d–f). Consequently, it was demonstrated that aminopyrifin perturbed this pathogen to infect the plant tissue with abnormal germ-tube morphology.

3. Cross-resistance in *Botrytis cinerea*

Cross-resistance of aminopyrifin to five practical fungicides was investigated in cucumber cotyledons using four strains of *B. cinerea* (Table 4).

The strain ma4-3 used as the standard strain was sensitive to all fungicides except diethofencarb. Strain 22 was sensitive to azoxystrobin and boscalid but resistant to benomyl, diethofencarb, and procymidone. Strain to0405 was tolerant to azoxystrobin and benomyl among the existing fungicides. Strain 24

Table 3. Effect of aminopyrifin on spore germination and germ tubes of *B. cinerea* on agar media

Tested fungicide	EC ₅₀ value (mg/L)	
	Spore germination	Germ-tube elongation
Aminopyrifin	>10	0.035

Table 4. Activity of aminopyrifin to resistant strains of *B. cinerea*

Tested fungicides	EC ₅₀ value (mg/L)			
	ma4-3 ^{a)}	22 ^{b)}	to0405 ^{c)}	24 ^{d)}
Aminopyrifin	0.83	0.43	0.83	0.49
Azoxystrobin	2.9	0.87	>100	2.7
Benomyl	1.1	>100	>100	>100
Boscalid	0.9	1.4	1.1	>100
Diethofencarb	>100	>100	12.0	0.31
Procymidone	4.9	>400	4.6	>400

^{a)} Isolate from strawberry, which was used as standard strain. ^{b)} Isolate from strawberry, which has a E198K mutation in *BenA/Mbc1* gene and a Q369P mutation in *BcOS-1/Daf1* gene. ^{c)} Isolate from tomato, which has a E198V mutation in *BenA/Mbc1* gene and a G143A mutation in *cytb* gene. ^{d)} Isolate from tomato, which has a E198A mutation in *BenA/Mbc1* gene, a I365S mutation in *BcOS-1/Daf1* gene, and a H272V mutation in *sdhB* gene.

was resistant to benomyl, boscalid, and procymidone. Therefore, there were no conventional fungicides to control these four strains. On the other hand, aminopyrifin showed high activity against all strains (EC₅₀ value: 0.43–0.83 mg/L). Therefore, it was demonstrated that the efficacy of aminopyrifin was not affected by strains resistant to azoxystrobin, benomyl, boscalid, diethofencarb, and procymidone (Table 4).

4. Preventive, curative efficacy and translaminar action against cucumber gray mold in the greenhouse

To investigate the biological properties of aminopyrifin on plants, the efficacy against gray mold was evaluated using cu-

cumber seedlings (Fig. 3). When aminopyrifin at 100 mg/L was treated 21.5 hr after inoculation, the control value was 50 (Fig. 3D). In contrast, control values of preventive (one day after treatment) and residual (8 days after treatment) efficacy were 85 at 1.6 mg/L and 100 at 25 mg/L, respectively (Fig. 3A and B). When aminopyrifin was applied to the adaxial surface of the cotyledon and inoculated onto the abaxial surface, the fungicide showed the efficacy of control value 92 at 100 mg/L (Fig. 3C). Thus, the result indicated that aminopyrifin had high preventive activity and translaminar action.

5. Field performance against gray mold of eggplant and powdery mildew of cucumber

The effectiveness of aminopyrifin against eggplant gray mold and cucumber powdery mildew in fields was examined.

As symptoms of gray mold were observed on eggplants before the first application, all diseased fruits were removed, and then the first application was performed. Afterward, the disease spread moderately; the disease frequency during this test in the untreated plot was 33.8%. Penthiopyrad at 100 mg/L, pyribencarb at 200 mg/L, iminoctadine albesirate at 150 mg/L, and iprodione at 500 mg/L controlled 91%, 83%, 68%, and 100%, respectively, against gray mold; aminopyrifin at 150 mg/L exhibited efficacy of 80% control (Fig. 4A).

In the cucumber field trial, powdery mildew rapidly spread, and the disease severity in the untreated plot on the day of the investigation was severe. While control values of both iminoctadine albesirate at 150 mg/L and mepanipyrim at 200 mg/L were above 90, that of aminopyrifin at 150 mg/L was 98 (Fig. 4B). It

Fig. 3. Biological properties of aminopyrifin against cucumber gray mold were examined in pot tests using cucumber seedlings. (A) Preventive efficacy: Fungicide solutions were sprayed onto whole plants, and spore suspensions were inoculated on the abaxial surface of the leaves one day after treatment. (B) Residual efficacy: Fungicide solutions were applied to whole plants. Eight days after treatment, spore suspensions were inoculated on the abaxial surface of the leaves. (C) Translaminar action: Fungicide solutions were sprayed on the upper side of the leaves. Spore suspensions were inoculated on the lower side of the leaves one day after application. (D) Curative efficacy: Spore suspensions were sprayed on the abaxial surface of the leaves. Solutions of the fungicides were applied on whole seedlings 21.5 hr after inoculation. Disease severity was investigated two days after inoculation. Efficacies were calculated in comparison with the untreated pots. Error bars represent standard deviation.

Fig. 4. Field performance of aminopyrifin against gray mold of eggplant and powdery mildew of cucumber. (A) Efficacy against eggplant gray mold was examined under the naturally infected condition. Fungicides were applied 5 times at 7-day intervals. Infected and healthy fruits were counted every week. The infection pressure was moderate. The concentrations of aminopyrifin (Ami), penhiopyrad (Pen), pyribencarb (Pyr), iminotadine albesirate (Imi), and iprodione (Ipr) were 150 mg/L, 100 mg/L, 200 mg/L, 150 mg/L, and 500 mg/L, respectively. (B) Efficacy against cucumber powdery mildew was tested under the naturally infected condition. The infection pressure was severe. Fungicides were applied four times at intervals of approximately 7 days. Investigation was conducted 7 days after the final treatment. The concentrations of aminopyrifin (Ami), iminotadine albesirate (Imi), and mepanipyrin (Mep) were 150 mg/L, 150 mg/L, and 200 mg/L, respectively.

was demonstrated that aminopyrifin at 150 mg/L was effective against gray mold and powdery mildew in the field.

Discussion

Aminopyrifin is a novel fungicide with a unique chemistry of 2-aminonicotinate. In this study, we characterized the biological properties of this new active ingredient, aminopyrifin, *in vitro* and *in vivo*.

As shown in Table 4, aminopyrifin showed high activity against strains of *B. cinerea* resistant to conventional fungicides, benzimidazole (benomyl), *N*-phenylcarbamate (diethofencarb), dicarboximide (procymidone), QoI (azoxystrobin), and SDHI (boscalid) without cross-resistance. This result suggests that aminopyrifin has a unique mode of action. Our previous study using *Neurospora crassa* indicated that aminopyrifin targets GWT-1, whose amino acid sequence of *B. cinerea* has high homology to that of *N. crassa*.⁴⁾ In agricultural situations, each strain of *B. cinerea* resistant to those fungicides has threatened stable crop production.^{1,2)} Problems of multi-resistance to these fungicides have also occurred recently.^{11–15)} As a fungicide with a novel mode of action, our discovery, aminopyrifin, may be useful for countering those problems.

Morphological investigation revealed that aminopyrifin induced a unique swollen shape in germ-tubes of *B. cinerea* (Fig. 2). The phenotype of germ-tubes of *B. cinerea* was similar to that of *Neurospora crassa*, which is mentioned within our previous report.⁴⁾ According to the Fungicide Resistance Action Committee (FRAC) Code List 2020, the following active ingredients were classified in each mode of the action group: benomyl (FRAC 1), procymidone (FRAC 2), oxpoconazole fumarate (FRAC 3), fenpropimorph (FRAC 5), boscalid (FRAC 7),

mepanipyrin (FRAC 9), diethofencarb (FRAC 10), azoxystrobin (FRAC 11), fludioxonil (FRAC 12), fenpyrazamine (FRAC 17), polyoxin (FRAC 19), and iminotadine albesirate (FRAC M 07). We compared the effect of these existing fungicides on germ-tubes with that of aminopyrifin on agar medium; however, germ-tubes swollen by aminopyrifin were not observed in the other fungicide-treated plot (data not shown). According to several reports, GWT-1 protein is localized in the membrane of the endoplasmic reticulum and catalyzes inositol acylation of phosphatidylinositol. Thus far, GPI-anchored enzymes related to the cell wall organization of fungi and yeast have been reported.^{16–19)} The deficient mutants of GPI-anchored proteins have also been reported to attenuate the pathogenicity of plant pathogens.²⁰⁾ It has been reported that E1210, the GWT-1 inhibitor for pharmaceutical use, inhibited the adherence of *Candida albicans* to polystyrene surfaces.²¹⁾ In Fig. 2B, normal appressorium formation was inhibited on aminopyrifin or M006-treated host epidermal cells with swollen and hyperbranched germ-tubes. Therefore, aminopyrifin may influence the cell wall integrity of pathogens *via* the inhibition of GPI-anchored protein maturation, which failed the invasions with the abnormal phenotype of germ-tubes.

GWT-1 inhibitors were well studied for pharmaceuticals.²²⁾ For instance, BIQ (1-[(4-butylphenyl)methyl]isoquinoline)^{23,24)} and manogepix (3-(3-{4-[(pyridin-2-yloxy)methyl]benzyl}isoxazol-5-yl)pyridin-2-amine)^{21,25,26)} are active against pathogens including *Candida*, *Cryptococcus*, and *Aspergillus* sp. The former is a quinoline compound, and the latter is a pyridine and isoxazole derivative and the prodrug of the compound, fosmanogepix, which is under clinical development. However, aminopyrifin is a novel compound with pyridine and ester moieties for agricultural use; its structure is unique from those of pharmaceutical compounds.

In addition to aminopyrifin's unique mode of action, this fungicide showed high activity against various economically important pathogenic fungi (Table 1 and 2).²⁷⁾ Furthermore, aminopyrifin exhibited high preventive, residual efficacies and also translaminar action, which contribute to efficient disease control in the field (Fig. 3). In fact, this fungicide was highly applicable as preventive use in the field against gray mold and powdery mildew (Fig. 4). In agricultural situations, farmers face problems of crop losses arising from various diseases and the appearance of pathogens resistant to fungicides. A novel fungicide, aminopyrifin, is anticipated to be one crop protection tool contributing to stable crop production.

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Electronic supplementary materials

The online version of this article contains supplementary material (Supplemental Fig. S1), which is available at <http://www.jstage.jst.go.jp/>

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