Original Article

Biological Properties of Fungitoxic Propargyl *N*-(6-ethyl-5-iodo-2-pyridyl)carbamate

Shigeru MITANI, Kanako NAKANO,* Norifusa MATSUO,*
and Terumasa KOMYOJI

Agrochemicals Development Laboratory, and *Organic Chemistry Research Laboratory,
Central Research Institute, Ishihara Sangyo Kaisha, Ltd.,
Nishishibukawa, Kusatsu 525, Japan

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Propargyl *N*-(6-ethyl-5-iodo-2-pyridyl)carbamate (PEIP) exhibited high fungitoxic activities against *Erysiphe graminis* f. sp. *tritici*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Pyricularia oryzae*, and *Rhizoctonia solani* at 0.1 ppm *in vitro* and 8 to 63 ppm *in vivo*. It also showed activity against benzimidazole-resistant isolates of *B. cinerea* as well as the sensitive isolates. PEIP possessed not only preventive activity, but also curative activity and translaminar action. The excellent activity of PEIP against *B. cinerea* and *Elsinoe ampelina* was also confirmed in field trials. PEIP induced morphological changes in the conidial germination and nuclear arrangement in germ-tubes of *B. cinerea* in a similar manner to that of benomyl, a benzimidazole fungicide. These results suggest that PEIP interferes with the cell division of this fungus.

INTRODUCTION

The benzimidazole resistance of plant pathogenic fungi has increased throughout the world, making it difficult to control many diseases with this class of fungicides.¹⁾ As a strategy to cope with the fungicide resistance of plant pathogens, the *N*-phenylcarbamate diethofencarb, which shows negatively correlated cross-resistance to resistant strains, has been introduced for practical use. But increased sensitivity to diethofencarb was limited to isolates with only high levels of benzimidazole resistance.²⁾

We discovered a family of pyridylcarbamate had high potency for the control of benzimidazole-resistant and -sensitive isolates of cucumber gray mold (*Botrytis cinerea*).³⁾ Among the compounds of this family, propargyl *N*-(6-ethyl-5-iodo-2-pyridyl)carbamate (PEIP) as shown in Fig. 1 had the most potent activity against both isolates. Moreover the compound had a cell division inhibitory activity similar to benzimidazoles and dietho-

fencarb. The excellent activity and interesting characteristic of this new compound encouraged us in further studies to know its biological properties.

The present paper describes some biological properties of PEIP including the fungicidal spectrum *in vitro* and the disease control efficacy due to the preventive, curative and systemic activities in field trials as well as pot tests. We also examined the mode of action of PEIP by using microscopic observation.

MATERIALS AND METHODS

1. Chemicals and Formulations

PEIP and diethofencarb were synthesized according to previously described methods,^{4,5)} and the structures of the compounds were confirmed by IR, Mass and ¹H NMR spectrometry.

The benzimidazole fungicide benomyl (50% wettable powder) and the 4',6-diamidino-2-phenylindole (DAPI, Wako Chemical Industries, Ltd.) were purchased.

PEIP and diethofencarb were further

Fig. 1 PEIP, propargyl *N*-(6-ethyl-5-iodo-2-pyridyl) carbamate.

formulated as 20% wettable powders as described before.⁴⁾

2. Plants and Pathogens

In glasshouse tests, cucumber (cv. Suyu), tomato (cv. Ponderosa), rice (cv. Koshihikari), wheat (cv. Norin No. 61) and oat (cv. Zenshin) seedlings were grown in a polyethylene pot (diameter: 7.5 cm). In case of cucumber plants, the experiments were performed with plants of one or two true leaf stage. Tomato, rice and oat plants were used at four to six leaf stage.

All the pathogens stored in the fungal bank of our company were used for *in vitro* and/or *in vivo* tests. We employed five kinds of *B. cinerea* isolate, which are highly resistant to benzimidazole (HR, S, S), to both of benzimidazole and dicarboximide (HR, R, S), to diethofencarb (S, S, R), to both of dicarboximide and diethofencarb (S, R, R), and intermediately resistant to benzimidazole and highly to diethofencarb (IR, S, R). *B. cinerea* (IR, S, R) was kindly gifted by Agricultural Technical Center of National Federation of Agricultural Co-operative Association (Zen-Noh).

3. Fungicidal Spectrum *in vitro*

To examine the antifungal spectrum *in vitro* of PEIP, we employed an agar dilution method. Each isolate was cultured on potato-sucrose agar (PSA) at 20 to 25°C. Mycelial disks (diameter: 4 mm) were then cut from the margins of the colonies and placed on the PSA plates containing PEIP in various concentrations. After incubation at their optimum temperature for an optimum period, colony diameter on PSA medium was measured to calculate EC₅₀ value.

4. Fungicidal Spectrum *in vivo*

To evaluate the preventive activity in the glasshouse, 10 ml of solution containing PEIP

was sprayed over the plant using a spray gun 24 hr before inoculation. The test plants were inoculated either by spraying aqueous spore suspensions (*Phytophthora infestans*, *Pseudoperonospora cubensis*, *Colletotrichum lagenarium*, *Cochliobolus miyabeanus* and *Pyricularia oryzae*) or by dusting the spores on the leaves (*Erysiphe graminis*, *Sphaerotheca fuliginea* and *Puccinia coronata*). Otherwise, mycelial disks of 4 mm in diameter were placed on the detached cucumber leaf (*Sclerotinia sclerotiorum* and *B. cinerea*), or the rice straws previously incubated with the sheath blight pathogen (*Rhizoctonia solani*) were used to inoculate between leaf sheath portions. The inoculated plants were incubated in the glasshouse for 3 to 11 days at 20 to 28°C.

To assess fungicidal activity, degree of disease development was observed visually and evaluated according to the following indices:

- 5: No lesion
- 4: Lesion area under 5% of treated leaves
- 3: Lesion area under 10% of treated leaves
- 2: Lesion area under 50% of treated leaves
- 1: Lesion area under 70% of treated leaves
- 0: Lesion area over 70% of treated leaves

With indices 5 to 3, the test compound was judged to be effective, and the minimum effective concentration (MEC) was determined.

5. Curative and Systemic Activity

The curative activity of PEIP for *C. lagenarium* was evaluated in the same manner as described in the preventive activity, except plants were inoculated 24 or 48 hr before the treatment. In this experiment, cucumber plants (the first true leaf) were inoculated with spore suspension of *C. lagenarium*. The inoculated plants were incubated in the glasshouse for 5 days.

In *B. cinerea*, we employed cucumber cotyledons and a paper disk method⁶⁾ with some modifications. Spore suspension in potato-sucrose broth (PSB) was dropped on a paper disk which was previously placed on the

detached cucumber leaf (the first true leaf). After incubating for 24 hr in a moist plastic case at 20°C, a leaf with the lesion was dipped into a PEIP aqueous solution for a minute, and incubated in a moist plastic case for 3 days.

Systemic activity from root to leaf was evaluated by pouring 20 ml of solution on the soil surface around the cucumber plant 48 hr before inoculation. Translaminar activity from leaf-upper-surface to leaf-under-surface was also evaluated. Chemical solution was sprayed on the upper-surface of the cucumber leaf 24 hr before inoculation. Mycelial disks of *B. cinerea* were inoculated on the under-surface of the detached leaf which had been sprayed on the upper side. The inoculated leaves were incubated in a moist plastic case for 3 days.

Except for the test of curative application against *B. cinerea*, the disease severity of cucumber plants was rated in a similar manner above. In the test of curative activity of *B. cinerea*, percentage of disease development was calculated by measuring the diameter of each lesion just before the chemical application (a day after inoculation) and at the end of the experiment (3 days after inoculation). Degree of disease development was evaluated according to the following indices:

- 5: No development
- 4: Lesion development under 5% of control leaves
- 3: Lesion development under 10% of control leaves
- 2: Lesion development under 50% of control leaves
- 1: Lesion development under 70% of control leaves
- 0: Lesion development over 70% of control leaves

6. Field Trials

PEIP was tested at the concentration of 250 and 500 ppm by a foliar spray at 1000 l/ha for the control of *B. cinerea* (curative activity) on kidney bean (cv. Himetebou) at an earlier stage of disease development and of 1000 ppm by a dormant-period treatment for *Elsinoe ampelina* (preventive activity) on grape (cv. Neomuscut). Trials were laid down as

randomized blocks, and replicated twice with 12 plants/plot for kidney bean or with 5 to 6 trees/plot for grape under natural infection. Disease development was observed 6 days after PEIP treatment (*B. cinerea*) or 27 days after treatment (*E. ampelina*). Percent of diseased plants or leaves (PDP or PDL) was determined by counting the number of diseased plants (kidney bean) or leaves (grape), and calculated by the following formula:

$$\text{PDP or PDL} = \frac{\text{number of diseased plants or leaves}}{\text{total number of plants or leaves}} \times 100$$

7. Observation of Spore Germination

A suspension of conidia of *B. cinerea* containing 10% PSB was mixed with each test compound at a final concentration of 1 ppm, and incubated at 20°C for 24 hr on a glass slide. Germination of conidia was observed by light microscopy (OLYMPUS BH-2 microscope).

8. Observation of Nuclei

Conidia of *B. cinerea* were suspended in sterile distilled water containing 10% PSB medium. After germination, each test compound was added, and 15 µl of the suspension was spread and incubated on the glass slide at 20°C for 24 hr. The suspension was air dried and stained with DAPI of 0.25 ppm in methanol solution. The stained preparations were observed under a fluorescence microscope (OLYMPUS AHB-TL-2) with HBO 200 W/2 light source. One excitation filter (UG-1, 365/10), one dichroic mirror (DM-400) and barrier filter (L420) were used.

RESULTS

1. Fungitoxic Spectrum

The fungitoxic activities of PEIP *in vitro* and *in vivo* (preventive activity) are summarized in Table 1. PEIP possessed a broad spectrum of activities against Ascomycetes, Basidiomycetes and Deuteromycetes with some exceptions and had a slight activity against Oomycetes.

It showed an especially high activity against *Monilinia fructicola*, *S. sclerotiorum*, *Ustilago nuda*, *B. cinerea*, *Cercospora beticola*, *Cercospora*

Table 1 Biological activity of PEIP in laboratory (*in vitro*) and glasshouse (*in vivo*) tests.

Pathogen	Minimum effective concentration (ppm)	
	<i>In vitro</i> ^{a)}	<i>In vivo</i> ^{b)}
Phycomycetes		
<i>Aphanomyces cochlioides</i>	>100	—
<i>Phytophthora infestans</i>	>100	>500
<i>Pseudoperonospora cubensis</i>	—	500
<i>Pythium aphanidermatum</i>	>100	—
Ascomycetes		
<i>Cochliobolus miyabeanus</i>	>100	>500
<i>Colletotrichum lagenarium</i>	1	31
<i>Elsinoe ampelina</i>	1	—
<i>Erysiphe graminis</i> f. sp. <i>tritici</i>	—	8
<i>Monilinia fructicola</i>	<0.1	—
<i>Mycosphaerella melonis</i>	1	—
<i>Pyrenophora graminea</i>	1	—
<i>Rosellinia necatrix</i>	1	—
<i>Sclerotinia sclerotiorum</i>	<0.1	31
<i>Sphaerotheca fuliginea</i>	—	250
<i>Valsa ceratosperma</i>	1	—
<i>Venturia nashicola</i>	1	—
Basidiomycetes		
<i>Corticium rolsii</i>	10	—
<i>Helicobasidium mompa</i>	10	—
<i>Puccinia coronata</i>	—	500
<i>Ustilago nuda</i>	<0.1	—
Deuteromycetes		
<i>Alternaria alternata</i>		
apple pathotype	10	—
<i>Botrytis cinerea</i> (HR, S, S) ^{c)}	<0.1	16
<i>Botrytis cinerea</i> (HR, R, S)	<0.1	16
<i>Botrytis cinerea</i> (S, S, R)	<0.1	16
<i>Botrytis cinerea</i> (S, R, R)	<0.1	16
<i>Botrytis cinerea</i> (IR, S, R)	0.1	63
<i>Cercospora beticola</i>	<0.1	—
<i>Cercospora kikuchii</i>	<0.1	—
<i>Cladosporium fulvum</i>	1	—
<i>Fusarium oxysporum</i> f. sp. <i>raphani</i>	1	—
<i>Helminthosporium sacchari</i>	>100	—
<i>Pyricularia oryzae</i>	<0.1	31
<i>Rhizoctonia solani</i>	<0.1	63
<i>Rhynchosporium secalis</i>	10	—
<i>Verticillium dahliae</i>	1	—

^{a)} Agar dilution method, EC₉₀ value.^{b)} Pot tests (preventive activity), EC₉₀ value.^{c)} Highly resistant to benzimidazoles (HR, S, S), to both of benzimidazoles and dicarboximides (HR, R, S), to diethofencarb (S, S, R), to both of dicarboximides and diethofencarb (S, R, R), and intermediately resistant to benzimidazoles and highly to diethofencarb (IR, S, R).

kikuchii, *P. oryzae* and *R. solani* at 0.1 ppm *in vitro*. PEIP had a similar degree of antifungal activity against (HR,S,S), (HR,R,S), (S,S,R) and (S,R,R) isolates of *B. cinerea*. Although PEIP showed activity against (IR,S,R) isolate, it was somewhat less sensitive than the other isolates. PEIP was also active against *C. lagenarium*, *E. ampelina*, *Mycosphaerella melonis*, *Pyrenophora graminea*, *Rosellinia necatrix*, *Valsa ceratosperma*, *Venturia nashicola*, *Cladosporium fulvum*, *Fusarium oxysporum*, and *Verticillium dahliae* *in vitro* at 1 ppm. On the other hand, an antifungal activity against *Aphanomyces cochlioides*, *Phytophthora infestans*, *Pythium aphanidermatum*, *Cochliobolus miyabeanus* and *Helminthosporium sacchari* was not observed even at 100 ppm.

In pot tests, PEIP was highly fungitoxic to *E. graminis* f. sp. *tritici* (MEC: 8 ppm), *B. cinerea* (MEC: 16 ppm against (HR,S,S), (HR,R,S), (S,S,R), and (S,R,R) isolates and 63 ppm against (IR,S,R) isolate), *C. lagenarium* (MEC: 31 ppm), *S. sclerotiorum* (MEC: 31 ppm), *P. oryzae* (MEC: 31 ppm), and *R. solani* (MEC: 63 ppm). Against *P. infestans* and *C. miyabeanus*, the control effects were not observed *in vivo* at 500 ppm.

2. Curative and Systemic Activity

PEIP possessed not only a preventive activity, but also a curative activity against *B. cinerea* and *C. lagenarium* on cucumber plants (Table 2). When the plants were sprayed 24 or 48 hr after inoculation at 125 ppm, disease development caused by *C. lagenarium* was

Table 2 Curative activity^{a)} of PEIP against *Botrytis cinerea* and *Colletotrichum lagenarium* on cucumber plants.

PEIP (ppm)	Treatment time after inoculation		
	<i>B. cinerea</i> (S,S,R) ^{b)}	<i>C. lagenarium</i>	
	24 hr	24 hr	48 hr
500	4	5	5
125	1	5	5
31	0	1	0

^{a)} With indices 5 to 3, the test compound was judged to be effective.^{b)} Sensitive to benzimidazoles and dicarboximides, highly resistant to diethofencarb.

Table 3 Systemic activity of PEIP against *Botrytis cinerea* on cucumber plants.

PEIP (ppm)	Roots to leaves	Leaf-upper-surface to leaf-under-surface
1000	1	5
500	0	5
125	—	1

With indices 5 to 3, the test compound was judged to be effective.

controlled perfectly. Good activity against *B. cinerea* was also observed at 500 ppm, however disease control was inferior to that against *C. lagenarium*.

Translaminar activity from leaf-upper-surface to leaf-under-surface and systemic activity from root to leaf were examined as shown in Table 3. Leaf-upper-surface treatment of PEIP gave complete control of *B. cinerea* at 500 and 1000 ppm. On the other hand, soil application of PEIP solution around the cucumber seedling did not show efficacy against *B. cinerea* at 1000 ppm.

3. Field Trials

Field trials revealed a good curative activity of PEIP against *B. cinerea* on kidney bean at 250 or 500 ppm (Fig. 2). When the solution of PEIP was applied for grape in a

Fig. 2 Control efficacy of PEIP against *Botrytis cinerea* on kidney bean in field trials.

Trial was laid down as replicated 2 times with 12 plants/plot under natural infection. PEIP treatment was done at the earlier stage of disease development. Six days after treatment, percent of diseased plants was determined by counting the number of diseased plants.

Fig. 3 Control efficacy of PEIP against *Elsinoe ampelina* on grape in field trials.

Trial was laid down as replicated 2 times with 5 to 6 trees/plot under natural infection. PEIP treatment was done at dormant period of plants. Twenty-seven days after treatment, percent of diseased leaves was determined by counting the number of diseased leaves.

dormant period, excellent control of *E. ampelina* was observed at 1000 ppm (Fig. 3).

4. Effect of PEIP on Germination

Influence of PEIP on germination of conidia of *B. cinerea* was observed under a microscope (Fig. 4). Although PEIP did not inhibit the spore germination, it induced morphological abnormalities such as swollen and distorted hyphal branching in both isolates of (S,S,R) and (HR,R,S) as shown in Fig. 4A and 4E. These morphological changes were quite similar to those of benomyl against isolate of (S,S,R) (Fig. 4B) and those of diethofencarb against (HR,R,S) (Fig. 4G).

5. Effect of PEIP on Nuclei

The treatment of germinating conidia of *B. cinerea* with 1 ppm of PEIP induced the formation of abnormal shape of nuclei in the isolates of (S,S,R) and (HR,R,S) (Fig. 5A and 5E). Quite similar phenomena were also observed in the treatment with benomyl on isolate of (S,S,R) (Fig. 5B) and with diethofencarb against (HR,R,S) (Fig. 5G).

DISCUSSION

Although the benzimidazole fungicides contributed to disease protection of broad varieties of important crops, emergence of resistance to this class of fungicides made it difficult to control many diseases.¹⁾ As a strategy to

cope with this resistance, the mixture of benzimidazole, carbendazim and diethofencarb

was introduced. But applications of the mixture resulted in selective effect on a population, so

Fig. 4

Fig. 5

that intermediate or weak benzimidazole resistant strains predominated in the fields.²⁾ Thus unsatisfactory control of the mixture was observed where the fungal population was dominated by the strains resistant to the mixture. Therefore the development of new chemicals for controlling both sensitive and resistant isolates has been needed. PEIP had a high activity against *B. cinerea* regardless of the level of benzimidazole resistance, suggesting that this compound has a high potency for the control of plant pathogens to cope with benzimidazole-resistance.

PEIP possessed not only a preventive activity, but also a curative activity against *B. cinerea* and *C. lagenarium* on cucumber plants. Systemic activity from leaf-upper-surface to leaf-under-surface was also observed. These curative and systemic properties are important for leading to the excellent control of the diseases in the fields.

Furthermore, phytotoxicity on varieties of crops, e.g. cucumber, tomato, kidney bean, rice, oat and grape was not observed at all when PEIP was treated at 500 ppm (data not shown). These results suggest that PEIP has a high potency to be a new fungicide for practical use.

Many systemic fungicides including benzimidazoles,^{7,8)} *N*-phenylcarbamates,^{9,10)} *N*-phenylformamidoximes,¹¹⁾ dicarboximides¹²⁾ and ergosterol biosynthesis inhibitors (EBIs)¹³⁾ are known to induce morphological change in fungi. Among them the primary mode of action of benzimidazoles is generally considered to inhibit microtubule assembly by binding to beta-tubulin.^{14,15)} *N*-Phenylcarbamates, *N*-phenylformamidoximes and a new series of compounds, *N*-phenylanilines exhibit similar biological properties to those of benzimidazoles.

These groups of compound show specific antifungal activity against highly benzimidazole-resistant strains,¹⁶⁻¹⁸⁾ and induced distorted and swollen-germ tubes of sensitive strains of *B. cinerea*.⁹⁻¹¹⁾ In *B. cinerea*, mitosis appeared to be arrestment after the treatments with *N*-phenylcarbamates, diethofencarb and methyl *N*-(3,5-dichlorophenyl)-carbamate (MDPC).^{9,10)} In *Venturia nashicola*, disordered configuration of nuclei in germinated conidia of sensitive strains was also observed by the treatment with a *N*-phenylformamidoxime, *N*-(3,5-dichloro-4-propynyl-oxyphenyl)-*N'*-methoxyformamidine (DCPF).¹⁹⁾ Recently, Fujimura *et al.* reported that selective fungitoxicity of diethofencarb to a benzimidazole-resistant strain was by binding of the compounds to tubulin in *Neurospora crassa*.^{20,21)}

PEIP was similar to benomyl and diethofencarb in having morphological effects on conidial germination and nuclear arrangement in germ-tubes. These results suggest that PEIP is most likely to inhibit cell division of the target fungus, *B. cinerea*. Pyridylcarbamates including PEIP might interfere with the formation or functions of microtubule in a manner similar to that of benzimidazoles, *N*-phenylcarbamates, *N*-phenylformamidoximes and/or *N*-phenylanilines. It should be emphasized that PEIP, benomyl and diethofencarb all belong to aromatic carbamates. Moreover PEIP had a similar pattern in the fungicidal spectrum to benzimidazoles, which is well known to have wide fungicidal spectrum. These fungicides were highly active on most Ascomycetes and Deuteromycetes, whereas they were less active on the Basidiomycetes and inactive on the Oomycetes.^{7,8)}

If the fungicidal activities of PEIP were due to its binding to tubulin, the active sites of

Fig. 4 Light photomicrographs of germinated conidia of *Botrytis cinerea* of (S, S, R) isolate (A, B, C and D) and (HR, R, S) isolate (E, F, G and H) ($\times 280$).

Conidia were incubated in the absence and the presence of 1 ppm of test compounds. A and E: treated with PEIP, B and F: treated with benomyl, C and G: treated with diethofencarb, D and H: control.

Fig. 5 Fluorescence photomicrographs of germinated conidia of *Botrytis cinerea* of (S, S, R) isolate (A, B, C and D) and (HR, R, S) isolate (E, F, G and H) ($\times 580$).

Conidia were incubated in the absence and the presence of 1 ppm of test compounds. A and E: treated with PEIP, B and F: treated with benomyl, C and G: treated with diethofencarb, D and H: control.

PEIP or of the target protein might be different from the other compounds as reported in the case of rhizoxin. The macrolide compound rhizoxin, which has a cell division inhibition activity, inhibited mycelial growth of *B. cinerea* and other fungi.^{22,23)} Rhizoxin was bound to beta-tubulin, but the binding site differed from benzimidazoles.²⁴⁾ Although the binding sites of PEIP was not clarified yet, PEIP would be the first aromatic carbamate which may have a cell division inhibition activity against both benzimidazole-sensitive and -resistant isolates.

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要 約

抗菌活性を有するプロパルギル *N*-(6-エチル-5-ヨード-2-ピリジル) カーバメートの作用特性

三谷 滋, 中野加奈子

松尾憲純, 光明寺輝正

プロパルギル *N*-(6-エチル-5-ヨード-2-ピリジル)-カーバメート (PEIP) は *Erysiphe graminis* f. sp. *tritici*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Pyricularia oryzae*, *Rhizoctonia solani* 等に高い活性を示し, その最低有効濃度は寒天平板希釈法で 0.1 ppm 以下であり, ポット試験ではおのおの 8~63 ppm であった. また PEIP はベンズイミダゾール感受性のみならず耐性の *B. cinerea* にも同様の高い効果を示した. また *B. cinerea* および *C. lagenarium* におのおの 500 および 125 ppm で治療効果を有していた. 移行性に関しては根からの浸透性は 1000 ppm でも認められなかったが, 葉表から葉裏への移行性は 500 ppm で認められた. また *B. cinerea* および *Elsinoe ampelina* に対する効果は圃場試験でも確認できた. *B. cinerea* の分生孢子発芽や, 発芽管中の核に対する PEIP の影響を調べたところ, 形態に異常が認められた. これらの作用はベノミルを感受性菌に処理したときのものと酷似していたことから, PEIP の作用点は有糸分裂阻害である可能性が示唆された.