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Original Article

Negatively Correlated Cross-resistance between Benzimidazole Fungicides and Methyl *N*-(3,5-Dichlorophenyl)carbamate*

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Benzimidazole-resistant field isolates of *Botrytis cinerea, Cercospora beticola, Fusarium nivale* and *Mycosphaerella melonis* showed an increased sensitivity to methyl *N*-(3,5-dichlorophenyl)carbamate (MDPC) on a nutrient medium as compared with the wild-type isolates. The difference in the sensitivity to MDPC between the resistant isolates and the wild-type ranged from about 8 times in *F. nivale* to about 80 times in *C. beticola*. In pot tests, MDPC was found to control gray mold and powdery mildew of cucumber, and *Cercospora* leaf spot of sugar beet caused by the resistant isolates, but not to be effective when the wild-type isolates were inoculated. Furthermore, MDPC showed systemic activity in plants and curative activity as well as preventive activity in controlling gray mold of cucumber. When mixed inoculation of the resistant isolate and the wild-type of *C. beticola* was made to the seedlings of sugar beet, mixed application of MDPC and benomyl was required to control the disease.

INTRODUCTION

Since the introduction of systemic fungicides in practice, resistance of phytopathogenic fungi to fungicides has become a serious problem in crop production.[1–4] In the field where resistant strains appeared, failure of disease control by the fungicides has been experienced in many locations. In order to cope with resistance of plant pathogens, it appears that one of the most effective ways is the use of fungicides to which resistant strains show negatively correlated cross-resistance.

Georgopoulos and Sisler[5] reported that carboxin-resistant *Ustilago maydis* showed an increased sensitivity to antimycin A as compared with the wild-type. Similarly, negatively correlated cross-resistance of phosphorothiolate-resistant isolates of *Pyricularia oryzae* was demonstrated to phosphoramidate compounds by Uesugi *et al.*[6] Van Tuyl *et al.*[7] also found that some thiabendazole-resistant isolates of *Aspergillus nidulans* appeared more sensitive to benomyl than the wild-type. More recently, Leroux and Gredt[8–10] reported that benzimidazole-resistant strains of *B. cinerea* and *Penicillium expansum* exhibited negatively correlated cross-resistance to herbicidal *N*-phenylcarbamates; barban, chlorpropham and chlorbufam. White and Thorn[11] pointed out that carboxin-resistant strains of *U. maydis* were more sensitive to thiophene carboxamide compounds than the wild-type. Furthermore, de Waard *et al.*[12–14] indicated an increased sensitivity of fenarimol-resistant isolates of *Penicillium italicum* and the other fungal species to fenpropimorph and dodine.

However, most of the compounds described above showed an increased fungitoxicity only

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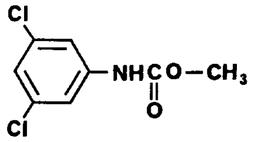

Fig. 1 MDPC, methyl *N*-(3,5-dichlorophenyl)-carbamate.

against resistant strains isolated in the laboratory, and negatively correlated cross-resistance was not demonstrated in field isolates. Although herbicidal *N*-phenyl carbamates were indicated to be toxic to several field isolates of *B. cinerea* and *P. expansum* resistant to benzimidazoles,[8-10] they were phytotoxic and not useful in practice. In this paper, we report another compound, *N*-(3,5-dichlorophenyl)-carbamate (MDPC, Fig. 1), which is more fungitoxic to benzimidazole-resistant field isolates than to the wild-type and exhibits protecting activity against the disease caused by the resistant strains with little phytotoxicity.

MATERIALS AND METHODS

1. *Isolates*

1.1 *B. cinerea*

The wild-type isolates, Bc-2, Bc-024, Bc-039 and Bc-058, and the benzimidazole-resistant isolates, Bc-044, Bc-074 and Bc-105, were isolated from orange, strawberry, tomato, cucumber and grape cultivated in various locations of Japan. The resistant isolate, Bc-11, was kindly provided by Dr. A. Kiso (Vegetable and Ornamental Crops Research Station, Kurume Branch).

1.2 *C. beticola*

The wild-type isolates, C-3-1-2 and Cer-5, were kindly supplied by National Institute of Agro-Enviromental Sciences and Dr. T. Sugimoto (Hokkaido National Agricultural Experiment Station), respectively. The wild-type isolate, Cb-024, and the benzimidazole-resistant isolates, Cb-016, Cb-021 and Cb-085, were isolated from sugar beet cultivated in Hokkaido. The resistant isolates, Cer-116 and Cer-175, were also provided by Dr. T. Sugimoto.

1.3 *M. melonis*

The wild-type isolate, No. 4, and the resistant isolate, No. 7, were kindly provided by Tottori Agricultural Experiment Station. The wild-type isolate, Dka-12, and the resistant isolate, Dka-2, were supplied by courtesy of Dr. S. Murakoshi (Kanagawa Horticultural Experiment Station).

1.4 *F. nivale*

The wild-type isolates, 3a and 81-FS-22-12, and the resistant isolates, 4-1 and T3-1, were kindly provided by Dr. I. Saito (Hokkaido Prefectural Kitami Agricultural Experiment Station).

2. *Culture Medium*

Isolates of *B. cinerea, M. melonis* and *F. nivale* were maintained on potato-dextrose agar (PDA) and *C. beticola* isolates on V-8 juice medium.

In all fungitoxicity studies *in vitro*, PDA was employed. For the inoculation to the plants, conidia of *C. beticola* were produced on tomato juice medium.

3. *Fungitoxicity Studies*

Wettable powder formulations of the test compounds were suspended in sterile distilled water to obtain desired concentrations. The solutions were then mixed well with melted PDA which was immediately poured into sterile plastic petri dishes. Mycelial plugs (5 mm in diameter) with the agar were placed on a center of the solidified plates and cultured at 18°C for *B. cinerea* and *F. nivale*, and 23°C for *C. beticola* and *M. melonis*. Colony diameter was measured after 20 days for *C. beticola* and 3 days for the other species.

4. *Chemicals*

MDPC was synthesized by reacting 3,5-dichlorophenyl isocyanate with methanol and formulated as a 50% wettable powder, which was employed throughout the experiments, unless otherwise stated. Benomyl was obtained as a commercial product of a 50% wettable powder.

5. *Pot Tests*

5.1 *Gray mold of cucumber*

Cotyledonous plants (cv., Sagami-hanjiro) grown for 8 to 10 days in a greenhouse were employed as host plants. Mycelial plugs (5 mm in diameter) of *B. cinerea* (Bc-074), which were cut off at the margin of a rapidly growing mycelial colony, were placed on a center of cotyledons for inoculation, before or after the application of the test compounds. For preventive treatment, the chemical solutions were sprayed on the plants 4 or 5 hr prior to inoculation. Curative application was made by spraying the chemical solutions 18 hr after inoculation. For a systemic test, the roots of cotyledonous plants were washed by tap water and dipped in the chemical solutions for 4 days at 18°C under fluorescent lamps before inoculation. The inoculated plants were incubated in a humid chamber in darkness at 18°C for 3 days. Except for the test of curative application, the disease severity of cotyledons was rated on the following indices; 0 (no symptom), 0.5 (slight infection under the inoculum), 1 (infection of less than 20% of leaf area), 2 (infection of less than 50%) and 4 (infection of more than 50%). Percentages of control (A) were obtained by the following equation;

$$A = 100 - \frac{\left(\begin{array}{c}\text{Sum of indices in} \\ \text{the treated plot}\end{array}\right)}{\left(\begin{array}{c}\text{Sum of indices in} \\ \text{the control plot}\end{array}\right)} \times 100$$

Curative activity was expressed as percentage of inhibition of lesion development by measuring diameter of leaf lesion just before the chemical application (18 hr after inoculation) and at the end of the experiment (3 days after inoculation).

5.2 Powdery mildew of cucumber

Cucumber seedlings (cv., Sagami-hanjiro) grown in a greenhouse for 2 weeks were sprayed with the chemical solutions to run-off. Spores of a benzimidazole-resistant strain of *Sphaerotheca fuliginea* were dusted on the leaves and the plants were cultured for disease development in a greenhouse. The photograph of the primary leaves was taken 12 days after inoculation.

5.3 Cercospora leaf spot of sugar beet

Sugar beet seedlings (cv., Monohill) cultured in a greenhouse for 6 weeks were sprayed with the chemical solutions to run-off. Conidial suspensions of the wild-type isolate, Cb-024, and/or the resistant one, Cb-085, were sprayed on the leaves for inoculation 1 day after the application. The inoculated plants were incubated for 8 days in a humid chamber in a greenhouse and the leaves were photographed.

RESULTS

1. Fungitoxicity of MDPC against the Benzimidazole-resistant Isolates and the Wild-type Isolates

MDPC was highly fungitoxic to benzimidazole-resistant field isolates of *B. cinerea, C. beticola, F. nivale* and *M. melonis,* whereas toxicity to the wild-type isolates was relatively low (Fig. 2). On the contrary, benomyl inhibited the growth of the wild-type isolates at low concentrations and exhibited low toxicity to the resistant isolates. Negatively correlated cross-resistance was clearly shown between MDPC and benomyl without exception among the isolates tested in this experiment.

Benzimidazole-resistant isolates of *B. cinerea* were about 50 times more sensitive to MDPC than the wild-type, when judged by the difference of ED_{50} values for inhibition of radial mycelial growth on PDA. Similarly, MDPC was about 80 times more toxic to benzimidazole-resistant isolates of *C. beticola* than the wild-type. The resistant isolates of *F. nivale* and *M. melonis* were also more sensitive to MDPC than to benomyl, but the difference in the sensitivity to MDPC between the resistant isolates and the wild-type was low; about 8 times for *F. nivale* and 11 times for *M. melonis.*

2. Control of Gray Mold

MDPC effectively controlled gray mold of cucumber caused by the resistant isolate, Bc-074, of *B. cinerea* in a pot test (Table 1). On the other hand, preventive spray of benomyl at a concentration of 200 μg/ml did not suppress the disease development of the resistant isolate-inoculated plants. Curative application of MDPC controlled the disease as effectively as preventive application. The chemical treatment to the roots of cucumber was also effective to prevent gray mold of the leaves, suggesting systemic movement of MDPC in plants.

3. Control of Powdery Mildew of Cucumber

When a benzimidazole-resistant strain of *S. fuliginea* was inoculated to cucumber seedlings, disease development was completely

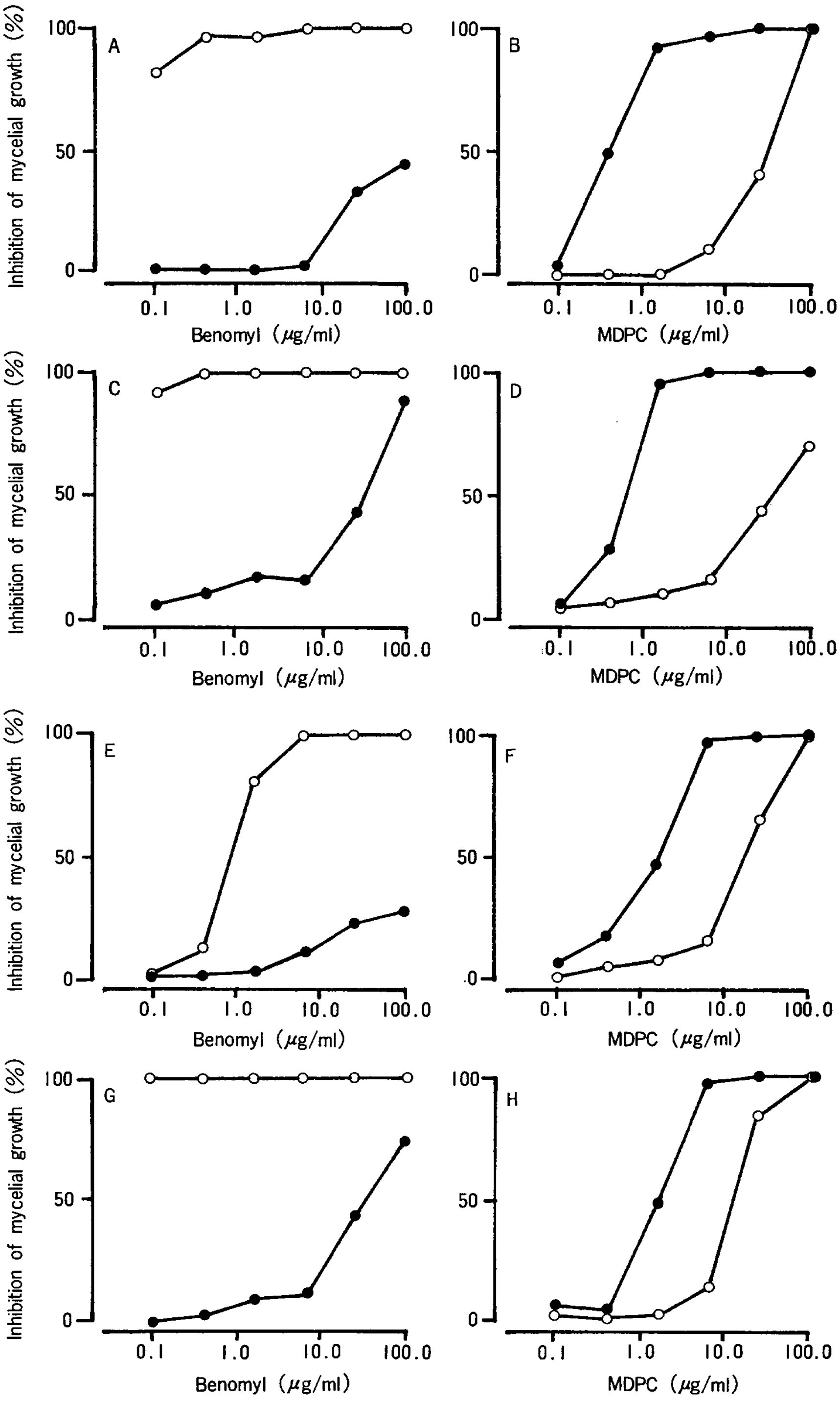

Fig. 2 Inhibition of radial mycelial growth of benzimidazole-resistant and wild-type isolates.

A, B: *B. cinerea* (○: mean value of the wild-type isolates, Bc-2, Bc-024, Bc-039 and Bc-058; ●: mean value of benzimidazole-resistant isolates, Bc-11, Bc-044, Bc-074 and Bc-105).

C, D: *C. beticola* (○: mean value of the wild-type isolates, C-3-1-2, Cer-5 and Cb-024; ●: mean value of benzimidazole-resistant isolates, Cb-016, Cb-021, Cer-116 and Cer-175).

E, F: *M. melonis* (○: mean value of the wild-type isolates, No. 4 and Dka-12; ●: mean value of benzimidazole-resistant isolates, No. 7 and Dka-2).

G, H: *F. nivale* (○: mean value of the wild-type isolates, 3a and 81-FS-22-12; ●: mean value of benzimidazole-resistant isolates, 4-1 and T3-1).

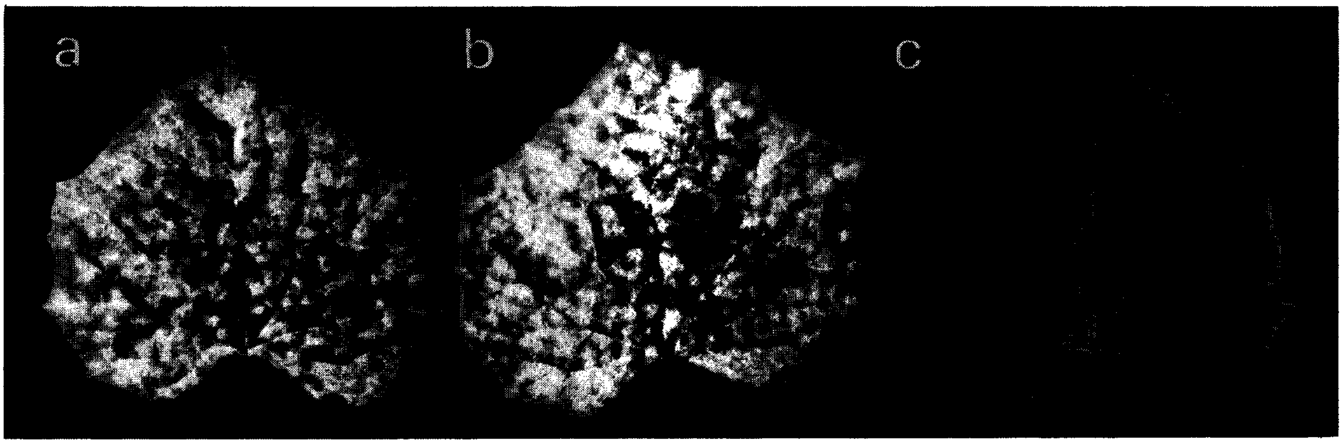

Fig. 3 Control of powdery mildew of cucumber caused by a benzimidazole-resistant strain.
a: untreated, b: benomyl-treated (100 μg/ml), c: MDPC-treated (100 μg/ml).

Table 1 Control of gray mold of cucumber caused by benzimidazole-resistant strain by MDPC.[a]

Concentration of MDPC (μg/ml)	Percentage of control		
	Preventive[b]	Curative	Systemic
200	95	71	—
50	59	57	98
12.5	12	0	88
3.1	—	—	85

[a] MDPC formulated as an emulsifiable concentrate was emloyed in this experiment.
[b] Type of test.

prevented by spraying MDPC at a concentration of 100 μg/ml (Fig. 3), whereas treatment with benomyl did not show any protection. Phytotoxicity of MDPC to cucumber seedlings was not observed in this experiment.

4. *Control of Cercospora Leaf Spot of Sugar Beet*

MDPC was also effective in controlling Cercospora leaf spot of sugar beet without phytotoxicity, when the resistant isolate was inoculated, but did not exert any effect on the disease development of the wild-type isolate-inoculated plants (Fig. 4). On the other hand, benomyl controlled the disease caused by the wild-type isolate but not the disease by the resistant one. When mixed inoculation of the wild-type and the resistant isolate was made at a ratio of 1 : 1, application of MDPC or benomyl alone did not suppress disease development and mixed application of both fungicides was required to obtain complete control of the disease.

DISCUSSION

MDPC is a derivative of 3,5-dichloroaniline and first reported to show only a limited fungitoxicity against the wild-type isolate of *Sclerotinia sclerotiorum*.[15] Among the family of derivatives of 3,5-dichloroaniline, dicarboximide fungicides, procymidone,[16] iprodione[17] and vinclozolin[18] were the most effective against *Botrytis* and *Sclerotinia* spp. and their activity was not influenced by benzimidazole-resistance of the strains. However, the present investigation indicated that MDPC was much more active against benzimidazole-resistant strains than the wild-type and negatively correlated cross-resistance was apparent between MDPC and benzimidazoles.

The present results further indicated that a fairly broad range of fungal species resistant to benzimidazoles showed negatively correlated cross-resistance to MDPC. This suggests that those fungal species acquire benzimidazole-resistance through the same mechanism of resistance. Moreover, great attention should be paid to the fact that all of the resistant isolates tested in the present experiments were field isolates. This raises the possibility of using MDPC to control the resistant strains in fields.

Herbicidal *N*-phenylcarbamates, barban,

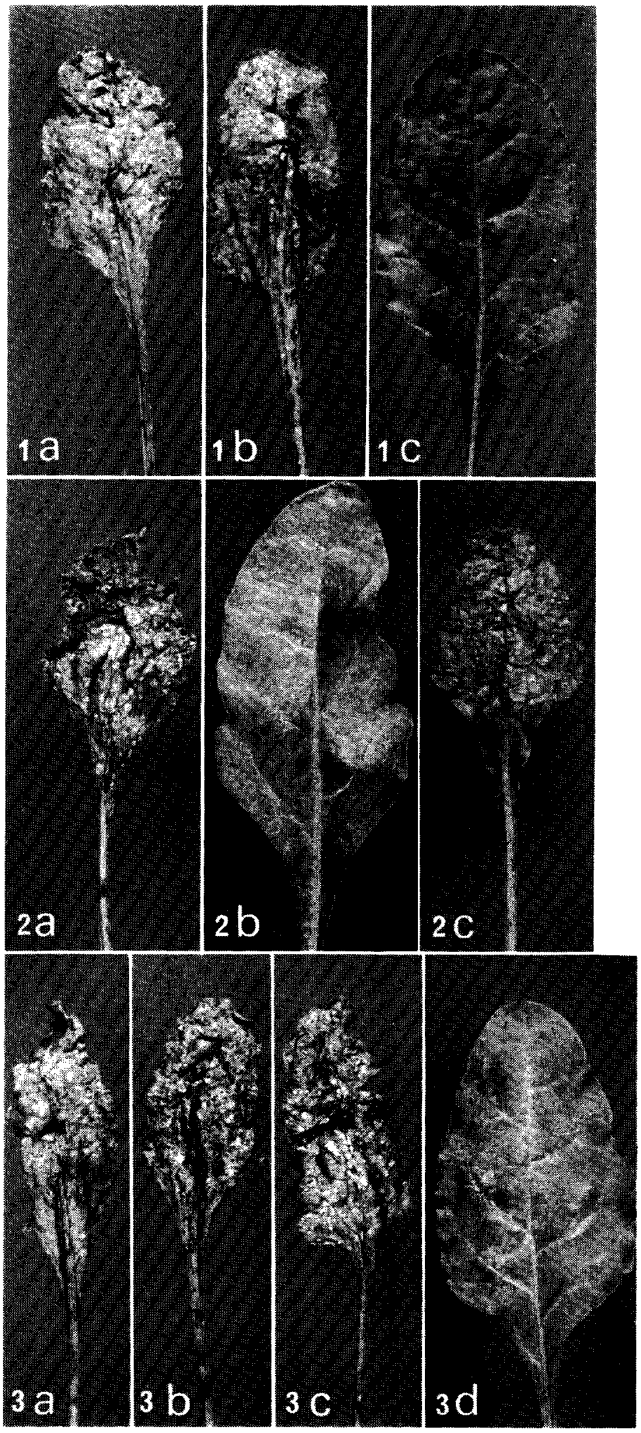

Fig. 4 Control of Cercospora leaf spot by benomyl and MDPC.

1a, 1b, 1c: inoculated with the wild-type isolate (Cb-024), 2a, 2b, 2c: inoculated with the benzimidazole-resistant isolate (Cb-085), 3a, 3b, 3c, 3d: inoculated with a mixture of Cb-024 and Cb-085, 1a, 2a, 3a: untreated, 1b, 2b, 3b: treated with MDPC (200 μg/ml), 1c, 2c, 3c: treated with benomyl (200 μg/ml), 3d: treated with a mixture of MDPC (100 μg/ml) and benomyl (100 μg/ml).

chlorpropham and chlorbufam are known to be mitotic inhibitors of plant cells.[19–21] Methyl benzimidazol-2-ylcarbamate, the conversion product of benomyl and thiophanate-methyl, is also proved to be a mitotic inhibitor of fungi.[22] Taking this similarity in the mode of action into consideration, Leroux and Gredt[8–10] tested fungitoxicities of *N*-phenylcarbamate herbicides to benzimidazole-resistant strains of *B. cinerea* and *P. expansum*, and found an increased sensitivity of the resistant strains to those compounds. Among *N*-phenylcarbamates, barban was reported to be the most effective against the resistant strains of *B. cinerea*. The present compound, MDPC, is however more fungitoxic to the resistant strains than barban and its phytotoxicity is much lower than that of barban.

Systemic activity of fungicides in plants is important to achieve effective control of the diseases in fields. MDPC was found to show systemic translocation in plants without phytotoxicity. Moreover, the compound inhibited further development of gray mold lesion on cucumber leaves by curative treatment. These properties may contribute high activity in the control of diseases, and MDPC or related compounds are useful candidates to cope with the resistance problem in practice.

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要　約

ベンズイミダゾール系殺菌剤および methyl *N*-(3,5-dichlorophenyl) carbamate 間における負相関交差耐性

加藤寿郎，鈴木恵子，高橋淳也，鴨下克三

Methyl *N*-(3, 5-dichlorophenyl) carbamate (MDPC) は，*Botrytis cinerea, Cercospora beticola, Fusarium nivale, Mycosphaerella melonis* のベンズイミダゾール耐性菌に対し，野生菌と比べて高い抗菌力を示し，ベンズイミダゾール系殺菌剤との間に負相関交差耐性が認められた．幼苗を用いた鉢試験でも，MDPC はベンズイミダゾール耐性菌によるキュウリ灰色かび病，キュウリうどんこ病，テンサイ褐斑病の発病を抑制する効果が認められた．また，植物体への浸透移行性および治療効果もあった．一方，テンサイ褐斑病菌と耐性菌を混合して接種した場合，ベノミルまたは MDPC の単独処理では十分な防除効果が認められず，効果を発揮するためには，両者の混合処理が必要であった．