灰色かび病菌における室内と圃場条件下でのプロサイミドンの耐性化の相違

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Difference in the Potential of *Botrytis cinerea* to Develop Resistance to Procymidone *in vitro* and in Field

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久田芳夫*・高木宏和*・川瀬保夫*・尾崎俊明*：灰色かび病菌における
室内と圃場条件下でのプロサイミドン耐性化の相違

Abstract

Under laboratory conditions, procymidone-resistant strains of *Botrytis cinerea* frequently emerged from both the conidia and the mycelia without UV irradiation or the treatment with other mutagenic agents, but no benomyl-resistant strain from the conidia. Contrarily, *B. cinerea* on rose plants in a greenhouse remained sensitive to procymidone even after spraying the fungicide 19 times during 3 years, but became highly resistant to benomyl after spraying the fungicide only 5 times. Procymidone-resistant strains obtained *in vitro* were inferior to the parent strain in their ability of sporulation and virulence. Resistant strain R-22 with relatively high virulence had a greatly reduced chance of survival in competition on the plant with the parent strain. The results suggest that procymidone resistance may be accompanied by decreased fitness and virulence, and resistant strains might be retarded or prevented from becoming dominant in the population in the field. Also, it can be concluded that laboratory experiments are quite limited to immediate indication of practical importance of fungicide resistance.

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Introduction

Procymidone (Sumilex ®) is a new class of fungicides with high effectiveness to control the plant diseases caused by *Botrytis* and *Sclerotinia* species[6]. Though procymidone is not released for a practical use in Japan yet, it is important to elucidate the potential of pathogenic fungi developing resistance to procymidone in order to enable the most effective use of the fungicide. The present study was conducted to know the potential of development of procymidone resistance in *Botrytis cinerea* which was exposed to the fungicide *in vitro* or in the field. The feature of procymidone resistance was discussed.

Materials and Methods

Fungi and Culture. *B. cinerea* isolated from strawberry, mandarin orange, eggplant, grape and cucumber were used in this work and they were designated as strain Bc-1, 2, 4, 5 and 9, respectively. The stock cultures were maintained on potato-sucrose agar medium. Potato-dextrose agar (PDA) contained extract of 200 g

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potato, 20 g glucose and 15 g agar per liter of distilled water ; Hislop's medium[7] contained 40 g glucose, 10 g peptone, 0.1 g KNO_3, 6.8 g KH_2PO_4, 2.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 20 mg $FeCl_3$ and 20 g agar per liter of distilled water. The fungi were cultured on these media at 25 C.

Fungicide. The fungicides used were 50 % wp product of procymidone and commercially available 50 % wp product of benomyl.

Pathogenicity test. Cucumber seedlings (cv. Sagami-hanjiro) at the primary leaf stage were employed for pathogenicity test of procymidone-resistant isolates obtained *in vitro* from strain Bc-2. A half of the leaf was inoculated with a resistant strain and the other half with the parent strain. The incidence of the disease was assessed after incubating the test plant for 3 days at 20 C. Virulence of resistant strains was expressed in terms of percent ratio of average diameter of the lesion affected by resistant strains to that affected by the parent strain.

Competition test. For competition test, procymidone-resistant isolate R-22 was selected because of its high virulence and large spore production. The plate of Hislop's medium or detached rose flower (cv. Peace) was inoculated with a suspension containing the conidia of R-22 and the parent strain at the rate of 20 to 1 in number. At intervals of 7 to 10-day incubation, 5 successive transfers were made by means of spore inoculation. In field test for the competition, rose plants (2 years) grown in a greenhouse were inoculated by spraying a mixed conidial suspension of R-22 and the parent strain on 15 May. The inoculum contained 6.48×10^8 conidia of R-22 and 3.3×10^7 of the parent strain in 225 ml. Rose flowers were harvested on 28 June and placed in a moist chamber. Single spore isolates obtained from the flowers were inoculated on the agar medium containing procymidone at a concentration of 500 μg/ml to examine their sensitivity to the fungicide.

Field trial for the development of resistance. The conidia of *B. cinerea* (Bc-2) were inoculated to the flowers of rose plants (cv. Peace, 3-6 years) grown in two greenhouses on 10 May 1976. Procymidone and benomyl were separately sprayed to a half number of rose plants (15 plants) in each greenhouse 19 times during 3-year experimental period, and the fungi were isolated from the plants at appropriate times (Table 1). Sensitivity of single spore isolates to the fungicides was estimated in the laboratory.

Table 1. The dates of fungicide spraying and fungal isolation in greenhouse test for development of resistance to procymidone or benomyl in *Botrytis cinerea* (Bc-2) on rose plant

	Spraying date[a]	Isolation date
1976	26/May, 2/Jun, 20/Oct, 16,24/Nov	14/Sep, 2/Nov, 5/Dec
1977	11,18,25/Apr, 5/May, 20,27/Jun, 7,14/Jul. 3,11,17/Oct, 7,21/Nov	29/Jun, 25/Nov
1978	4/Jul	26/Sep

a) Procymidone was sprayed at a concentration of 250 μg/ml throughout this test, and benomyl at the concentrations of 250 μg/ml (26/May/1976 to 25/Apr/1977), 500 μg/ml (5/May to 11/Oct) and 1,000 μg/ml (17/Oct to 21/Nov).

Results

Training of the mycelium to resistance

The training of the mycelium of *B. cinerea* (Bc-2) to tolerate procymidone and

benomyl was made in two ways. The first involved successive transfers of the mycelium to Hislop's medium containing the fungicides at the concentrations to inhibit 50 or 90 % of hyphal growth. The second method consisted of incubating the mycelium opposite to the filter paper containing the fungicides at a growth-inhibitory dose on the agar medium and then transfering the mycelium at growth-inhibited zone of mycelial colony to new plates with the fungicide-containing filter paper. Incubation period for transfer was 5–10 days. Even after 20 transfers on the former tests and 5 transfers on the latter, there was no change in the sensitivity of the fungus to either fungicides.

Development of resistant strains from the conidia

Large number of the conidia of *B. cinerea* was inoculated to Hislop's medium containing procymidone or benomyl at the various concentrations. From the conidia of strain Bc-2, procymidone-resistant colonies emerged at the frequency of about 200×10^{-8} independent on the concentration of the fungicide (Table 2). On the other hand, no development of benomyl-resistant strains was observed on the agar media containing benomyl at the concentrations of 0.8 to 500 μg/ml. Procymidone-resistant strains also extensively developed from other 6 strains of *B. cinerea* at the frequency of 17 to 347 $\times 10^{-8}$.

Table 2. *In vitro* development of fungicide-resistant strains from the conidia of *Botrytis cinerea* (Bc-2)

Fungicide	Concentration (μg/ml)	No. of colonies developed/5×10^7 conidia[a]		Frequency ($\times 10^{-8}$)
		Test 1	Test 2	
Procymidone	500	105	72	177
	100	110	97	207
	20	85	88	173
Benomyl	500	0	0	0
	20	0	0	0
	0.8	0	0	0

a) The number of colonies was counted after 3 days of incubation.

Development of resistant strains from the mycelium

The mycelial disc (0.5 cm in diameter) obtained from the colony of *B. cinerea* grown on PDA was inoculated on PDA containing procymidone at a concentration of 500 μg/ml. In the presence of procymidone mycelial growth from the inoculated disc did not occur during 2-day incubation, but thereafter at a considerably high frequency growing mycelium appeared (Table 3). Free from fungicide, the mycelium of *B. cinerea* grew wholly over the medium in a petri dish 3 days after inoculating the mycelial disc. The mycelia produced in the presence of procymidone by prolonged incubation were resistant to procymidone. The frequency was not related to the concentration of procymidone exposed (Table 4). Similarly, procymidone-resistant strains developed from the tissue of eggplant fruits (3-4 mm pieces in size) infected with *B. cinerea* (Table 5).

Biological properties of resistant isolates

Fifty-six isolates resistant to procymidone were obtained *in vitro* from the conidia of *B. cinerea* (Bc-2). The rate of hyphal growth, sporulation and sclerotium for-

Table 3. *In vitro* development of resistant strains from the mycelium of *Botrytis cinerea* under selection pressure of 500 μg/ml of procymidone

Fungal strain	No. of discs developing resistant hyphae/No. of mycelial discs inoculated[a)]	
	Test 1	Test 2
Bc-1	3/20	4/20
Bc-2	4/20	2/20
Bc-4	0/20	1/20
Bc-5	9/20	9/20
Bc-9	1/20	0/20

a) The number of discs developing resistant hyphae was counted after 8 days of incubation.

Table 4. *In vitro* development of resistant strains from the mycelium of *Botrytis cinerea* (Bc-2) exposed to various concentrations of procymidone

Concentration of procymidone (μg/ml)	No. of discs developing resistant hyphae/No. of mycelial discs inoculated[a)]
500	8/50
50	4/36
5	8/45

a) The number of discs developing resistant hyphae was counted after 8 days of incubation.

Table 5. *In vitro* development of resistant strains from the tissue of eggplant fruit infected with *Botrytis cinerea* under selection pressure of 500 μg/ml of procymidone

Fungal strain	No. of pieces developing resistant hyphae/No. of inoculated pieces of tissue affected[a)]	
	PDA medium	Hislop's medium
Bc-2	1/20	1/20
Bc-5	6/20	2/20
Bc-9	2/20	6/20

a) The number of pieces developing resistant hyphae was counted after 8 days of incubation.

mation of these isolates were examined on Hislop's medium containing procymidone at a concentration of 100 μg/ml. Besides, their virulence to plant was tested on cucumber seedlings. Although resistant strains differed considerably from each other in the characteristics, they were generally inferior to the parent strain in biological properties examined. To take the case of virulence, 20 resistant strains were non-pathogenic and the virulence of 23 resistant strains was less than 50% of that of the parent strain. There was no resistant strain superior to the parent strain in virulence. As for sporulation and sclerotium fomation, 20 resistant strains did not form conidia and 12 did not sclerotia, respectively. Properties of 8 isolates with relatively high virulence were summarized in Table 6. All of 44 resistant strains examined had retained their resistance on PDA without the fungicide during about 2 years.

Competition of a resistant strain with the parent strain

As represented in Table 7, the relative population of the resistant strain was remarkably diminished on Hislop's medium and resistant strain disappeared on rose flower by 5 transfers. In field test for the competition, no procymidone-resistant strain was detected in 98 isolates sampled from rose flowers about 6 weeks after mixed inoculations.

Field trial for the development of resistance

Even after 19 sprays of procymidone, the distribution pattern of fungicide sensitivity of 72 isolates from rose flowers was the same to that of original strain Bc-2 (Fig. 1). In the treatment of benomyl, the development of resistant *B. cinerea* was already observed after 3 sprays. About 80 % of 139 isolates was highly resistant to benomyl after 5 sprays, and all of 50 isolates after 18 sprays.

Table 6. Biological properties of 8 procymidone-resistant isolates of *Botrytis cinerea* which have relatively high virulence[a)]

Isolate	Rate of mycelial extension (mm/day)	Sporulation[b)]	Sclerotium formation	Relative virulence[c)]
R-9	14	+	+	76
R-12	19	++ (9.5×10^5)	+	66
R-15	10	+	+	88
R-18	14	+	+	64
R-20	8	++ (14×10^5)	+	66
R-21	8	+	+	71
R-22	12	++ (5.8×10^5)	+	76
R-37	12	++ (4.0×10^5)	−	62
Parent strain (Bc-2)	12	+++ (41×10^5)	+	100

a) Resistant strains were incubated on Hislop's medium containing procymidone at a concentration of 100 μg/ml.
b) Figure in parentheses refers to the mean conidial number formed per petri dish.
c) Virulence was expressed in terms of percent ratio of average diameter of the lesion infected with resistant strain to that infected with the parent strain.

Table 7. The competition of procymidone-resistant strain R-22 with the parent strain (Bc-2) of *Botrytis cinerea* on the medium and rose flower after mixed inoculation[a)]

Transfer	No. of resistant isolates/No. of total isolates			
	Medium		Rose flower	
	Test 1	Test 2	Test 1	Test 2
1 Time	—	—	68/100	113/115
5 Times	15/100	9/78	0/100	0/100

a) Mixed inoculum of resistant strain R-22 and the parent strain was prepared in the ratio of 20 to 1 with respect to the conidial density.

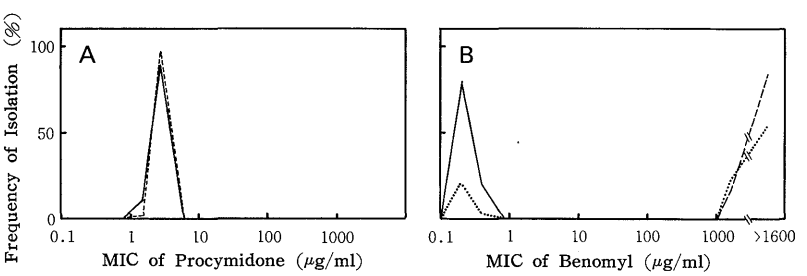

Fig. 1. Fungicide sensitivity of *Botrytis cinerea* isolates from rose plants sprayed with procymidone (A) or benomyl (B) in a greenhouse.
——— Strain Bc-2 (original strain)
..... 139 Isolates after 5 sprays
—·—·— 50 Isolates after 18 sprays
- - - - - 72 Isolates after 19 sprays

Discussion

Under laboratory conditions, procymidone-resistant strains of *B. cinerea* frequently emerged from both the conidia and the mycelia without the treatment of UV irradiation or other mutagenic agents, though it did not acquire any resistance by training of the mycelium. A lag period of incubation over 3 days was needed for the resistant hyphae to start visible growth from the inoculated mycelium in the agar medium or in the fruit tissue. This is characteristic to development of procymidone-resistant strains from the mycelium. Other workers also found that procymidone-resistant strains of *B. cinerea* developed from the conidia[12] and the mycelia[11,13] at a high frequency in the laboratory.

Meyer and Parmeter[10] and Webster *et al.*[14] suggested heterokaryosis as a possible mechanism of fungicide resistance in multinucleate fungi such as *Thanatephorus cucumeris* and *B. cinerea*. Fungicide-resistant sectors frequently appear due to heterokaryosis. Frequency of development of procymidone resistance was higher than would be expected from spontaneous gene mutation, not depending on the concentration of procymidone. Also, resistance was stable in the absence of procymidone, suggesting genetically determined resistance rather than adaptive-type. These results lead to a possibility that heterokaryosis might serve as the mechanism of procymidone resistance, but further investigation is needed to elucidate it.

Procymidone was sprayed against *B. cinerea* (Bc-2) on rose plants in a greenhouse 19 times during 3 years, but sensitivity of the fungi isolated finally was similar to that of the original strain. In the treatment of benomyl under the same conditions, benomyl-resistant strains readily developed after spraying 3 times. Under laboratory conditions, however, we failed to obtain benomyl-resistant strains from *B. cinerea* (Bc-2) in contrast with the case of procymidone. These incompatible results indicate that resistance problems to fungicide under practical conditions could not be deduced from the results of laboratory experiments. Wolfe[15] and Dekker[1,2] concluded laboratory experiments were quite limited to immediate indication of practical importance of fungicide resistance. Dekker pointed out three factors important to a resistant strain becoming dominant in the field ; (1) the characteritics of resistant strains, (2) the type of disease, (3) the selection pressure exerted by fungicide. It is considered that the first factor is important to elucidate the potential of *B. cinerea* to develop resistance to procymidone in practice. All of the procymidone-resistant strains obtained *in vitro* had less virulence and ability to sporulate than the parent strain. Moreover, the resistant strain disappeared in competition with the parent strain on the plant without the treatment of procymidone. Procymidone resistance may be accompanied by decreased fitness and virulence, similarly to triforine resistance. Fuchs *et al.*[3] reported that all of the triforine-resistant strains of *Cladosporium cucumerinum,* which were induced by UV irradiation, had decreased virulence, sporulation and germination of spores. Problems of resistance to this fungicide have never experienced in practice. In this way, procymidone-resistant strains might be also retarded or prevented from becoming dominant in the population in the field. Difficulty of development of fungicide resistance is influenced by the nature of pathogen (generation time and ability of reproduction etc.), selection pressure by fungicide (level and frequency of application etc.) and the type of host plant. The potential of development of procymidone resistance in practical use must be further investigated with use of many fungal strains under various conditions. Hirota and Kato[4] reported that *B. cinerea* isolated from the eggplant which was sprayed with procymidone at a concentration of 250 μg/ml 30 times every 6 days was entirely

sensitive to the fungicide.

Iprodione, which is a new fungicide having cyclic *N*-(3,5-dichlorophenyl) imide in its chemical structure, induced *in vitro* resistant strains in *B. cinerea* and they showed cross-resistance to procymidone[5,9,12]. Kotani[8] reported the rapid development of iprodione resistance in *B. cinerea* on eggplants grown in a greenhouse, where two sprays of the fungicide at a concentration of 500 μg/ml were performed unsatisfactorily against this disease. This result also suggests the possibility that development of resistance to procymidone would readily occur in practice, and disagrees with our results. However, he obtained iprodione-resistant strains of *B. cinerea* by placing a piece of the infected tissue of fruits harvested from eggplants in the greenhouse onto agar media containing 50 μg/ml of the fungicide and incubating for several days. This is identical to the procedure that we obtained *in vitro* procymidone-resistant strains from *B. cinerea*. Assay method of procymidone-resistant strains seems worthy of further examination.

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和　文　摘　要

灰色かび病菌における室内と圃場条件下でのプロサイミドン耐性化の相違

久田芳夫・高木宏和・川瀬保夫・尾崎俊明

室内試験で，灰色かび病菌（*Botrytis cinerea*）の分生胞子，菌糸および罹病組織などから，プロサイミドン耐性菌がかなりの高頻度で検出された。同様に，分生胞子からベノミル耐性菌発生の検討を行ったところ，その出現は全く認められなかった。ハウス栽培したバラの灰色かび病菌一菌株を対象に，3年間にわたり19回薬剤散布を行い，その間に分離した灰色かび病菌の薬剤感受性を調べたところ，室内試験結果とは逆に，プロサイミドン耐性菌の発生は全く認められなかったにもかかわらず，ベノミル耐性菌は3回散布後頃から認められ始め，5回散布後では分離菌の約80%が高度耐性菌となっていた。プロサイミドンに対する室内耐性菌は，感受性の親株に比べ病原性，胞子形成能などが劣っていた。また，比較的病原性の高い室内耐性菌を使って親株との競合力を調べたところ，室内耐性菌は植物体上できわめて劣性であった。以上の結果から，プロサイミドン耐性菌は，薬剤圧により宿主植物体上で出現したとしても，その個体群の中で優性になり難いのではないかと考えられる。また，耐性菌発生の難易を，限られた室内実験によってのみ推定することは困難であることが示唆された。